



Australian Government

Department of Agriculture, Fisheries and Forestry

Review of Published Tests to Detect Pathogens in Veterinary Vaccines Intended for Importation into Australia

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Executive summary

Australia has a favourable animal health status. To maintain this favourable status Australia adopts a risk based approach to the management of animal biosecurity. This is particularly important in the case of veterinary vaccines because they may not be subjected to microbiologically lethal treatment during production and if contaminated, could bring about the widespread dissemination of serious pathogens and associated diseases such as foot-and-mouth disease. Imported vaccines present inherently high biosecurity risks due to the direct exposure of large numbers of live animals to these products. Therefore, imported veterinary vaccines undergo a detailed and rigorous technical assessment of the biosecurity risks. Imported veterinary vaccines are strictly controlled and products are tested for extraneous agents using sensitive methods in accordance with Australia's requirements for the importation of veterinary vaccines.

Australia requires that the most sensitive and reliable test methods are used to prevent contamination of imported veterinary vaccines with extraneous agents. This review provides clarification on the acceptable tests for extraneous agents required to meet Australia's import policy for veterinary vaccines. This will improve the efficiency of assessments for the importation of veterinary vaccines by consolidating the relevant scientific literature and assessing the suitability of these test methods for the detection of extraneous agents. Australia's import requirements have not changed as a result of this review.

Australia's requirements for the importation of veterinary vaccines are contained in the *Australian Quarantine Policy and Requirements for the Importation of Live and Novel Veterinary Bulk and Finished Vaccines (1999)* and *Specific Quarantine Requirements for the Importation of Inactivated Veterinary Vaccines (1997)*. These include lists of animal pathogens (Annexes 1–3) that the Australian Government Department of Agriculture, Fisheries and Forestry (DAFF) may require country freedom from or testing for as part of the import assessment process.

Annex 1 consists of major exotic notifiable animal disease pathogens as listed by the World Organisation for Animal Health (OIE) (e.g. foot-and-mouth disease and classical swine fever). Annex 2 includes exotic animal transmissible spongiform encephalopathies (e.g. bovine spongiform encephalopathy and scrapie). Annex 3 includes other significant exotic animal pathogens, more virulent exotic strains of endemic animal pathogens, and endemic animal pathogens that are common contaminants of veterinary vaccines. Annexes 1–3 have been updated taking into account current valid taxonomy (see appendix 1).

Animal Biosecurity Branch of DAFF reviewed published test methods suitable for the reliable detection of Annex 1 and 3 extraneous agents in imported veterinary vaccines. The animal biosecurity risks associated with exotic animal transmissible spongiform encephalopathies are managed through measures other than testing. The review included general¹ test methods in accordance with the relevant sections of the *United States Code of Federal Regulations*, Title 9: *Animals and Animal Products*, part 113 (9 CFR 113) and the European Pharmacopoeia. These international standards are primarily intended for generic screening to detect non-specified contaminating bacteria, mycoplasma, fungi and viruses. The standards thus require only that the sensitivity of the culture system used for extraneous agent testing is representative of the pathogens found in Europe and the United States for the source and target species. Australia recognises that veterinary vaccines meeting European and United States standards may not be tested for extraneous

¹ General test methods are designed to detect a wide range of common bacteria, mycoplasma and fungi using media that will grow most contaminants. Viral contaminants can be detected using an appropriate range of cell lines and checking for cytopathic effects, inclusion bodies and haemadsorption.

agents exotic to Australia, so that testing for specific pathogens of concern to Australia is required to protect Australia's favourable animal health status.

This report details the findings of this review and describes published test methods that are considered to be reliable and sensitive for detecting extraneous agents in vaccines and vaccine raw materials provided they are validated in accordance with Australia's requirements for importation of veterinary vaccines. There may be other validated unpublished test methods that are equally reliable and sensitive, including some test methods that remain commercial-in-confidence and are unpublished. The Biological Imports Program², a program within DAFF will assess these on a case-by-case basis.

Some pathogens in Annex 1 and 3 affect multiple animal species and are listed under each species or species group in updated Annexes 1–3 (appendix 1).

The review concluded that the following Annex 1 and 3 extraneous agents can be reliably cultured using general test methods in accordance with 9 CFR 113 but detection or identification is unreliable, therefore specific testing³ is required. Footnotes have been added to qualify the inclusion of the pathogens in each list below. These lists are for summary purposes only and the individual chapters for each pathogen should be referred to for the details.

Bovine parainfluenza virus 3⁴
Bovine parvovirus⁵
Bovine respiratory syncytial virus⁶
*Brucella canis*⁷
*Brucella suis*⁸
Canine adenovirus 1, 2⁹
Equid herpesvirus 1, 2, 3 and 4¹⁰
Equine arteritis virus
Murine adenovirus
Ovine adenovirus (OAdV)
Peste-des-petits-ruminants virus
Porcine adenovirus (PAdV)
Porcine haemagglutinating encephalomyelitis virus
Rabies virus¹¹
Salmonella Enteritidis
Salmonella Gallinarum
Salmonella Pullorum¹²

The review concluded that the following Annex 1 and 3 extraneous agents can be reliably detected by primary culture alone using general test methods in accordance with 9 CFR 113 and therefore specific testing is not required.

² On behalf of the Australian Director of Quarantine.

³ Specific test methods offer optimum conditions for the detection and identification of specific pathogens.

⁴ Provided the BEK or BEL cell lines are used.

⁵ If FA test performed between 8–16 hours.

⁶ Provided that FA is performed with a polyclonal antiserum directed against BRSV whole virus antigen.

⁷ The generalised methods described in European Pharmacopoeia monograph 2.6.12 are not suitable.

⁸ The generalised methods described in European Pharmacopoeia monograph 2.6.12 are suitable.

⁹ Provided the MDCK or MDCK-SP cell lines are used for isolation.

¹⁰ Provided that primary isolation is in equine foetal kidney cells or equine fibroblasts.

¹¹ If combined with primary isolation in Neuro-2a cells.

¹² The method in European Pharmacopoeia monograph 2.6.24 is not suitable.

Lumpy skin disease virus¹³
Rinderpest virus
Vesicular stomatitis virus

The review concluded that the following Annex 1 and 3 extraneous agents can be reliably detected by primary culture alone using test methods other than those described as general test methods in 9 CFR 113 and therefore specific testing is not required.

African horse sickness virus
Burkholderia mallei
Caprine and ovine pox virus
Rift Valley fever virus

The review concluded that the following Annex 1 and 3 extraneous agents cannot be reliably cultured by primary culture using general test methods in accordance with 9 CFR 113. Other test methods are acceptable for primary culture but detection is unreliable, therefore specific testing is required.

African swine fever virus
Akabane virus
Anatid herpesvirus 1¹² (duck enteritis virus, duck plague herpesvirus)
Avian adenoviruses¹² (all viruses in the genus Aviadenovirus and duck adenovirus A (egg drop syndrome virus))
Avian encephalomyelitis virus¹²
Avian influenza virus
Avian leukosis virus¹²
Avian nephritis virus 1 and 2¹²
Avian orthoreovirus¹² (avian reovirus)
Babesia caballi
Bluetongue virus
Border disease virus
Bovine adenovirus
Bovine ephemeral fever virus
Bovine herpesvirus 1
Bovine herpesvirus 2
Bovine herpesvirus 4
Bovine viral diarrhoea virus 1 and 2
*Brucella abortus*⁷
*Brucella melitensis*⁷
Canine distemper virus
Canine parvovirus
Chicken anaemia virus
Classical swine fever virus
*Coxiella burnetii*⁷
Duck viral hepatitis virus 1¹²
Ectromelia virus
Epizootic haemorrhagic disease virus
Equine adenovirus

¹³ Provided LT cells or OA3.Ts cells are used.

Equine encephalitis viruses (eastern equine encephalitis virus (EEEV), western equine encephalitis virus (WEEV) and Venezuelan equine encephalitis virus (VEEV))
 Equine influenza virus
 Felid herpesvirus 1 (feline rhinotracheitis virus)
 Feline calicivirus
 Feline infectious peritonitis virus
 Feline panleukopaemia virus
 Foot-and-mouth disease virus
 Fowlpox virus
*Francisella tularensis*⁷
 Hantaan virus
Histoplasma capsulatum var. *farcinosum*
 Horse pox virus
 Infectious bronchitis virus¹²
 Infectious bursal disease virus¹²
 Infectious laryngotracheitis virus¹²
 Japanese encephalitis virus
Leptospira interrogans var. *canicola*⁷
 Louping ill virus
 Lymphocytic choriomeningitis virus
 Marek's disease virus 1 and 2¹² (gallid herpesvirus 2 and 3)
*Mycoplasma agalactiae*¹⁴
Mycoplasma capricolum subsp. *capripneumoniae*¹⁴
*Mycoplasma gallisepticum*¹⁵
*Mycoplasma hyopneumoniae*¹⁴
Mycoplasma mycoides subsp. *mycoides* SC¹⁵ (MmmSC)
*Mycoplasma synoviae*¹⁵
 Newcastle disease virus
 Orf virus
 Porcine circovirus 2
 Porcine epidemic diarrhoea virus
 Porcine parvovirus
 Porcine teschovirus 1 (polioencephalomyelitis virus)
 Pseudorabies virus (Aujeszky's disease virus, suid herpesvirus 1)
 Rabbit fibroma virus (Shope fibroma virus)
 Rabbit haemorrhagic disease virus (rabbit calicivirus)
 Reticuloendotheliosis virus¹⁶
 Rotavirus (bovine and porcine)
 Sendai virus
 Swine influenza virus
 Swine pox virus
 Swine vesicular disease virus
Taylorella equigenitalis
Theileria equi
 Theiler's murine encephalomyelitis virus
Treponema paraluis-cuniculi

¹⁴ The generalised culture method described in European Pharmacopoeia monograph 2.6.7 *Mycoplasmas* is not acceptable.

¹⁵ The generalised culture method described in European Pharmacopoeia monograph 2.6.7 *Mycoplasmas* is acceptable.

¹⁶ The method described in European Pharmacopoeia 2.6.24 is acceptable for culture of REV; however, specific testing is required as some isolates are non-cytolytic.

Turkey rhinotracheitis virus¹² (avian metapneumovirus, avian pneumovirus)
West Nile virus

The review concluded that the following Annex 1 and 3 extraneous agents cannot be reliably cultured or detected by primary culture using any method including general test methods in accordance with 9 CFR 113, therefore specific testing is required.

Bovine immunodeficiency virus
Bovine leukaemia virus
Caprine arthritis encephalitis virus
Ehrlichia canis
Equine infectious anaemia virus
Feline immunodeficiency virus
Feline leukaemia virus
Jaagsiekte sheep retrovirus (ovine pulmonary adenocarcinoma virus, pulmonary adenomatosis virus)
Neorickettsia risticii
*Ornithobacterium rhinotracheale*⁷
Trypanosoma evansi
Porcine reproductive and respiratory syndrome virus
Porcine respiratory coronavirus
Visna/maedi (maedi-visna) virus
Transmissible gastroenteritis virus

Master and working seeds, cell lines, substrates, and other materials of animal origin must be free from extraneous agents. There are no perfect test methods currently available. However, continuing to use test methods with identified limitations when there are more reliable and sensitive tests available, does not meet Australia's requirements for the importation of veterinary vaccines.

In some circumstances it may take veterinary vaccine manufacturers time to review and update test methods used to detect extraneous agents in imported veterinary vaccines. Importers of veterinary vaccines should consult with the Biological Imports Program to establish a reasonable timeframe to adopt and validate more reliable and sensitive tests where appropriate. Specific test methods approved for use in veterinary vaccines registered in Europe and the United States, and with a history of safe use in Australia will be taken into account.

Applications to import new veterinary vaccines should include validated test methods that are up to date. Specific test methods approved for use in veterinary vaccines registered in Europe and the United States will be taken into account.

Acronyms and abbreviations

µg	microgram
µl	microlitre
3201	IL-2-independent feline T4 lymphoma
4647	Green monkey kidney
9 CFR	<i>United States Code of Federal Regulations Title 9: Animals and Animal Products</i>
A549tTA	human lung carcinoma
A-72	canine adenocarcinoma
AA	<i>Aedes albopictus</i>
AA C3/36	clone of <i>Aedes albopictus</i> cell line
AAdV	avian adenovirus
AAdV-I	avian adenovirus group I
AAdV-II	avian adenovirus group II
AC-EIA	antigen capture enzyme immunoassay
AC-ELISA	antigen capture enzyme linked immunosorbent assay
AEV	avian encephalomyelitis virus
AGID	agar gel immunodiffusion
AGMK	African green monkey kidney
AGP	agar gel precipitation
AHSV	African horse sickness virus
AIV	avian influenza virus
AK-D	feline lung (ATCC no. CCL-150)
ALV	avian leukosis virus
AMOS	acronym for (<i>Brucella</i>) <i>abortus</i> , (<i>Brucella</i>) <i>melitensis</i> , (<i>Brucella</i>) <i>ovis</i> and (<i>Brucella</i>) <i>suis</i>
AMOS-ERY PCR	multi-locus AMOS PCR targeting the <i>ery</i> locus of <i>Brucella</i> spp.
AMV	avian myeloblastosis virus
ANV	avian nephritis virus
API	analytical profile index
AP-PCR	arbitrarily primed PCR
ASFV	African swine fever virus
ATCC	American Type Culture Collection
ATI	acidophilic-type inclusion body
B95a	marmoset B lymphoblastoid
BAdV	bovine adenovirus
BAE	bovine arterial endothelial cells
BCYE	buffered charcoal yeast extract agar
BDV	border disease virus
BEC	bovine endothelial cells
BEFV	bovine ephemeral fever virus
BEK	bovine embryonic/foetal/calf kidney
BEL	bovine embryonic lung
BETC	bovine embryonic tracheal cells
BFS	bovine foetal spleen
BGM/BGM-70	baby grivet monkey kidney/subclone of baby grivet monkey kidney
BHK/BHK-W12/BHK-21/BHK-89	baby hamster kidney
BH-RSV	<i>env</i> -defective Rous sarcoma virus

BHV-1	bovine herpesvirus 1 (infectious bovine rhinotracheitis virus)
BHV-2	bovine herpesvirus 2
BHV-4	bovine herpesvirus 4
BIV	bovine immunodeficiency virus
BK	primary calf kidney cells
BLF	buffalo lung fibroblast
BLGFP	CRFK cells transfected with BLV LTR
BLV	bovine leukaemia virus
BNM	bovine foetal nasal mucosa
BOMAC	bovine macrophage
BPI-3V	bovine parainfluenza virus 3
BPV	bovine parvovirus
BPW	buffered peptone water
BRSV	bovine respiratory syncytial virus
BSC-1	subclone of African green monkey kidney epithelial cell line
BT	bovine turbinate cells
BTR	bovine thymic
BTV	bluetongue virus
BVDV	bovine viral diarrhoea virus/bovine pestivirus
C/E	chickens/CEF with genotype susceptible to infection with exogenous avian leukosis virus and resistant to infection with endogenous avian leukosis virus
C/O	chickens/CEF with genotype susceptible to infection with endogenous and exogenous avian leukosis virus
C13	baby hamster kidney cell line transformed with SV40/SV28
CA	capsid protein
<i>ca</i>	genetic locus encoding capsid protein
CAdV	canine adenovirus
CAEV	caprine arthritis encephalitis virus
CAM	chorioallantoic membrane
CAV	chicken anaemia virus
CCC	continuous line of cat cells
CCL64-RCDV	mink lung cells persistently infected with racoon-origin CDV
CCT	canine malignant histiocytosis
cDNA	complementary DNA
CDV	canine distemper virus
CEF	chicken embryo fibroblast
CEK	chicken embryo kidney
CELi	chicken embryo liver
CER	chicken embryo-related (hybrid of CEF and BHK-21)
CFA	complement fixation assay
CFU	colony forming units
CH	chemiluminescent hybridization
CH-SAH	chicken hepatoma
cIBDV	classical infectious bursal disease virus
CK	canine kidney
CKC	chicken kidney cells
CM	carboxymethyl
CMK	cebus monkey kidney
COFAL	complement fixation assay for avian leukosis
COFAR	complement fixation procedure for assay of avian REV

COS	simian CV-1 cell line transformed with SV40
CPAE	calf pulmonary artery endothelial
cpBVDV	cytopathic BVDV
CPE	cytopathic effect
CPV	canine parvovirus
CRFK	Crandell feline kidney/ Crandell-Rees feline kidney
CRIB	a bovine diarrhoea virus-resistant clone of MDBK cells
crmB	cytokine response modifier B
CRT-2	calf kidney
CSFV	classical swine fever virus
CTC	primary bovine calf testicular cells
DBS-FR _h L-2	foetal rhesus monkey diploid lung
DC	dendritic cells
dCTP	d(eoxy)- + c(ytidine) + t(ri)p(hosphate)(One of the two pyrimidine nucleotides used to synthesize DNA)
DEAE-dextran	diethylaminoethyl-dextran
DEF	duck embryo fibroblast
DEK	duck embryo kidney
DEL	duck embryo liver
Detroit-6	human bone marrow carcinoma
DEV	duck enteritis virus
DF-K	dog kidney fibroblast
DH-82	transformed canine macrophage/monocyte
DI	defective interfering
DJRK	mutant rabbit kidney
DLS	dimer linkage structure (5' region of genome of retroviruses where genomic RNA dimerization occurs)
DNA	deoxyribonucleic acid
DOBV	Dobrava virus
DVHV	duck viral hepatitis virus 1
EAdV	equine adenovirus
EAdV-1	EAdV serotype 1
EAdV-2	EAdV serotype 2
EAV	equine arteritis virus
ECE	embryonated chicken eggs
EDC	equine dermal cells
<i>Eco</i> RI	Restriction endonuclease <i>Eco</i> RI
EDE	embryonated duck eggs
EEEV	eastern equine encephalitis virus
EFK	equine foetal kidney
EGFP	enhanced green fluorescent protein
EHDV	epizootic haemorrhagic disease virus
EHV	equid herpesvirus
EHV-1	equid herpesvirus 1
EHV-2	equid herpesvirus 2
EHV-3	equid herpesvirus 3
EHV-4	equid herpesvirus 4
EIA	enzyme immunoassay
EIAV	equine infectious anaemia virus
EID ₅₀	median egg infective dose
EIV	equine influenza virus

EK 269	equine kidney
ELD ₅₀	median embryo lethal dose
ELISA	enzyme linked immunosorbent assay
EM	electron microscopy
ENV	envelope protein
<i>env</i>	envelope gene
ERIC-PCR	enterobacterial repetitive intergenic consensus sequence PCR
ESK	swine embryo kidney
ETCC	equine transitional cell carcinoma
F	fusion (<i>F</i> when referring to the gene)
FA	immunofluorescence assay (fluorescent antibody test (FAT))
FBL	foetal bovine lung
FBS	foetal bovine serum
Fc3Tg	feline tongue (ATCC no. CCL-176)
FCoV	feline coronavirus
FCV	feline calicivirus
FE	feline embryo
FEF	feline embryonic fibroblast
FEK	foetal equine kidney
FeLV	feline leukaemia virus
FFA	focus forming assay
FFU	focus forming unit
fg	ficogram
FHV-1	felid herpesvirus 1 (feline rhinotracheitis virus)
FIPV	feline infectious peritonitis virus
FISH	fluorescence <i>in situ</i> hybridisation
FITC	fluorescein isothiocyanate
FIV	feline immunodeficiency virus
FLK	foetal lamb kidney
FMDV	foot-and-mouth disease virus
fnRT-PCR	fluorogenic nuclease real-time RT-PCR
FPV	feline panleukopaenia virus
FRET	fluorescence resonance energy transfer
FS-L3	porcine kidney epithelial
FTA	fluorescent treponema antigen
GAdV-1	goat adenovirus 1
GAG	retroviral polyprotein
<i>gag</i>	genetic locus encoding GAG polyprotein
<i>gB</i>	glycoprotein B
GBK	Georgia bovine kidney
<i>gC</i>	glycoprotein C
GC	goat cells
<i>gp</i>	glycoprotein
<i>gG</i>	glycoprotein G
<i>gH</i>	glycoprotein H
GSA	group specific antigen
<i>gX</i>	glycoprotein X
H	haemagglutinin
H&E	haematoxylin and eosin
HA	haemagglutination assay

HEK 293T	human embryonic kidney cells transformed with human adenovirus 5
HEL	human embryonic lung
HeLa	human cervical carcinoma
Hep-2	human laryngeal carcinoma
HEV	haemagglutinating encephalomyelitis virus
HI	haemagglutination inhibition
HmLu-1	hamster lung
HPV	horse pox virus
HRT-18	human rectal adenocarcinoma
HTNV	Hantaan virus
HVT	turkey herpesvirus
IBDV	infectious bursal disease virus
IB-RS-2	porcine kidney
IBV	infectious bronchitis virus
IC	intracranial
ICA	immunochromatography assay
IEM	immunolabelling electron microscopy
IFA	indirect immunofluorescence assay
IFFA-3	hamster embryo
IgG	immunoglobulin G
IgM	immunoglobulin M
IHA	immunohistochemical assay
ILTV	infectious laryngotracheitis virus
IMPACT	infectious microbe PCR amplification test
IN	intranuclear
IP	immunoperoxidase
IRES	internal ribosomal entry site
ISH	<i>in situ</i> hybridisation
IV	intravascular
J774	murine macrophage
JEV	Japanese encephalitis virus
Jinet	cynomolgus monkey kidney
JSRV	Jaagsiekte sheep retrovirus
KB	human nasopharyngeal carcinoma
kDa	kilo Dalton
KK	kitten kidney
KSE6	swine kidney epithelial
L	C3H mouse fibroblasts
L929	mouse fibroblast
LAMP	loop-mediated isothermal amplification
LAT	latency associated transcript
LCMV	lymphocytic choriomeningitis virus
LIV	louping ill virus
LK	lamb kidney
LLC-MK2	rhesus monkey kidney
LLC-RK1	rabbit kidney
LMH	chicken hepatocellular carcinoma
LPS	lipopolysaccharide
L-RNA	large viral RNA segment
LSA-1	feline T-lymphoblastoid

LSCC-RP9/LSCC-RP12	chicken B-lymphoblastoid cell lines transformed by Rous-associated virus 2
LSDV	lumpy skin disease virus
LT	lamb testis
LTR	long terminal repeat
LUX-PCR	light upon extension fluorogenic real-time PCR
M	molar (unit of concentration)
MA-104	foetal rhesus monkey kidney
<i>M</i>	Genetic locus encoding matrix protein
mAb	monoclonal antibody
MAP	mouse antibody production
MARC-145	monkey kidney
MAT	microscopic agglutination test
MDBK	Madin–Darby bovine kidney
MDBK-SY	bovine kidney cell line/subclone of MDBK
MDCC-CU147	Marek’s disease virus transformed chicken T lymphoblastoid
MDCC-MSB1	Marek’s disease virus transformed chicken T lymphoblastoid
MDCK	Madin–Darby canine kidney
MDCK-SP	subclone of MDCK
MDM	monocyte-derived macrophages
MDV	Marek’s disease virus
ME	primary Swiss mouse embryo
MGB	minor groove binding
MK	monkey kidney
ml	millilitre
ML	myeloblastic leukaemia
MM	multiple myeloma
<i>MmmSC</i>	<i>Mycoplasma mycoides</i> subsp. <i>mycoides</i> small colony (SC) type
M-MSV	Moloney-murine sarcoma virus
MOI	multiplicity of infection
MQ-NCSU	chicken macrophage
MRC-5	human secondary lung fibroblast (ATCC no. CCL-171)
M-RNA	medium viral RNA segment
MS	monkey spleen
MSC-PCR	assay for identification of the <i>Mycoplasma mycoides</i> small colony type from the mycoides cluster
MuAdV	murine adenovirus
MVV	visna/maedi (maedi–visna) virus
MYA-1	feline T-lymphoblastoid
MYC-PCR	PCR assay targeting the 16SrRNA sequence of <i>Mycoplasma mycoides</i> subsp. <i>mycoides</i> SC
N	nucleocapsid protein
<i>N</i>	nucleocapsid gene
NAT	nucleic acid amplification technique
ncpBVDV	non-cytopathic BVDV
NDV	Newcastle disease virus
Neuro-2a	murine neuroblastoma (ATCC no. CCL-131)
NIL-2	hamster embryo
NPA	Non-producer cell activation
NPT _r	newborn pig tracheal
NSK	newborn swine kidney

NSP	non-structural protein
<i>NSP</i>	genetic locus encoding non-structural protein
OA3.Ts	ovine testis
OAdV	ovine adenovirus
OFT	ovine foetal turbinate
OIE	World Organisation for Animal Health
OMK	owl monkey kidney
ORF	open reading frame
P	phosphoprotein
PAdV	porcine adenovirus
PAN-PCR	particle associated nucleic acid polymerase chain reaction
PBF	plasma blocking factor
PBL	peripheral blood lymphocytes
PBMC	peripheral blood mononucleocytes
PC4	physical containment level 4
PCR	polymerase chain reaction
PCR-DGGE	polymerase chain reaction denaturing gradient gel electrophoresis
PCR-RE	polymerase chain reaction restriction endonuclease digestion of PCR product
PCR-REA	polymerase chain reaction restriction enzyme analysis
PCR-RFLP	polymerase chain reaction restriction fragment length polymorphism
PCV-1	porcine circovirus 1
PCV-2	porcine circovirus 2
PEDV	porcine epidemic diarrhoea virus
PERT	PCR-based reverse transcriptase activity assay (product enhanced reverse transcriptase activity)
PEV	polioencephalomyelitis virus
PFU	plaque-forming unit
pg	picogram
PHA	phytohaemagglutinin
PK-15	porcine kidney
PLK	primary lamb keratinocytes
PMA	phenotypic mixing assay
<i>pol</i>	genetic locus encoding polymerase protein
PPLO	pleuropneumonia-like organism nutrient agar
PPRV	peste-des-petits-ruminants virus
PPV	porcine parvovirus
PRRSV	porcine reproductive and respiratory syndrome virus
PRV	pseudorabies virus
PS-Y15	clone of swine kidney
PUUV	Puumala virus
PVM	pneumonia virus of mice
QC-PCR	quantitative competitive polymerase chain reaction
qRT-PCR	quantitative reverse transcription polymerase chain reaction
QT-35	quail fibrosarcoma
R(-)Q	Japanese quail cell line transformed with envelope defective and high titre Rous sarcoma virus
RAPD	rapid amplified polymorphic DNA
RBC	red blood cell

r-BK	Razi bovine kidney
RCV	rabbit calicivirus
REP-PCR	repetitive extragenic palindromic element PCR
REV	reticuloendotheliosis virus
RFLP	restriction fragment length polymorphism
RIA	radioimmunoassay
RIF	resistance inducing factor
RK/RK-13	rabbit kidney
RNA	ribonucleic acid
RPR	rapid plasma regain card test
rRNA	ribosomal RNA
RSV	Rous sarcoma virus
RT	reverse transcriptase
RT-LAMP	RT loop-mediated isothermal amplification
RT-PCR	reverse transcription polymerase chain reaction
RT-PCR-RE	RT-PCR restriction endonuclease
RV	rotavirus
RVFV	Rift Valley fever virus
S phase	Interphase synthesis phase of cell cycle
SAT	serum agglutination test
SB	subclone of Chinese hamster fibroblast V79 cells
SC	selenite cystine
Seg-1	segment 1
SEK	primary ovine kidney
SEOV	Seoul virus
SERS	surface-enhanced Raman scattering
SeV	Sendai virus
SFV	Shope fibroma virus
SIRC	Statens Seruminstitut rabbit cornea
SIV	swine influenza virus
SJPL	St Jude porcine lung epithelial
SK	primary swine kidney
SK-15	swine kidney
SK-6	swine kidney
SK-K	swine kidney
SLAM	signalling lymphocyte activation molecule
SN	serum neutralization
SNP	single nucleotide polymorphism
SPC	sphingosylphosphorylcholine
SPF	specific pathogen free
SPV	swine pox virus
SQMC	squirrel monkey kidney, intestine and lung cells
SQMK	squirrel monkey kidney
s-RCV	smooth RCV
S-RNA	small viral RNA segment
ST	primary swine testicle
SV28	simian virus 28
SV40	simian virus 40
SVDV	swine vesicular disease virus
<i>Tax</i>	trans-activator x (x is undefined)
<i>Tax</i> -RE	<i>Tax</i> response element

TCID ₅₀	median tissue culture infective dose
TET	tetrathionate
TGA	tellurite glycine agar
TGEV	transmissible gastroenteritis virus
TK (<i>tk</i>)	thymidine kinase
TMA	tissue microarray technology
TMEV	Theiler's murine encephalomyelitis virus
TOC	tracheal organ cultures
TRV	turkey rhinotracheitis virus
UL	unique long
UTR	un-translated region
VDRL	Venereal Disease Research Laboratory
VEEV	Venezuelan equine encephalitis virus
Vero	African green monkey kidney
Vero F6	Vero cells transfected with the gene encoding herpes simplex virus-1 entry protein glycoprotein-H (gH)
Vero-DST	transgenic Vero
vIBDV	variant infectious bursal disease virus
VN	virus neutralisation
<i>VP</i>	viral protein gene
VSV	vesicular stomatitis virus
vvIBDV	very virulent infectious bursal disease virus
WEEV	western equine encephalitis virus
WI-38	human foetal lung (ATCC no. CCL-75)
WNV	West Nile virus
× g	times gravity

Introduction

Australia has a favourable animal health status. To maintain this favourable status Australia adopts a risk based approach to the management of animal biosecurity. This is particularly important in the case of veterinary vaccines because they may not be subjected to microbiologically lethal treatment during production and if contaminated, could bring about the widespread dissemination of serious pathogens and associated diseases or the emergence of new diseases through host adaptation and amplification of contaminating microorganisms. Imported vaccines present inherently high biosecurity risks due to the direct exposure of large numbers of live animals to these products. Historically, there are many documented examples of vaccination programs that have resulted in the introduction and establishment of new infectious agents into animal populations in a number of countries.

A decision by DAFF to permit imports depends upon a detailed and rigorous technical assessment of the raw materials, their processing and the testing of final product. The production of imported vaccines is strictly controlled and products are tested for adventitious agents (including pathogens of biosecurity concern) using reliable and sensitive methods. Extraneous agents are microorganisms that have been unintentionally introduced into the manufacturing process of a biological product that is used in the production of a vaccine. This includes bacteria, fungi, mycoplasmas, rickettsia, protozoa, parasites, transmissible spongiform encephalopathy (TSE) agents, and viruses. Due to various factors such as dilution and low levels of agent in the source material, contaminated veterinary vaccines are likely to have very low concentrations of the agent concerned. Therefore, the tests used to detect extraneous agents must be as sensitive as possible. For many pathogens the isolation efficiency in a particular culture system is not the same for low concentrations as opposed to high concentrations of the agent, and is dependent on the multiplicity of infection (MOI). Many culture systems that are suitable for diagnosis of active infection or growth of laboratory cultures may not be suitable for amplification of low level contaminants.

Australia's requirements for the importation of veterinary vaccines are contained in the *Australian Quarantine Policy and Requirements for the Importation of Live and Novel Veterinary Bulk and Finished Vaccines (1999)* and *Specific Quarantine Requirements for the Importation of Inactivated Veterinary Vaccines (1997)*. These documents include lists of animal pathogens (exotic, exotic strains and endemic) that DAFF may require country freedom from or testing for as part of the assessment process. There are three lists and these are referred to in the documents as Annexes 1–3.

Annex 1 includes the major exotic animal pathogens and all are OIE notifiable disease pathogens such as foot-and-mouth disease and classical swine fever. Annex 2 includes exotic animal TSEs such as bovine spongiform encephalopathy (BSE) and scrapie. Annex 3 includes other significant exotic animal pathogens, more virulent exotic strains of endemic animal pathogens and endemic animal pathogens that are common contaminants of veterinary vaccines. The animal biosecurity risks associated with exotic animal transmissible spongiform encephalopathies are managed through measures other than testing.

All applications to import veterinary vaccines are assessed on a case-by-case basis as no vaccine is the same. Most imported veterinary vaccines may meet European and/or United States requirements and undergo testing for various extraneous agents. However, Australia's requirements are in variance in many aspects to these due to Australia's different and favourable animal health status. This means that additional testing for extraneous agents of vaccine master seeds (bacteria, mycoplasma and viruses), master cell seeds and production materials derived from animal materials is generally required.

The veterinary vaccine requirements refer to testing specified in the European Pharmacopoeia or 9 CFR 113. The European Pharmacopoeia and 9 CFR 113 specify testing for the majority of potential contaminating extraneous agents by a generic protocol of primary isolation in a limited number of cell lines. This generic protocol is not sufficiently sensitive for the detection of most pathogens listed in Annex 1 and Annex 3. The following generalised problems exist:

- The limited number of cell lines specified for primary isolation of extraneous agents does not accommodate the requirements of each pathogen for the use of the most highly sensitive cell lines.
- The generalized culture protocol does not accommodate the optimal conditions for each pathogen with regards to culture times, number of passages, incubation temperatures, media and media supplement characteristics. For some pathogens the sensitivity of culture isolation is greatest with the use of specialised culture techniques; for example suspension cultures using micro-bead carriers or centrifugal inoculation techniques in microtitre plates. For other pathogens the mitotic state of the cells, confluency of the cell cultures, or passage age of the cells may significantly influence sensitivity of isolation.
- Detection by generic primary isolation in cell culture may not be sufficiently sensitive to detect the low levels of contaminating viruses that may be present in veterinary vaccines.

The European Pharmacopoeia general monograph *Vaccines for veterinary use* and 5.2.4 *Cell cultures for the production of veterinary vaccines* describe generalised methods for extraneous viral testing using cell culture monolayers that are sensitive to the virus being tested, and detection using specific testing methods that are sensitive to the viral agent. The monographs do not specify details of a culture system or assay for the viruses in Annex 1 and 3, simply that the methods should be sensitive. Australia's requirement for specific testing is consistent with the European Pharmacopoeia.

The European Pharmacopoeia monographs *Vaccines for veterinary use*, 2.6.12 *Microbiological examination of non-sterile products: microbial enumeration tests* and 2.6.13 *Microbiological examination of non-sterile products: test for specified microorganisms* describe generalised methods for microbiological testing and selective methods of testing for specified microorganisms.

9 CFR 113 contains standard requirements in relation to veterinary vaccines. This includes requirements in relation to extraneous agents testing.

There are numerous test methods available for specific testing of pathogens; however, they vary in their sensitivity and specificity, and they may not be fit for the intended purpose. A test method suitable for diagnosing a disease in a country where that disease is endemic may not be suitable for detecting low titres of exotic pathogens in a veterinary vaccine. There is considerable uncertainty as to what test methods are suitable for specific testing of veterinary vaccines for Annex 1 and 3 pathogens. However, from an Australian biosecurity perspective, test methods should be as sensitive as possible to reduce the chance of false negative results and contamination of imported veterinary vaccines with extraneous agents.

This review provides clarification on the acceptable tests for extraneous agents required to meet Australia's import policy for veterinary vaccines. The efficiency of assessments undertaken for the importation of veterinary vaccines will improve through the consolidation of the relevant scientific literature and assessment of the suitability of these test methods for the detection of extraneous agents. Australia's import requirements have not changed as a result of this review.

Some test methods may initially identify a contaminating agent to genus level and further tests are required to identify to species level. This review provides specific tests to detect and identify agents

to species level. It is recognised that it may not be necessary to proceed to species level when it is clear that there is contamination with an extraneous agent.

Retroviruses

Retroviruses represent a particular challenge in regard to regulation of vaccines in general and live vaccines in particular because the contamination risk is not reduced through inactivation of the vaccine. The challenges for veterinary vaccines are twofold. Annex 1 and 3 includes a number of exogenous¹⁷ retroviruses that require specific testing under Australia's veterinary vaccines requirements. Live and inactivated veterinary vaccines may also be contaminated with endogenous¹⁸ retroviruses and these may not readily be detected by routine extraneous agent testing.

Endogenous retroviruses are generally not pathogenic in their original hosts; however, some can induce disease. For example, endogenous retroviruses from AKR strains of mice induce lymphoma in their hosts. Certain endogenous retroviruses infect new hosts and induce diseases; there has been an instance in which an endogenous retrovirus from Asian rodents infected Gibbon apes and induced lymphoma (Nowinski and Hays 1978). The koala retrovirus induces neoplastic diseases and immune suppression in the new host (Tarlington et al. 2008).

Mice, pigs, cats, primates and chickens are known to have infectious endogenous retroviruses and cell lines from these animals are used to manufacture live attenuated and inactivated veterinary vaccines. Several live attenuated vaccines are produced using cells which are known to produce infectious endogenous retroviruses; however, the risks of infection by endogenous retroviruses from xenospecies have not been addressed as a safety issue by vaccine manufacturers and regulatory bodies.

In April 2010, the European Medicines Agency's (EMA) Committee for Medicinal Products for Veterinary Use (CVMP) reviewed (EMA:CVMP 2010) the presence of feline endogenous retrovirus RD114 in some live attenuated vaccines commercially available in Europe for use in animals. This was a result of the publication of an article (Miyazawa et al. 2010) on the detection of feline replication-competent endogenous retrovirus RD114 in some live attenuated cat and dog vaccines commercially available in Europe (United Kingdom) and in Japan.

It was not considered acceptable to have vaccine batches on the market containing unwanted live virus particles, without trying to investigate and correct this issue.

Regulatory bodies require assessment of retroviral status as part of the virological assessment of vaccine and biopharmaceutical products for administration to humans. Retroviral contamination is a general safety concern because the capacity of retroviruses for random integration of retroviral DNA into the host genome means retroviruses have inherent mutagenic potential. The international biopharmaceutical standard for retroviral detection is reverse transcriptase (RT) activity assay, as all retroviruses contain RT enzyme within the virion and encode RT in the viral genome. Recently the European regulatory body, Agence Française de Sécurité Sanitaire des Produits de Santé has published evidence that the PCR-based RT activity assay or product enhanced RT activity (PERT) assay has one million fold greater sensitivity for detection of retroviruses in vaccines than conventional RT assays in use in the Europe and the United States (André et al. 2000). The

¹⁷ Exogenous retroviruses are transmitted horizontally by infection and they infect somatic cells but not germ line cells.

¹⁸ Endogenous retroviruses are retroviruses that have been integrated into germ line cells and are inherited by offspring from parents. Endogenous retroviruses may produce infectious virions and replication may be dependent on the biological environment.

application of this methodology has identified retroviral contamination in a large number of final vaccine batches produced from both avian and mammalian cell lines. The assessment of veterinary vaccines for use in Australia currently does not require assessment of retroviral status.

The Review of Published Tests to Detect Pathogens in Veterinary Vaccines Intended for Importation into Australia undertaken by Animal Biosecurity Branch covers the detection of exogenous retroviruses listed in Annex 1 and 3. The review has identified a PERT assay that should be used as the initial diagnostic assay (or general test) for retrovirus detection in conjunction with PCR for detection of proviral DNA. Attempted isolation, confirmation and identification of retrovirus positives is then required by culture isolation, animal isolation or sequencing of PCR products.

Animal Biosecurity Branch has considered the implications associated with getting a positive result for 'retrovirus' when using the PERT assay as a general test. Until there is a review on the biosecurity risks associated with endogenous retroviruses, advice to the Biological Imports Program will be that a positive result as described should not result in rejection of a veterinary vaccine import application provided specific tests are conducted to identify the retrovirus that has been detected.

Scope

This review is intended to provide guidance to DAFF and veterinary vaccine importers on suitable test methods for the reliable detection of extraneous agents in imported veterinary vaccines as listed in Annex 1 and 3. This includes the suitability of the relevant general test methods for the detection of extraneous agents described in 9 CFR 113 and the European Pharmacopoeia.

The test methods reviewed are limited to those published in peer reviewed journals and other reputable sources and do not take into account whether the test method is validated in accordance with Australia's requirements for the importation of veterinary vaccines.

Some test methods remain commercial-in-confidence and are therefore unpublished. These will continue to be assessed on a case-by-case basis in accordance with Australia's requirements for the importation of veterinary vaccines contained in the *Australian Quarantine Policy and Requirements for the Importation of Live and Novel Veterinary Bulk and Finished Vaccines (1999)* and *Specific Quarantine Requirements for the Importation of Inactivated Veterinary Vaccines (1997)*.

Commonwealth Scientific and Industrial Research Organisation's (CSIRO) Australian Animal Health Laboratory (AAHL) is a national facility whose major role is to diagnose emergency animal disease outbreaks. AAHL plays a vital role in maintaining Australia's capability to quickly diagnose exotic and emerging animal diseases. This is achieved through ongoing research programs to develop the most sensitive, accurate and timely diagnostic tests, which are critical to the success of any eradication campaign in the event of a disease outbreak. AAHL also undertakes research to develop new diagnostic tests, vaccines and treatments for both exotic and endemic animal diseases of national importance.

This review does not include the test methods used by AAHL to test veterinary vaccines for extraneous agents. AAHL's validated test methods are assessed by the Biological Imports Program as suitable for the reliable detection of extraneous agents in imported veterinary vaccines as listed in Annex 1 and 3. AAHL's test methods will be reassessed by the Biological Imports Program whenever test methods are updated.

Current requirements

Australia's requirements for the importation of veterinary vaccines are contained in the *Australian Quarantine Policy and Requirements for the Importation of Live and Novel Veterinary Bulk and Finished Vaccines (1999)* and *Specific Quarantine Requirements for the Importation of Inactivated Veterinary Vaccines (1997)*.

Imported veterinary vaccines are assessed to ensure that master and working seeds, cell lines, substrates, and other materials of animal origin are free from extraneous agents. The Biological Imports Program bases its assessment on factors such as the country of origin, processing, treatment and testing of the vaccine for extraneous agents listed in Annex 1 and 3.

Annex 1 is a list of pathogens exotic to Australia which pose such a major economic and social threat that sourcing of potentially contaminated products from affected countries (or OIE defined regions) will not be considered unless the product is effectively sterilised.

Annex 3 is a list of other animal diseases which are either exotic pathogens other than those in Annex 1 or exotic strains of an endemic pathogen or are potential contaminants of economic or social concern to Australia. During assessment, the Biological Imports Program may also identify other potential contaminants of concern.

All raw materials of animal origin used in the production of vaccines to be imported into Australia must be free of extraneous agents. They must be tested for bacteria, fungi and mycoplasma using sensitive and accurate techniques. Unless effectively sterilised, they must also be tested for the pathogens listed in Annexes 1 and 3 as appropriate to the species of origin using validated test methods.

Review of Annex 1 and 3 pathogens

Viruses — mammalian

African horse sickness virus

Family *Reoviridae*, genus *Orbivirus*

Primary isolation of African horse sickness virus (AHSV) is recommended in primary calf kidney (BK), marmoset B lymphoblastoid (B95a cells), BHK-21 or African green monkey kidney (Vero) cells. The test sample should be inoculated onto the cell monolayers in roller bottles in a small volume and incubated for 1 hour to allow adsorption of virus prior to the addition of maintenance medium. Inoculated cultures should be incubated for 8–10 days between passages and maintained for at least 4 passages. AHSV is readily detected by cytopathic effect (CPE) in infected cultures within 2–8 days (Laviada et al. 1992; Laviada et al. 1993; OIE 2010).

Specific testing for AHSV is not required as the virus is readily detected by CPE following culture isolation.

The generalised culture methods of 9 CFR 113.52¹⁹, 113.53²⁰ and 113.55²¹ will not meet the growth requirements of AHSV and will not be sufficient for the primary isolation of AHSV. The cell lines recommended for the primary isolation of AHSV are consistent with the 9 CFR guidelines. However, the specific growth requirements of AHSV will not be achieved using the 9 CFR protocol, as the timing and number of passages of infected cultures are suboptimal.

The European Pharmacopoeia general monograph *Vaccines for veterinary use* and 5.2.4 *Cell cultures for the production of veterinary vaccines* describe generalised methods for extraneous agent testing using cell culture monolayers that are sensitive to the agent being tested, and detection using specific testing methods that are sensitive to the viral agent. The monographs do not specify details of a culture system or assay for AHSV detection, simply that the methods should be sensitive.

Conclusion

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 are not acceptable.

Primary isolation of AHSV in BK, BHK-21, B95a or Vero cell lines is recommended. Cultures require 1 hour adsorption incubation, incubation for 8–10 days between passages and maintenance for at least 4 passages.

Specific testing is not required for AHSV.

African swine fever virus

Family *Asfarviridae*, genus *Asfivirus*

¹⁹ Requirements for cell lines used for production of biologics.

²⁰ Requirements for ingredients of animal origin used for production of biologics.

²¹ Detection of extraneous agents in Master Seed Virus.

Primary isolation of African swine fever virus (ASFV) is in primary porcine leukocyte or porcine bone marrow cultures, newborn swine kidney (NSK) or newborn pig tracheal (NPTr) cell lines. NSK and NPTr cells are highly sensitive for primary isolation of ASFV (Ferrari et al. 2003). Inoculated cultures should be maintained for 7–10 days between passages, and at least 3 passages are required. ASFV can be detected by CPE at 7–10 days after infection; however, confirmation of CPE by haemagglutination assay (HA) or immunofluorescence assay (FA) is required for reliable detection. A proportion of ASFV strains are non-haemadsorbing and these strains are more difficult to detect by CPE. Non-haemadsorbing strains with inapparent CPE can be detected by ASFV-specific polymerase chain reaction (PCR) (OIE 2010; Thomson et al. 1979).

Specific testing is required for ASFV because detection of culture isolated virus by CPE is problematic. Specific testing for ASFV is by HA, FA or PCR. Only PCR is sufficiently sensitive to detect all isolates of ASFV. Specific testing is required on the final passage together with earlier (first or second passage) samples.

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 will not meet the specific growth requirements of ASFV and will not be sufficient for the primary isolation of ASFV. The generalised protocols do not meet the requirements of ASFV for susceptible cell lines, the number of culture passages or length of culture incubations. The 9 CFR 113.34: *Detection of hemagglutinating viruses* will detect some but not all ASFV contaminating isolates. The 9 CFR guidelines do not include specific testing for ASFV.

The European Pharmacopoeia general monograph *Vaccines for veterinary use* and 5.2.4 *Cell cultures for the production of veterinary vaccines* describe generalised methods for extraneous agent testing using cell culture monolayers that are sensitive to the agent being tested, and detection using specific testing methods that are sensitive to the viral agent. The monographs do not specify details of a culture system or assay for ASFV detection, simply that the methods should be sensitive.

Conclusion

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 are not acceptable.

Primary isolation of ASFV in primary porcine leukocyte or porcine bone marrow cultures, NSK or NPTr cell lines is recommended. Cultures should be maintained for 7–10 days between passages, and at least 3 passages are required.

Specific testing is required for ASFV on the final and one earlier (first or second passage) culture samples by FA and PCR.

Akabane virus

Family *Bunyaviridae*, genus *Orthobunyavirus*

Akabane virus infects a wide spectrum of susceptible cell lines. Primary isolation is recommended in hamster lung cells (HmLu-1) or by intracranial (IC) inoculation of suckling mice (1–2 days old) (Cybinski and Muller 1990; Eto et al. 1991; Kurogi et al. 1976). HmLu-1 cells are equally as permissive as IC inoculation of suckling mice for Akabane virus isolation and the virus grows to approximately 1.5 log higher titres than in Vero cell culture (Kurogi et al. 1976). Akabane virus can be isolated in *Aedes albopictus* (AA) cell cultures; however, growth in AA cells is very dependent on optimizing incubation temperatures. Baby hamster kidney-W12 (BHK-W12), porcine kidney

(PK-15), bovine embryonic kidney (BEK), bovine thymic (BTR) and rabbit kidney (RK-13) cell lines are reported to have low sensitivity to infection with Akabane virus. However, there is 1 report of successful isolation of some strains of Akabane virus in baby hamster kidney-21 (BHK-21) cell line that could not be isolated in AA cells or by IC inoculation of suckling mice (Cybinski and Muller 1990).

Some field strains are reported to have poor growth in culture prior to culture adaptation (Cybinski and Muller 1990). Culture adaptation is achieved by a 2 step isolation procedure in which there is either initial IC inoculation of suckling mice or inoculation of AA cells, followed by culture isolation in a susceptible detector cell line such as HmLu-1, BHK-W12, PK-15, BEK, BTR and RK-13 in which CPE is evident. This method increases the sensitivity of detection for some field strains of Akabane virus.

Serum used to supplement the maintenance medium must be shown to be free of specific antibody against Akabane virus that may neutralize virus infectivity.

The inoculum should be adsorbed onto the monolayer in a minimal volume for a 2 hour period prior to addition of the maintenance medium in order to ensure adequate virus attachment and entry. Akabane virus is rapidly cytolytic in cell culture: CPE extends to 30% of a cell monolayer at 24 hours, 50% at 48 hours and 100% by 72 hours. Peak viral titres occur at 48 hours and fall by 72 hours due to virus instability in acidic medium at 37 °C. Provision should therefore be made in the protocol for passaging of cultures no later than 48 hours after primary inoculation to avoid any reduction in viral titre (Hoffmann and St George 1985).

Combination of general culture isolation with specific testing is recommended in order to increase the sensitivity of detection and increase the likelihood of detection given unpredictable inhibition of virus growth in culture.

Reverse transcription polymerase chain reaction (RT-PCR) for the S gene of Akabane virus has wide conservation across all known strains and is very sensitive with a detection limit of $10^{1.0}$ median tissue culture infective doses (TCID₅₀) per ml. RT-PCR combined with primary isolation is therefore an optimal method for extraneous agent testing for Akabane virus (Ohashi et al. 2004).

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 for extraneous agent testing are not adequate without the modification to the culture conditions as described above. The 9 CFR guidelines do not require specific testing for Akabane virus.

The European Pharmacopoeia general monograph *Vaccines for veterinary use* and 5.2.4 *Cell cultures for the production of veterinary vaccines* describe generalised methods for extraneous agent testing using cell culture monolayers that are sensitive to the agent being tested, and detection using specific testing methods that are sensitive to the viral agent. The monographs do not specify details of a culture system or assay for Akabane virus detection, simply that the methods should be sensitive.

Conclusion

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 are not acceptable.

Primary isolation by a 2-stage process is recommended. The first stage of the isolation is by IC inoculation of suckling mice or inoculation of AA cells, and the second stage is in a detector cell line such as HmLu-1, BHK-W12, PK-15, BEK, BTR or RK-13. The generalised culture protocol

should be modified to include incubation for 2 hours to allow adsorption, and cultures should be passaged no later than 48 hours after inoculation.

Specific testing is required by RT-PCR targeting the S gene.

Bluetongue virus

Family *Reoviridae*, genus *Orbivirus*

Primary isolation of bluetongue virus (BTV) is possible in BHK-21, Vero, calf pulmonary artery endothelial (CPAE), mouse fibroblast (L929) and clone of *Aedes albopictus* (AA C3/36) cell lines. BHK-21 and CPAE cells are highly susceptible to infection with both cell culture adapted and field BTV strains (McLaughlin et al. 2003; OIE 2010).

Primary isolation of BTV is problematic, especially for field strains that are not culture adapted (Clavijo et al. 2000). Culture adaptation can be achieved by initial inoculation of embryonated chicken eggs (ECE), followed by passaging of ECE tissue homogenates in AA cell culture, and then inoculation of susceptible cell lines. ECE should be inoculated by the intravascular (IV) route as it is 1000 times more sensitive than yolk sac inoculation. Organs collected from the inoculated ECE are the heart, kidney, brain and liver. Pooled organs are homogenised, clarified and resuspended as a 10% solution for inoculation of cell culture. Arboviruses typically grow to higher titres in AA cell culture but do not produce CPE and an indicator system is required. Therefore 2 serial passages in mammalian cell culture are required.

Virus growth in cell culture can be inhibited by blood or serum components either introduced in the inoculum or in serum supplemented culture medium. BTV becomes sequestered within pockets of erythrocytes. Sequestered virus in primary inoculum containing blood components must be freed by sonication prior to inoculation. Virus infectivity is known to be neutralized by serum antibodies in serum supplemented culture medium. Inhibition of virus growth is avoided by using serum free medium.

The adsorption phase is critical for BTV infection of the monolayer. One hour adsorption is required in a minimal volume (depending on the surface area of the monolayer used) with rocking to ensure continuous movement of inoculum over the monolayer.

BTV is readily detected by CPE, evident as cytolysis and plaque formation 2–5 days after culture inoculation (Housawi et al. 2004).

RT-PCR amplification of the ribonucleic acid (RNA) genome segments 5 or 7 (highly conserved in all 24 serotypes) is widely used to identify BTV and is highly sensitive and specific (Anthony et al. 2007; Aradaib et al. 1998a). Specific BTV RT-PCR combined with cell culture isolation would provide optimal sensitivity for detection of infectious, replicating BTV. A number of alternative immunological and virus neutralizing assays have been developed for BTV antigen detection.

A Taqman real-time RT-PCR has been developed targeting BTV segment 1 (Shaw et al. 2007). The flanking primers are specific for sequence conserved in an alignment of 132 BTV isolates representing a wide geographic range and including all 24 serotypes. Two fluorogenic probes are used to detect isolates either of western or eastern origin. The assay sensitivity and specificity is high and repeatability was good for all isolates except for 2 African BTV-9 isolates for which inconsistent results were obtained.

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 for extraneous agent testing will not be sufficient for primary isolation of BTV. The 9 CFR 113.47: *Detection of extraneous viruses by the fluorescent antibody technique* stipulates FA testing for BTV in infected monolayers. The sensitivity of the FA test for BTV is not sufficient for extraneous agent testing.

The European Pharmacopoeia general monograph *Vaccines for veterinary use* and 5.2.4 *Cell cultures for the production of veterinary vaccines* describe generalised methods for extraneous agent testing using cell culture monolayers that are sensitive to the agent being tested, and detection using specific testing methods that are sensitive to the viral agent. The monographs do not specify details of a culture system or assay for BTV detection, simply that the methods should be sensitive.

Conclusion

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 are not acceptable.

FA testing for BTV as described in 9 CFR 113.47: *Detection of extraneous viruses by the fluorescent antibody technique* is not sufficient for detection of BTV.

A 3 stage primary isolation for BTV is recommended. The initial stage is isolation in ECE, followed by inoculation of AA culture, and then 2 passages in detector cell lines. Suitable detector cell lines are HmLu-1, BHK-21 or CPAE. Modifications required to a generalised culture protocol are sonication of the inoculum, use of serum free medium and adsorption incubation.

Specific testing is recommended by either RT-PCR amplification of the RNA genome segment 5 or 7 or Taqman real-time RT-PCR targeting BTV segment 1 (Seg-1). Two fluorogenic probes are used to detect isolates of western or eastern origin respectively. Molecular testing by either RT-PCR or Taqman real-time RT-PCR is required both prior to inoculation of ECE and at the final culture passage.

Border disease virus

Family *Flaviviridae*, genus *Pestivirus*

For border disease virus (BDV) refer to entry for bovine viral diarrhoea virus 1 & 2 (bovine pestiviruses).

Bovine adenovirus

Family *Adenoviridae*, genus *Mastadenovirus* and *Atadenovirus*

Bovine adenovirus (BAdV) comprises 2 subgroups; subgroup 1 encompasses conventional BAdV of the genus *Mastadenovirus* and includes serotypes 1, 2, 3 and 9; and subgroup 2 encompasses non-conventional BAdV classified together with ovine adenovirus (OAdV) isolate 7 in the genus *Atadenovirus*, and includes serotypes 4, 5, 6, 7, 8 and 10 (Adair 1979; Adair et al. 1983; Horner et al. 1989).

BAdVs of subgroup 1 are readily isolated on first passage in Vero cells, peripheral blood lymphocytes (PBL), BEK, primary bovine calf testicular cells (CTC) and bovine turbinate cells (BT). Virus growth is evident by CPE in culture, which consists of cellular rounding and clumping and refractile intracellular inclusions. Detection is confirmed by FA testing for the common group antigen (Cole 1970).

BAdVs of subgroup 2 are slow growing, do not replicate in BEK cells and can only be isolated after 3–10 passages in CTC. CPE is indistinct and covers a maximum of 50 % of the infected monolayer (Horner et al. 1989; Smyth et al. 1999).

Subgroup 2 BAdVs do not share cross-reactive group antigens with other mammalian adenoviruses of the genus *Mastadenovirus*. Conventional FA will not identify subgroup 2 BAdV and FA using specific antiserum to subgroup 2 is required.

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 for extraneous agent testing will not be sufficient for primary isolation of subgroup 2 adenoviruses. The guideline 9 CFR 113.47: *Detection of extraneous viruses by the fluorescent antibody technique* describing the FA on infected cell monolayers is sufficient for conventional subgroup 1 BAdV detection. The FA is not sufficient for detection of subgroup 2 BAdV.

The European Pharmacopoeia general monograph *Vaccines for veterinary use* and 5.2.4 *Cell cultures for the production of veterinary vaccines* describe generalised methods for extraneous agent testing using cell culture monolayers that are sensitive to the agent being tested, and detection using specific testing methods that are sensitive to the viral agent. The monographs do not specify details of a culture system or assay for BAdV detection, simply that the methods should be sensitive.

Conclusion

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 are not acceptable.

Primary isolation of BAdV in CTC is recommended. Modifications to the general culture conditions should be the inclusion of 3–10 culture passages.

FA for BAdV as described in 9 CFR 113.47: *Detection of extraneous viruses by the fluorescent antibody technique* is sufficient for conventional subgroup 1 BAdV detection but is not sufficient for detection of subgroup 2 BAdV.

Specific testing is required by FA for the common group antigen and also using antiserum specific to subgroup 2 BAdV.

Bovine ephemeral fever virus

Family *Rhabdoviridae*, genus *Ephemerovirus*

Bovine ephemeral fever virus (BEFV) can be isolated in BHK-21, primary calf kidney cells (BK), HmLu-1, Vero, monkey spleen (MS), BEK and AA cell lines. Susceptible primary cell cultures are: calf kidney, lung, spleen, and testicle; bovine kidney, testis and synovial cells; and hamster lung. BEFV can be isolated by IC inoculation of suckling mice or inoculation of ECE (Elamin and Spradbrow 1978; Gard et al. 1988; Hoffmann and St George 1985; Matumoto et al. 1970; Murphy et al. 1972; Sato et al. 1975; Tzipori 1975a; Tzipori 1975b).

Primary isolation of BEFV in culture gives unreliable results and is variable both within and between viral strains. Improved detection can be achieved by either initial inoculation of AA cells, IC inoculation of suckling mice, or IV inoculation of ECE, followed by at least 2 passages in susceptible cell lines.

Cell cultures should be incubated for at least 7 days between passages then passaged and maintained a further 7–10 days. CPE is apparent 4–7 days after inoculation of the culture. Neurotropic strains of BEFV produce highly variable plaques that can be difficult to detect. Specific testing is required because primary isolation is unreliable. FA is recommended using rabbit antiserum against group specific antigen.

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 for extraneous agent testing will not be sufficient for primary isolation of BEFV. The 9 CFR guidelines do not include an FA for BEFV.

The European Pharmacopoeia general monograph *Vaccines for veterinary use* and 5.2.4 *Cell cultures for the production of veterinary vaccines* describe generalised methods for extraneous agent testing using cell culture monolayers that are sensitive to the agent being tested, and detection using specific testing methods that are sensitive to the viral agent. The monographs do not specify details of a culture system or assay for BEFV detection, simply that the methods should be sensitive.

Conclusion

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 are not acceptable.

A 2-stage primary isolation of BEFV is recommended. The first stage of isolation is by either initial inoculation of AA cells, IC inoculation of suckling mice, or IV inoculation of ECE, followed by at least 2 passages in susceptible cell lines (BHK-21, BK, HmLu-1, Vero, MS, or BEK). Cell cultures should be maintained for at least 7 days then passaged and maintained a further 7–10 days.

Specific testing is required by FA using rabbit antiserum against group specific antigen.

Bovine herpesvirus 1

Family *Herpesviridae*, genus *Varicellovirus*

Bovine herpesvirus 1 (BHV-1) is also known as infectious bovine rhinotracheitis virus. Primary isolation of BHV-1 is possible in primary or secondary cultures of BEK, bovine embryonic lung (BEL), bovine oviduct, BT, CTC, bovine embryonic tracheal cells (BETC), murine oviduct, and cell lines such as BEK, Madin–Darby bovine kidney (MDBK) and Vero. In bovine origin cells CPE is typically evident at 2–7 days after inoculation onto culture. Cowdry intranuclear inclusions and syncytia formation are observed in infected cultures. Infected cells should be incubated for 14 days and undergo 2 passages to ensure detection. For the second passage the cell culture is freeze–thawed and clarified and the supernatant is inoculated onto fresh monolayers. Cell lines recommended by the OIE are primary or secondary BEK, BEL or CTC, or cell lines MDBK and BEK. CPE in primary BEK or BT cells is more readily identified than in infected BEK or MDBK cell lines (Crandell et al. 1978; Forman et al. 1982; Hall and Minocha 1977; Peterson and Goyal 1988).

Cell culture isolation of BHV-1 is in general unreliable and there are a number of factors influencing the efficacy of isolation (Jones 2003; Jones et al. 2006; Jones and Chowdhury 2007).

At low levels of BHV-1 in the test material, low yields of virus are produced, CPE is undetectable and latent infection is accompanied by recovery of cell culture growth and phenotype. A study of

BHV-1 replication in BEK cells found that productive infection occurred for $\text{MOI} \geq 1.0$, and latent, non-cytopathic infection occurred for $\text{MOI} \leq 0.5$ (Michalski and Hsiung 1976).

Induction of interferon in the culture system will inhibit BHV-1 infection and prevent the development of CPE. Interferon induction may be induced by vaccine viral antigens in test materials, or may occur due to low levels of virus infection (Peek et al. 2004).

Primary cell cultures from calves or embryos with latent BHV-1 infection when used for extraneous agent testing will be resistant to cytolitic infection with BHV-1.

An incubation to allow virus adsorption is recommended for 1 hour. Corticosterone treatment of BT cell cultures at a final concentration of 10^{-8} M increases BHV-1 viral titres from infected cells by 10–12 times. Serum supplements added to the growth medium should be free of anti-BHV-1 antibodies. Primary cells grown for more than 1 week have been shown to have reduced sensitivity for BHV-1 and the titres recovered are reduced by 90%.

Specific testing methods are recommended in addition to general culture detection due to the low sensitivity of detection by primary isolation (there are many examples where infectious virus has not been detected by primary isolation). Specific detection methods are virus neutralisation (VN), FA, PCR or real-time PCR (Terpstra 1979).

PCR methods have far greater sensitivity than other methods but specificity can be problematic. A PCR has been developed against the unique long (UL) region of BHV-1 that can detect as little as 3-5 deoxyribonucleic acid (DNA) copies/50 μ l (van Engelenburg et al. 1993). Another PCR has been developed targeting the thymidine kinase (*tk*) gene of BHV-1 that can detect 1 TCID₅₀/50 μ l. A light upon extension fluorogenic real-time PCR (LUX-PCR) method has been developed that overcomes some of the problems with specificity; however, this method has not as yet been standardised sufficiently for recommendation. The LUX-PCR can detect 0.04 TCID₅₀ BHV-1 in cell culture and is 3 logs more sensitive than the PCR (Chen et al. 2007; Moore et al. 2000).

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 for extraneous agent testing do not provide the optimal growth conditions for BHV-1 and will not be sufficient for primary isolation of BHV-1. The 9 CFR protocols do not require specific testing for BHV-1.

The European Pharmacopoeia general monograph *Vaccines for veterinary use* and 5.2.4 *Cell cultures for the production of veterinary vaccines* describe generalised methods for extraneous agent testing using cell culture monolayers that are sensitive to the agent being tested, and detection using specific testing methods that are sensitive to the viral agent. The monographs do not specify details of a culture system or assay for BHV-1 detection, simply that the methods should be sensitive.

Conclusion

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 are not acceptable.

Primary isolation of BHV-1 in primary or secondary cultures of BEK, BEL, BT, or CTC is recommended. Test material should be inoculated onto fresh monolayers, adsorption incubation for 1 hour is required, cultures should be maintained for 14 days and undergo 3 passages. Serum supplements in the medium must be free of BHV-1 reactivity.

Specific testing for BHV-1 is required by PCR.

Bovine herpesvirus 2

Family *Herpesviridae*, genus *Simplexvirus*

Bovine herpesvirus 2 (BHV-2) can be isolated in the following susceptible cell lines: Vero, BK, CTC, foetal skin and organ cultures of bovine teat skin. Bovine foetal skin and CTC are the most sensitive cell culture systems for isolation of BHV-2.

BHV-2 is cytolytic at 12–48 hours with peak virus found 36 hours after inoculation. CPE is evident as the formation of giant syncytial cells and intranuclear inclusions (Sterz and Ludwig 1972).

Incubation temperature of infected cultures has a profound effect on BHV-2 growth. BHV-2 should be isolated at 30–32 °C, not 35–37 °C as is standard for extraneous agent testing (Letchworth, III et al. 1982; Letchworth and Carmichael 1982; Letchworth and Carmichael 1984).

Serum used to supplement the maintenance medium must be shown to be free of specific antibody against BHV-2 that may neutralize virus infectivity.

The inoculum should be adsorbed onto the monolayer in a minimal volume for a 2 hour period prior to addition of the maintenance medium in order to ensure adequate virus attachment and entry.

In vitro growth of BHV-2 is highly sensitive to the relative concentrations of amino acids arginine, histidine and lysine. Depletion of arginine or histidine in the culture medium (as might occur with infrequent replacement of the medium) will result in cessation of BHV-2 growth; whereas supplementation with lysine will result in inhibition of BHV-2 (supplementation of growth medium with lysine is a common practice in generalized cell culture protocols).

Low MOI in cell cultures (probable scenario with extraneous agent testing) or incubation at temperatures above 35 °C can result in persistent, non-lytic infection with latent virus and associated growth transformation of the host cells. Latent BHV-2 infection will not be detectable by CPE and requires molecular diagnostic techniques (Russell et al. 1987).

Specific testing by a shuttle PCR that amplifies the glycoprotein B (gB) gene has been shown to be of equivalent sensitivity to nested PCRs and to be more sensitive than the *tk* PCR (De-Giuli et al. 2002; Imai et al. 2002). Cell culture followed by PCR will increase the sensitivity of detection and increase the likelihood of detecting low-level, persistent *in vitro* infections.

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 for extraneous agent testing do not meet the growth requirements of BHV-2 and will not be sufficient for primary isolation of BHV-2. The 9 CFR guidelines do not include specific testing for BHV-2.

The European Pharmacopoeia general monograph *Vaccines for veterinary use* and 5.2.4 *Cell cultures for the production of veterinary vaccines* describe generalised methods for extraneous agent testing using cell culture monolayers that are sensitive to the agent being tested, and detection using specific testing methods that are sensitive to the viral agent. The monographs do not specify details of a culture system or assay for BHV-2 detection, simply that the methods should be sensitive.

Conclusion

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 are not acceptable.

Primary isolation of BHV-2 in CTC or bovine foetal skin cells is recommended. Culture conditions should be modified to incubation at 30–31 °C, 2 hours adsorption and serum used to supplement the cell culture medium must be shown to be free of anti-BHV-2 neutralizing antibody.

Specific testing is required by PCR targeting the gB gene.

Bovine herpesvirus 4

Family *Herpesviridae*, genus *Rhadinovirus*

Bovine herpesvirus 4 (BHV-4) can be isolated in a wide spectrum of cell cultures (Egyed 1998). Primary cell cultures of cattle, sheep, goats, dogs, cats, rabbits, pigs, and primary chicken kidneys are susceptible to BHV-4 infection. Susceptible cell lines include mink lung, ferret kidney, Crandell feline kidney (CRFK), owl monkey kidney (OMK), squirrel monkey kidney (SQMK), squirrel monkey kidney, intestine and lung (SQMC), cebus monkey kidney (CMK), Vero, bovine macrophage cells (BOMAC), MDBK, RK, human foetal lung (WI-38 (ATCC no. CCL-75)), human secondary lung fibroblast (MRC-5 (ATCC no. CCL-171)), human giant-cell glioblastoma and goat (GC).

The MDBK, BEK, bovine foetal spleen (BFS) cells and Georgia bovine kidney (GBK) cells are the most commonly used culture systems for propagation of BHV-4. In one study to determine the relative infectivity of different cell lines BHV-4 growth was highest in MDBK, primary glioblastoma giant-cells and in MRC-5 cell lines. Recently the bovine arterial endothelial (BAE) cell line has been investigated for susceptibility to BHV-4 and was found to have 1000 times greater sensitivity to BHV-4 infection than the more commonly used MDBK cell line (Donofrio and van Santen 2001; Dubuisson et al. 1992; Egyed et al. 1996; Egyed 1998; Lin et al. 1997; Michalski and Hsiung 1976).

Virus infection of cell culture can result in persistent latent infection which is reversible by treatment with dexamethasone, sodium butyrate or phorbol esters. Inoculation of dexamethasone into test cultures may be considered to ensure detection of persistent or latent infection.

Adsorption of virus for 2 hours on the cell monolayer is required for virus attachment and entry. Plaques appear at 5–7 days and are more easily identified using agar overlays. Plaque formation is more readily identified in BAE cell lines than in MDBK cells.

BHV-4 replication is dependent on the S phase of the cell cycle and therefore infected cell cultures must be rapidly dividing. Monolayers cultivated for an extended time have a reduced capacity to support BHV-4 replication. It is recommended that monolayers are inoculated within 24 hours after passaging. For any particular working stock of cells the growth rate will fluctuate according to the passage history and culture conditions and it cannot be assumed all working stocks of susceptible cell types will be sensitive to BHV-4. Cultures that are not rapidly dividing may fail to detect primary isolation of BHV-4.

Isolation of BHV-4 is difficult because it replicates slowly and CPE is barely discernable in most cell lines. Specific molecular assays for BHV-4 are recommended together with primary isolation in

cell culture. In one study, cell culture isolation detected only 1/15 positives whereas PCR detected all 15 positives (Boerner et al. 1999; Naeem et al. 1991).

PCR amplification of the polyreplicative *EcoRI* L-fragment of BHV-4 has very high sensitivity and can detect as little as 1 fg of DNA or 1 genome in 500 cultured cells. A second nested PCR has been developed that amplifies the BHV-4 *tk* gene. The *tk* nested PCR has better specificity and less cross reactivity when compared to the PCR against the polyreplicative *EcoRI* L-fragment and comparable sensitivity. A third diagnostic PCR has been developed that amplifies BHV-4 gB.

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 for extraneous agent testing do not meet the growth requirements of BHV-4 and will not be sufficient for primary isolation of BHV-4. The 9 CFR generalised culture protocol specifies inoculation of monolayers that have been established over 5–7 days, whereas BHV-4 requires rapidly dividing cells inoculated within 24 hours of passaging. The 9 CFR guidelines do not include specific testing for BHV-4.

The European Pharmacopoeia general monograph *Vaccines for veterinary use* and 5.2.4 *Cell cultures for the production of veterinary vaccines* describe generalised methods for extraneous agent testing using cell culture monolayers that are sensitive to the agent being tested, and detection using specific testing methods that are sensitive to the viral agent. The monographs do not specify details of a culture system or assay for BHV-4 detection, simply that the methods should be sensitive.

Conclusion

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 are not acceptable.

Primary isolation of BHV-4 in the BAE cell line is recommended. The cultures for inoculation should be rapidly dividing and inoculated within 24 hours after passaging. An adsorption step is required for 2 hours prior to cultivation.

Specific testing is required by nested PCR targeting the BHV-4 *tk* gene.

Bovine immunodeficiency virus

Family *Retroviridae*, genus *Lentivirus*

Virus isolation is difficult and unreliable and currently successful isolation has been reported for only 3 isolates of bovine immunodeficiency virus (BIV). Susceptible cell lines for BIV are foetal bovine lung (FBL), MDBK, BFS, and primary bovine peripheral blood mononucleocytes (PBMC) (Bouillant et al. 1989; Heaton et al. 1998). In many cases cell cultures are identified as permissive to infection with BIV but infection does not result in release of infectious virus.

BIV infection requires adsorption incubation for 2 hours as a suspension culture followed by washing and plating for establishment of the monolayer. CPE is evident by the formation of syncytia (multi-nucleate giant cells) in cell culture 4–9 days after inoculation (Onuma et al. 1990).

Specific testing is recommended because of the difficulty of primary isolation. Available assays for BIV are PCR for proviral DNA, PCR-based reverse transcriptase (RT) activity assay or product enhanced reverse transcriptase (PERT) assay, FA and enzyme linked immunosorbent assay (ELISA) for capsid (CA) antigen (Baron et al. 1995; Orr et al. 2003).

PCR amplification of the DNA sequence encoding the RT gene can be detected in cell culture from 24 hours. There are 5 published PCR assays for BIV: dimer linkage structure (DLS) *env*, DLS *pol*, *gag*, and two assays targeting *pol*. A published study comparing the 5 available assays showed that the DLS nested *pol* assay for provirus had the best performance for sensitivity (80 %) and specificity (86 %) but still generated false negatives and the performance of the assay was not adequate for a diagnostic assay (Nadin-Davis et al. 1993; Nash et al. 1995; Suarez et al. 1995; Suarez and Whetstone 1997). The other PCRs performed poorly with regards to sensitivity and specificity.

A PERT assay has been applied to the detection of BIV in biological products with very high sensitivity (André et al. 2000; Kashanchi et al. 2002; Moore et al. 1996; Silver et al. 1993).

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 for extraneous agent testing will not be sufficient for primary isolation of BIV. The 9 CFR guidelines do not include the required cell lines for BIV isolation and do not include specific testing to increase the sensitivity of detection.

The European Pharmacopoeia general monograph *Vaccines for veterinary use* and 5.2.4 *Cell cultures for the production of veterinary vaccines* describe generalised methods for extraneous agent testing using cell culture monolayers that are sensitive to the agent being tested, and detection using specific testing methods that are sensitive to the viral agent. The monographs do not specify details of a culture system or assay for BIV detection, simply that the methods should be sensitive.

Conclusion

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 are not acceptable.

Primary isolation of BIV is not recommended.

Specific testing is required. A PERT assay is required as the initial diagnostic assay for BIV detection in conjunction with PCR for detection of proviral DNA. Attempted isolation, confirmation and identification of retrovirus positives is then required by culture isolation, animal isolation or sequencing of PCR products.

Bovine leukaemia virus

Family *Retroviridae*, genus *Deltaretrovirus*

Primary isolation of bovine leukaemia virus (BLV) is very difficult and there is no suitable culture method for extraneous agent testing. The method recommended by the OIE for primary isolation of BLV is by co-cultivation of BLV infected lymphocytes from the blood with indicator cell lines. Extraneous agent testing of cell lines for BLV infection could be performed by co-cultivation and mitogen stimulation with cytokines to induce formation of provirus. In general co-cultivation is not a suitable method for the testing of biological products. Following isolation by co-culture, BLV can be propagated by inoculation of foetal lamb kidney (FLK) cells or human embryonic kidney cells transformed with human adenovirus 5 (HEK 293T), but direct inoculation of these cell lines has poor sensitivity for primary isolation (Van Den Broeke et al. 2001; Willer et al. 1987).

The *deltaretrovirus* indicator cell line BLGFP has been developed for sensitive detection of BLV. BLGFP was created by stable transfection of CRFK cells with a construct encoding enhanced green fluorescent protein (EGFP) under the control of the regulatory element BLV long terminal repeat

(LTR). Infection with BLV results in trans-activator x (*Tax*) protein expression which binds to the *Tax* response element (*Tax*-RE) in the LTR and drives EGFP expression. EGFP expression in indicator cell lines can be assayed by flow cytometry or immunofluorescence detection. To increase infection rates the inoculation of BLV test material onto BLGFP is performed by a technique of spinoculation. Spinoculation involves coating of wells with L-lysine and centrifugation of cells and viral test material to promote adhesion and transduction of cells by virus. Using this technique the very low levels of *in vitro* retrovirus infectivity can be detected by reporter EGFP expression. This technique has promise for application to extraneous agent testing but has not been evaluated for this application and therefore cannot be recommended (Jewell and Mansky 2005).

BLV growth in persistently infected FLK cells is inhibited by bovine interferon γ . Induction of interferon γ may occur on inoculation of test material containing other viral antigens such as vaccine antigens and thereby further inhibit BLV detection (Niermann and Buehring 1997; Sentsui et al. 2001; Wyatt et al. 1989).

BLV infection of cell culture induces a poorly characterised 155 kDa, plasma blocking factor (PBF) that is responsible for blocking transcription and translation of viral proteins and establishing latency. PBF is responsible in part for the difficulty in cell culture isolation of BLV (van den Heuvel et al. 2005).

Specific testing is therefore recommended for BLV detection. ELISA and agar gel immunodiffusion (AGID) can be used for viral antigen. An antigen capture (AC-ELISA) using monoclonal antibodies (mAbs) BLV-gp51/6A12 and BLV-p24/X48 has been shown to have the greatest sensitivity for BLV detection in serum. A number of BLV AC-ELISA kits are available commercially (Ban et al. 1990; González et al. 2007).

A PCR directed against BLV proviral DNA has greater sensitivity than serological methods. PCRs targeting the *gag*-p24 gene and *pol* (reverse transcriptase) gene and *env* gene have been described. The most sensitive method for molecular detection is reported to be the nested PCR directed against the *env* gene encoding gp51; however, the test sensitivity and specificity are not adequate in an applied setting. The threshold for detection using the nested PCR is 5–10 target DNA molecules per sample. A Taqman real-time PCR targeting the *pol* gene has been developed. The detection threshold is 6 genomic copies and is therefore equivalent to the threshold obtained for the nested PCR. The Taqman assay has increased sensitivity and specificity for the detection of BLV infection when compared to the nested PCR, ELISA and AGID (Ballagi-Pordány et al. 1992; Beier et al. 1998; Lew et al. 2004; Venables et al. 1997).

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 for extraneous agent testing will not be sufficient for primary isolation of BLV. The 9 CFR guidelines do not include specific testing for BLV or generic testing for retroviruses.

The European Pharmacopoeia general monograph *Vaccines for veterinary use* and 5.2.4 *Cell cultures for the production of veterinary vaccines* describe generalised methods for extraneous agent testing using cell culture monolayers that are sensitive to the agent being tested, and detection using specific testing methods that are sensitive to the viral agent. The monographs do not specify details of a culture system or assay for BLV detection, simply that the methods should be sensitive.

Conclusion

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 are not acceptable.

Culture isolation of BLV is not recommended.

Specific testing is required. A PERT assay is required as the initial diagnostic assay for BLV detection combined with testing by the real-time Taqman PCR for proviral DNA targeting the *pol* gene (André et al. 2000; Graves et al. 1977; Reichert et al. 1992). Attempted isolation, confirmation and identification of retrovirus positives is required by culture isolation, animal isolation or sequencing of PCR products.

Bovine parainfluenza virus 3

Family *Paramyxoviridae*, genus *Respirovirus*

Susceptible cell lines for bovine parainfluenza virus 3 (BPI-3V) are Vero, CRFK, MDBK, FLK, BEL, BT, calf kidney (CRT-2), CTC, BEK and BFS. The most susceptible culture systems and the ones most often used are BEK or BEL. The defining differences between human parainfluenza virus 3 and BPI-3V are that BPI-3V has restricted growth in monkey cells and cultivation of BPI-3V does not require proteases (Castleman et al. 1991; Hemmatzadeh and Haghighi 2007; Ozdarendeli et al. 1997; Rulka 1992; Rulka et al. 1993; van der Maaten 1969).

CPE is evident from 2 days after infection as giant cells, formation of syncytia, and intra-nuclear and intra-cytoplasmic, small, eosinophilic, granular inclusions. It is recommended to culture for 10 days (Fulton and Root 1978).

There are both cytopathic and non-cytopathic isolates of BPI-3V. CPE is an unreliable means of detection for non-cytopathic isolates and the sensitivity of detection by CPE alone is inadequate for very low levels of infection. BPI-3V infectivity may be inhibited by induction of interferons within the culture system. Therefore specific testing is recommended.

FA is the most commonly used assay for specific testing of BPI-3V. Systematic studies comparing testing by either haemadsorption or FA indicates FA is the only sensitive and reliable method for detection of BPI-3V, and the sensitivity of FA is reported to be at least 3 times greater than haemadsorption for virus isolated in BEL (Toth and Jankura 1990).

In a number of other studies of BPI-3V both FA and HA have been used and in all cases FA has achieved greater sensitivity than HA. Studies examining the molecular mechanism of the HA for BPI-3V have found that fluctuation of HA activity for infected cultures was due to the fluctuation in viral fusion (F) and HA surface antigen with changes in the viral growth kinetics (Schmidt et al. 2000). FA positive cells appear as early as 0.5 hours after inoculation. A PCR based on the matrix (*M*) gene has not been standardised for diagnostic purposes.

The generalised culture methods described by 9 CFR 113.52, 113.53 and 113.55 are suitable for isolation of BPI-3V provided the recommended sensitive cell lines BEK or BEL are used. The 9 CFR guidelines do not require specific testing for BPI-3V.

The European Pharmacopoeia general monograph *Vaccines for veterinary use* and 5.2.4 *Cell cultures for the production of veterinary vaccines* describe generalised methods for extraneous agent testing using cell culture monolayers that are sensitive to the agent being tested, and detection using specific testing methods that are sensitive to the viral agent. The monographs do not specify details of a culture system or assay for BPI-3V detection, simply that the methods should be sensitive.

Conclusion

The generalised culture methods described by 9 CFR 113.52, 113.53 and 113.55 are suitable for isolation of BPI-3V provided the recommended sensitive cell lines BEK or BEL are used.

Primary isolation of BPI-3V in BEK or BEL is recommended. Cultures should be maintained for 10 days.

Specific testing for BPI-3V is required by FA because there are both cytopathic and non-cytopathic isolates of BPI-3V. CPE is an unreliable means of detection for non-cytopathic isolates and the sensitivity of detection by CPE alone is inadequate for very low levels of infection.

Bovine parvovirus

Family *Parvoviridae*, genus *Bocavirus*

Susceptible cell lines for primary isolation of Bovine parvovirus (BPV) are CTC, BK, BEK, buffalo lung fibroblast (BLF), BETC and BEL.

BPV isolation is highly dependent on rapidly dividing cell cultures of low passage number. Inoculation should occur onto subconfluent monolayers 18–24 hours after seeding of a 70% monolayer. Seeding density of cells and MOI of infection are also critical to virus growth and therefore infection should be by inoculation of a dilution series of the test virus.

Adsorption for 1 hour is required for adequate virus attachment and entry.

Serum supplementation of culture medium should be free of parvovirus reactive antibody that will neutralize inoculated virus (Durham and Johnson 1985; Lubeck and Johnson 1976; Wosu 1987).

Virus detection is evident by the development of CPE 20–48 hours after inoculation. CPE is characterised by cytolytic plaques and intra-nuclear inclusions.

FA is recommended to ensure adequate sensitivity of detection. Cells become positive by FA 8–16 hours after infection; however, detection declines after 16 hours.

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 for extraneous agent testing do not provide the required culture conditions for isolation of BPV and will not be sufficient for primary isolation of BPV. The protocol described by 9 CFR 113.47: *Detection of extraneous viruses by the fluorescent antibody technique* includes recommendations for FA for BPV. The 9 CFR guidelines do not specify that FA should be performed at 8–16 hours and testing later than this increases the likelihood of false negative results (Allander et al. 2001; Mengeling et al. 1986).

The European Pharmacopoeia general monograph *Vaccines for veterinary use* and 5.2.4 *Cell cultures for the production of veterinary vaccines* describe generalised methods for extraneous agent testing using cell culture monolayers that are sensitive to the agent being tested, and detection using specific testing methods that are sensitive to the viral agent. The monographs do not specify details of a culture system or assay for BPV detection, simply that the methods should be sensitive.

Conclusion

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 are not acceptable.

Primary isolation of BPV in CTC, BK, BEK, BLF, BETC or BEL cell lines is recommended. Test material should be inoculated as a dilution series onto rapidly dividing subconfluent monolayers within 24 hours of seeding. Adsorption incubation for 1 hour is required and serum used to supplement the growth medium should be free of parvovirus reactivity.

FA for BPV as described in 9 CFR 113.47: *Detection of extraneous viruses by the fluorescent antibody technique* is sufficient for BPV detection provided that FA is performed at 8–16 hours.

Specific testing by FA is required on infected monolayers 8–16 hours after infection.

Bovine respiratory syncytial virus

Family *Pneumovirinae*, genus *Pneumovirus*

Bovine respiratory syncytial virus (BRSV) can replicate in primary bovine and ovine cell cultures derived from the respiratory tract, and in the following cell lines; BT, chicken embryo-related (CER), Vero, MDBK and CRIB (a bovine viral diarrhoea virus-resistant clone of MDBK cells). CER and CRIB have the highest sensitivity for BRSV isolation and Vero cells have significantly lower sensitivity (two logs lower than other susceptible cell lines) (Flores and Donis 1995; Harrison and Pursell 1985; Spilki et al. 2006a; Spilki et al. 2006b; Taylor et al. 1984).

Primary isolation of BRSV requires inoculation of monolayers that are 85% confluent and requires an adsorption step of 1 hour. Maintenance medium should be supplemented with serum that is free of BRSV antibody so that virus neutralization does not occur. CPE can be detected at 3–4 days after inoculation by the formation of syncytia. For some strains CPE appears only after 7–10 days and 3 passages are recommended.

Because of the low sensitivity of culture detection for BRSV specific testing is recommended in addition to virus isolation. Nested RT-PCR amplification of either the glycoprotein G (gG), fusion (F) or nucleocapsid (N) genes of BRSV, or real-time Taqman RT-PCR amplification of BRSV F gene have greater sensitivity than primary isolation in culture alone. Studies examining the relative sensitivity of the different nested RT-PCRs have not been done; however, a study comparing the sensitivity of the nested RT-PCR for BRSV G gene against the real-time Taqman RT-PCR found no difference in sensitivity for these assays (Deplanche et al. 2007; Hakhverdyan et al. 2005; Valarcher et al. 1999).

Specific testing for BRSV is required by nested or real-time Taqman RT-PCR targeting the F gene of BRSV.

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 for extraneous agent testing do not provide optimal growth conditions for BRSV and will not be sufficient for primary isolation of BRSV. The 9 CFR 113.47: *Detection of extraneous viruses by the fluorescent antibody technique* protocol includes FA for BRSV. FA assay has sufficient sensitivity when performed with a polyclonal antiserum directed against BRSV whole virus antigen.

The European Pharmacopoeia general monograph *Vaccines for veterinary use* and 5.2.4 *Cell cultures for the production of veterinary vaccines* describe generalised methods for extraneous agent testing using cell culture monolayers that are sensitive to the agent being tested, and detection using specific testing methods that are sensitive to the viral agent. The monographs do not specify

details of a culture system or assay for BRSV detection, simply that the methods should be sensitive.

Conclusion

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 are not acceptable.

Primary isolation of BRSV in CER or CRIB cell lines is recommended. Test material should be inoculated onto monolayers that are 85% confluent and adsorption for 1 hour is required. Serum supplements should be free of BRSV reactivity. Cultures should be incubated for 7–10 days and 3 passages are required.

FA for BRSV as described in 9 CFR 113.47: *Detection of extraneous viruses by the fluorescent antibody technique* is sufficient for BRSV detection provided that FA is performed with a polyclonal antiserum directed against BRSV whole virus antigen.

Specific testing for BRSV is required either by FA assay as described in 9 CFR 113.47 or by nested or real-time Taqman RT-PCR targeting the F gene of BRSV.

Bovine viral diarrhoea virus 1 and 2

Family *Flaviviridae*, genus *Pestivirus*

Bovine viral diarrhoea virus (BVDV), also referred to as bovine pestivirus, can replicate in the following primary cell cultures: BEL, CTC, BT, bovine foetal nasal mucosa (BNM), BK, bovine endothelial cells (BEC), bovine alveolar macrophages, PBL, lamb testis (LT) and primary ovine kidney (SEK). Cell lines susceptible to BVDV infections are Razi bovine kidney (r-BK), Vero, MDBK, GBK, human lung carcinoma (A549tTA) and bovine kidney subclone of MDBK (MDBK-SY). Primary isolation is most commonly performed in MDBK, BT, or BK cells (Flores and Donis 1995).

Foci of CPE first develop from 3 days after inoculation of BVDV in cell culture and obvious plaque formation is seen at 5–8 days. Infection of stationary, confluent monolayers (as described by the 9 CFR protocol) has been shown to be only 64% as sensitive as inoculation of incomplete monolayers that will become confluent 7 days after inoculation. Three passages are recommended for the primary isolation of BVDV.

A number of factors influence primary isolation of BVDV, including the biotype of virus, growth activation state, co-infection or super-infection with other viruses and contamination of the culture system (Donis and Dubovi 1987; Gong et al. 1998; Hewicker-Trautwein et al. 1992; Johnson and Rosenbusch 1990; Stringfellow et al. 2005).

There are 2 biotypes of BVDV based on CPE in cultured bovine cells. Cytopathic BVDV (cpBVDV) produces CPE on MDBK, BT, or BK cells by apoptosis, whereas non-cytopathic BVDV (ncpBVDV) establishes persistent non-cytolytic infections without evidence of CPE. The cpBVDV and ncpBVDV biotypes replicate equally well; however, CPE is evident only for the cpBVDV. Cell death in cpBVDV infected cultures occurs by apoptosis and cellular and nuclear fragments are visible in culture monolayers. The cpBVDV and ncpBVDV often coexist in infected bovine cells. The cpBVDV is generated from the ncpBVDV by recombination with cellular or viral sequences or by mutation of the non-structural protein (*NSP*) 2 gene. Cell lines infected with ncpBVDV become refractive to the development of CPE due to cpBVDV although both viruses co-replicate and are

maintained in culture. Isolates of ncpBVDV that do not produce CPE in standard cell lines do produce CPE under some culture conditions and some isolates of ncpBVDV have been shown to develop CPE in BEC. Detection of either ncpBVDV or cpBVDV based on CPE alone is not reliable and specific testing is required (Fulton et al. 2000; Fulton et al. 2002; Liebler-Tenorio et al. 2004; Ridpath et al. 1994; Ridpath et al. 2000; Saito et al. 2004).

Combined mitogen stimulation of bovine PBL or of MDBK cell cultures with phytohaemagglutinin (PHA) and polycation has been shown to increase the sensitivity for detection of BVDV.

Induction of interferon in inoculated cultures enhances the maintenance of persistent non-cytopathic BVDV infection. This is of significance when testing viral seed stocks for contamination with BVDV as the viral stock may induce interferon and inhibit the detection of super-infecting BVDV (Nakamura et al. 1995; Ohmann and Babiuk 1988; Peek et al. 2004).

Interference assays have been developed to detect ncpBVDV based on the suppression of interferon induction by ncpBVDV leading to enhancement of superinfecting viral infections with cpBVDV, Newcastle disease virus or vesicular stomatitis virus in resistant cell lines. The reported sensitivity of these assays is approximately equivalent to that of FA using polyclonal serum (Patel and Shilleto 2003).

Persistent infection of cultures with BVDV does not involve defective interfering (DI) particles.

Contamination by BVDV of primary bovine cell cultures and bovine serum supplements used in maintenance medium occurs at a frequency of 10–50% and can interfere with the capacity of the culture system to identify BVDV for the purpose of extraneous agent testing. When contaminated foetal bovine serum FBS is used in maintenance medium for a range of bovine and ovine cell cultures it is reported that approximately 50% of the cells become infected on the first passage after exposure to BVDV (Fulton et al. 2003; Rossi et al. 1980; Studer et al. 2002; Wellemans and Van Opdenbosch 1987).

One report found 50% of commercial vaccines tested (n=32) by RT-PCR were contaminated with 3–6 logs of BVDV due to FBS contamination during cultivation. Studies of BHV-1 vaccine contaminated with BVDV that subsequently resulted in an outbreak of BVDV in bovine herds was not detected by routine batch testing of the vaccine and serum and multiple samples were required to be tested for detection of the contamination. Testing was by FA using polyclonal antiserum on inoculated monolayers and ELISA.

A number of specific assays have been developed for the diagnosis of BVDV infection. FA have been described using fluorescein-conjugated polyclonal anti-BVDV serum and also based on a number of anti-BVDV mAbs. FA detects both ncpBVDV and cpBVDV biotypes. FA based on the mAb D89 has been shown to detect 75% of positives by 5 days after inoculation and 100% by 10–20 days.

A dot-hybridization assay developed with a 1.1 kb complementary DNA (cDNA) prepared from BVDV genomic RNA was found to be 10–100 times more sensitive than both culture isolation and FAs for BVDV, and detected all isolates examined. A study comparing FAs using polyclonal anti-BVDV serum found the assay to have 86.3% sensitivity. By comparison an immunoperoxidase (IP) assay had 90% sensitivity and a RT-PCR to detect the 5' untranslated region (UTR) detected 100% of BVDV isolates from cell culture. A single tube real-time Taqman RT-PCR based on the BVDV 5' UTR has been developed with a lower detection threshold of 100 genome copies. A nested RT-PCR also based on the 5' UTR was found to have a very low detection limit of 10^3 TCID₅₀/ ml of

BVDV. The nested RT-PCR whilst able to detect very low levels of virus genome has also been found to have very low specificity due to the detection of non-infectious genome in inactivated biologicals. RT-PCR based on the 5' UTR can detect BVDV genotypes 1 and 2. A comparison of FA with AC-ELISA found FA to have 97% sensitivity compared to 90% sensitivity for the ELISA. MAbs D89 specific for BVDV E2 (gp53) protein and specific for NS3 (p80) protein are commonly used for BVDV FA (Antonis et al. 2004; Barlic-Maganja and Grom 2001; Boulanger et al. 1991; El-Kholy et al. 1998; Givens et al. 2001; Gogorza et al. 2005; Greiser-Wilke et al. 1991; Liess et al. 1993; Potgieter and Brock 1989; Ridpath and Bolin 1998; Tsuboi and Bielanski 2005).

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 for extraneous agent testing do not provide optimal growth conditions for BVDV and will not be sufficient for primary isolation of BVDV. The 9 CFR 113.47: *Detection of extraneous viruses by the fluorescent antibody technique* protocol includes FA for BVDV. FA for BVDV is not sufficiently sensitive for extraneous agent testing.

The European Pharmacopoeia general monograph *Vaccines for veterinary use* and 5.2.4 *Cell cultures for the production of veterinary vaccines* describe generalised methods for extraneous agent testing using cell culture monolayers that are sensitive to the agent being tested, and detection using specific testing methods that are sensitive to the viral agent. The monographs do not specify details of a culture system or assay for BVDV detection, simply that the methods should be sensitive.

Conclusion

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 are not acceptable.

Primary isolation of BVDV in BEC, MDBK, BT or BK cells is recommended. Cells should be seeded to establish 70% monolayers at the time of inoculation and should be mitogen stimulated with either PHA or polycation. All medium supplements and cell lines used for the assay must be tested as free of BVDV contamination or reactivity. Three culture passages are required.

FA for BVDV as described in 9 CFR 113.47: *Detection of extraneous viruses by the fluorescent antibody technique* is not sufficiently sensitive for extraneous agent testing.

Specific testing for BVDV is required by nested RT-PCR or real-time Taqman RT-PCR targeting the 5' UTR of BVDV.

Porcine isolates of BVDV

Porcine isolates of pestivirus belong predominantly to the 2 major subgroups classical swine fever virus 1 (CSFV-1 (type species Brescia strain)) or CSFV-2 (type species Alfort strain). There are a number of variant porcine pestivirus isolates grouped together in subgroup CSFV-3. Pestiviruses are not highly host specific and BVDV and BDV have infrequently been isolated from porcine hosts, whilst CSFV are restricted to porcine hosts. CSFV and BVDV have been shown by infection inhibition/competition studies to bind to the same cellular receptor in bovine cell culture using the E2 surface glycoprotein; however, the pestivirus strain differences in receptor avidity are responsible for differences in infectivity (Hulst and Moormann 1997). CSFV is able to utilise an alternative E3 method of cellular entry in porcine culture systems that is believed to contribute to its restricted host tropism. Pestivirus isolates (of all subgroups) originating from porcine hosts develop host tropism and therefore primary culture of these isolates will be most sensitive in a porcine cell

culture system rather than a bovine cell culture system irrespective of whether the isolate is characterised as CSFV, BVDV or BDV.

The standard for primary isolation of porcine isolates of BVDV is in PK-15 cells or primary swine testicle (ST) cells. The most sensitive method of culturing porcine pestivirus isolates is reported to be PK-15 cells seeded onto cover-slips. CPE is evident within 3 days but is frequently difficult to discern, in particular for non-cytopathic isolates (OIE 2010).

Specific testing for porcine isolates of BVDV is required to confirm culture isolation.

Fluorescence staining has been developed using both polyclonal antiserum and mAbs. The FA is problematic due to a high incidence of false negatives. An AC-ELISA has been developed which has a similar problem with poor sensitivity (Shannon et al. 1993).

Conventional RT-PCRs targeting the 5' UTR of porcine pestiviruses have been developed that have comparable sensitivity to the ELISA (Hofmann et al. 1994; McGoldrick et al. 1998). A single tube nested RT-PCR and a real-time Taqman RT-PCR have been developed and are reported to have higher sensitivity than primary isolation in culture and FA combined (McGoldrick et al. 1998).

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 for extraneous agent testing will not be sufficient for primary isolation of porcine isolates of BVDV. The 9 CFR guidelines do not require specific testing for porcine isolates of BVDV.

The European Pharmacopoeia general monograph *Vaccines for veterinary use* and 5.2.4 *Cell cultures for the production of veterinary vaccines* describe generalised methods for extraneous agent testing using cell culture monolayers that are sensitive to the agent being tested, and detection using specific testing methods that are sensitive to the viral agent. The monographs do not specify details of a culture system or assay for porcine pestiviruses detection, simply that the methods should be sensitive.

Conclusion

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 are not acceptable.

Primary isolation of porcine isolates of BVDV in PK-15 cells is recommended.

FA for porcine isolates of BVDV as described in 9 CFR 113.47: *Detection of extraneous viruses by the fluorescent antibody technique* is not sufficiently sensitive for extraneous agent testing.

Specific testing is required for porcine isolates of BVDV by real-time Taqman RT-PCR.

Canine adenovirus 1 and 2

Family *Adenoviridae*, genus *Mastadenovirus*

Primary isolation of canine adenovirus 1 and 2 (CAV-1 and CAV-2) can be performed in Madin–Darby canine kidney (MDCK), subclone of MDCK (MDCK-SP), canine kidney (CK), Vero, cynomolgus monkey kidney (Jinet), dog kidney fibroblast (DF-K), canine adenocarcinoma (A-72), PK-15 and primary swine kidney (SK) cell cultures. MDCK and MDCK-SP cells are the most susceptible culture systems for CAV (Cavalli et al. 1993). A-72 cells have low susceptibility to CAV infection and only low titres are produced (Binn et al. 1980). CAV infection of JINET and

Vero cell lines does not result in obvious CPE (Murakami et al. 1977). Growth on PK-15 and SK requires prior adaptation by passaging in MDCK and CPE is not obvious in this culture system (Ferreira 1971). CPE is evident as cytolysis and Cowdry type B intra-nuclear, basophilic, inclusion bodies 36-48 hours after infection, and plaque appear in monolayers after 7 days (Cavalli et al. 1993). The inclusions produced by CAdV types 1 and 2 are different (Adair 1979). Type 1 viruses produce multiple refractile eosinophilic inclusions whereas those for type 2 viruses are large granular and non-refractile.

MDCK cell cultures have considerable heterogeneity and clonal purification and expansion of subclones of MDCK can result in very different culture phenotypes (Bagust and Dennet 1977; Bendheim 1973; Nakazato et al. 1989). MDCK cultures respond to culture conditions by selection for altered phenotypes and this in turn can influence the susceptibility of cultures to infection and the expression of CPE. MDCK-SP has been adapted to growth in serum-free medium and clonally expanded for use in vaccine propagation, and is of equivalent susceptibility for CAdV-1 and CAdV-2 infection (Mochizuki 2006).

Serial passage of CAdV can result in internal deletions or DI particles forming as a part of the process of culture adaptation (Sira et al. 1989). The DI particles can inhibit propagation of the virus and interfere with detection of contaminating virus.

Large and small plaque variants of CAdV have been characterised, with large plaque variants being more infectious and growing to higher titres (Tohya et al. 1989). Small plaque variants are difficult to detect by CPE in primary cultures.

Specific testing is required because of the difficulty of culture detection for CAdV. Specific testing is possible by HA, serum neutralization (SN), FA, ELISA, and PCR. ELISA and SN are reported to give 98% agreement for detection of serum antibodies in infected dogs; however, these tests are not useful for extraneous agent detection.

A PCR has been developed for detection of CAdV-2 targeting the fibre gene and a second PCR targets the capsid (*ca*) gene and amplifies both CAdV-1 and CAdV-2 (Erles et al. 2004). A second PCR for CAdV has been developed targeting the E2 gene and applied in several clinical studies (Benetka et al. 2006; Chvala et al. 2007). A nested PCR and a short primer nested PCR have been developed targeting the hexon gene of CAdV (Boomkens et al. 2005). A PCR has been developed targeting the E3 gene and flanking region for detection of types 1 and 2 CAdV (Hu et al. 2001). For all 4 molecular assays developed there has not been systematic validation and nor has the specificity and sensitivity been determined.

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 for extraneous agent testing will be sufficient for primary isolation of CAdV provided the MDCK or MDCK-SP cell lines are used for isolation. The 9 CFR guidelines do not include an FA for CAdV.

The European Pharmacopoeia general monograph *Vaccines for veterinary use* and 5.2.4 *Cell cultures for the production of veterinary vaccines* describe generalised methods for extraneous agent testing using cell culture monolayers that are sensitive to the agent being tested, and detection using specific testing methods that are sensitive to the viral agent. The monographs do not specify details of a culture system or assay for CAdV detection, simply that the methods should be sensitive.

Conclusion

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 for extraneous agent testing are acceptable provided the MDCK or MDCK-SP cell lines are used for isolation.

Primary isolation of CAdV in MDCK or MDBK-SP is recommended.

Specific testing is required for CAdV by FA.

Canine distemper virus

Family *Paramyxoviridae*, genus *Morbillivirus*

Primary isolation of canine distemper virus (CDV) is possible in Vero, transgenic Vero (Vero-DST), CK, green monkey kidney (4647), human cervical carcinoma (HeLa), canine malignant histiocytosis (CCT), MDCK, BHK-21, human laryngeal carcinoma (Hep-2), mutant rabbit kidney (DJRK), B95a, mink lung cells persistently infected with racoon-origin CDV (CCL64-RCDV), feline embryo (FE), chicken embryo fibroblast (CEF), bovine fibroblasts, primary canine bladder epithelial cells, and glioma cell cultures (Chen et al. 2000; Yamaguchi et al. 2005). Cell culture adapted strains of CDV and the attenuated Onderstepoort vaccine strain are most readily propagated in Vero, MDCK, or CEF cell cultures and these are most sensitive for CDV isolation. Isolation of field strains of CDV in commercial, immortalised cell lines is unreliable and insensitive. Vero-DST is a stable transgenic Vero cell line that has been developed expressing canine signalling lymphocyte activation molecule (SLAM), the CDV receptor molecule (Lan et al. 2005; Lan et al. 2006). The Vero-DST cell line is a sensitive culture system for the isolation of field isolates of CDV and results in a higher proportion of released as opposed to cell-associated virus in infected cultures.

Serum factors can be inhibitory to CDV growth and therefore serum-free maintenance medium should be used for cultivation (Mochizuki 2006; Tian et al. 1998). Adsorption incubation for 1 hour is required for effective infection of monolayers. CDV growth requires incubation at 33 °C for 8 days for roller bottles or microcarriers, and 10 days for stationary cultures (Mironova et al. 1990). The viral titres obtained are at least 1 log higher when grown in roller bottles or attached to microcarriers (for infection of Vero cells or CEF), than for infection of stationary monolayers (Lesko et al. 1993). Eight days after infection, CPE is evident as cellular necrosis, formation of syncytia and inclusion bodies.

Culture isolation is problematic and is reported to be unreliable for some field strains of CDV. Persistent non-cytolytic infection occurs frequently during culture propagation and reduces the sensitivity of using CPE for detection of CDV (Iwatsuki et al. 1999; Metzler et al. 1984; Stettler et al. 1997; Zurbriggen et al. 1993). Persistent non-cytolytic infection is not associated with DI particles as all mRNAs and proteins are continuously expressed during non-cytolytic infection, but are associated with virus transmission via cell-cell junctions and an absence of viral budding. Therefore specific assays detecting viral nucleic acid or viral protein will be effective in detecting persistent culture infections. However, DI particles are reported to occur for some attenuated strains of CDV such as Onderstepoort.

Specific testing is required for CDV as culture isolation is problematic. Specific testing for CDV is possible by FA, *in situ* hybridisation (ISH), immunochromatography assay (ICA), Dot enzyme immunoassay (EIA), AC-ELISA, VN, RT-PCR, and real-time RT-PCR.

ISH assays using digoxigenin labelled probes have been developed but their application has been limited to detection of vaccine strains of CDV in Vero cell culture or canine tissue infection (Rzezutka and Mizak 2003; Zurbriggen et al. 1993).

FA and ISH have been developed for the detection of CDV using monoclonal anti-CDV antibodies (Damián et al. 2005; Hentschke 1995; Jozwik and Frymus 2005). The FA has low sensitivity and limited application.

An ISH assay has been developed using tissue microarray technology (TMA) to facilitate screening of large numbers of samples (Hammer et al. 2007). This assay is intended as a supplementary screening assay rather than as a stand-alone diagnostic assay.

An AC-ELISA has been developed using hyperimmune antiserum raised in a goat against CDV infected Vero cells (Gemma et al. 1996; Soma et al. 2003). The detection limit of the assay is 100 TCID₅₀/μl and the assay is reported to have low levels of cross reactivity with measles virus. A dot EIA has been developed using hyperimmune anti-CDV serum (Ramadass and Latha 2001). Both the AC-ELISA and the EIA have been applied to diagnosing clinical infection in dogs but have not been validated for extraneous agent testing of biological materials.

RT-PCRs have been developed for CDV targeting various regions of the genome, including the nucleocapsid protein (N), phosphoprotein (P), haemagglutinin (H), and F genes of CDV (Calderon et al. 2007; Demeter et al. 2007). RT-PCR-RE assays target the heterogeneity in the H and F genes are useful for strain discrimination and epidemiological analysis, whereas assays targeting the conserved N and P gene are widely used for diagnosis of CDV. Nested RT-PCRs have been developed targeting the N and P genes (Jozwik and Frymus 2005; Rzezutka and Mizak 2002). The detection limits of the RT-PCR and nested RT-PCRs targeting the N gene were 10 and 0.1 TCID₅₀ respectively and the assays showed 100% specificity (Jozwik and Frymus 2005). A RT-PCR has been developed using a universal primer set targeting the P gene and capable of detecting all morbilliviruses, including CDV (Barrett et al. 1993).

A real-time Taqman RT-PCR has been developed for CDV targeting the P gene (Scagliarini et al. 2007). The Taqman assay is highly sensitive for CDV detection in cell culture and can detect as little as 10 copies/μl. The assay has very low inter- and intra-assay variability and is specific for CDV. The assay was successfully validated in experimental infections and also against 30 CDV strains. A RT loop-mediated isothermal amplification (RT-LAMP) assay has been developed targeting the N gene of CDV (Cho and Park 2005). The assay sensitivity and specificity are reported to be 100% and 93.3% respectively, and the detection limit of the RT-LAMP method was 10⁻¹TCID₅₀/ml (100 times more sensitive than the RT-PCR). The assay has been validated for the detection of 50 canine samples. A real-time Taqman RT-PCR has been developed targeting the N gene of CDV, with a reported detection limit of 100 copies (Elia et al. 2006). The real-time RT-PCRs developed have all been validated for canine field samples and culture infections; however, they have not been systematically compared for sensitivity and specificity.

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 for extraneous agent testing do not provide optimal growth conditions for CDV and will not be sufficient for primary isolation of CDV. The 9 CFR 113.47: *Detection of extraneous viruses by the fluorescent antibody technique* protocol includes an FA for CDV. The FA for CDV is not sufficiently sensitive for extraneous agent testing.

The European Pharmacopoeia general monograph *Vaccines for veterinary use* and 5.2.4 *Cell cultures for the production of veterinary vaccines* describe generalised methods for extraneous

agent testing using cell culture monolayers that are sensitive to the agent being tested, and detection using specific testing methods that are sensitive to the viral agent. The monographs do not specify details of a culture system or assay for CDV detection, simply that the methods should be sensitive.

Conclusion

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 are not acceptable.

Primary isolation of CDV in Vero-DST, MDCK or B95a cell culture is recommended. Cultures should be incubated in serum-free maintenance medium at 33 °C for 8 days in either roller bottles or using microcarriers, and adsorption incubation for 1 hour is required.

FA for CDV as described in 9 CFR 113.47: *Detection of extraneous viruses by the fluorescent antibody technique* is not sufficiently sensitive for extraneous agent testing.

Specific testing is required for CDV by real-time Taqman RT-PCR targeting the P gene or the RT-LAMP assay targeting the N gene of CDV.

Canine parvovirus

Family *Parvoviridae*, genus *Parvovirus*

Primary isolation of canine parvovirus (CPV) is in canine or feline cell lines MDCK, feline embryonic fibroblast (FEF), A-72, IL-2-independent feline T4 lymphoma (3201), or CRFK. The A-72 cell line is reported to be the most susceptible to CPV infection (Aubert et al. 1980; Horiuchi 1992; Horiuchi et al. 1994; Truyen and Parrish 1992). Freshly seeded CRFK cells with a high mitotic index are more sensitive to CPV infection than established monolayers. Detection sensitivity is enhanced by seeding low concentrations of cells and optimal MOI. CPE is evident as cytolysis, detachment and rounding of infected cells and intra-nuclear inclusions 2–3 days after inoculation (Joshi et al. 1998). Confirmation of CPE is by staining of the inclusions using an FA.

Specific testing is required for CPV to increase the detection sensitivity. Specific testing is possible by FA, HA, sandwich ELISA, EIA, PCR, and real-time Taqman PCR.

In one study HA performed with culture supernatant was reported to be more sensitive than FA for inclusions and vice versa in a different study (Senda et al. 1987). ELISA, EIA and HA are reported to be equivalent in sensitivity, and this is lower than that for culture isolation of CPV alone (Joshi et al. 2001; Martinello 1996).

A PCR has been developed targeting the VP2 coding region (Senda et al. 1995). PCR detection has been shown to increase the sensitivity of detection of CPV 2–4 logs above that of culture in CRFK cells alone (Senda et al. 1995). A nested PCR targeting the VP2 coding region has been shown to double the sensitivity of detection for CPV above that for the classical PCR (Mizak and Rzezutka 1999). The published reports on sensitivity of tests for CPV relate to studies using faecal detection and the results therefore cannot be directly translated to extraneous agent testing.

A real-time Taqman PCR has been developed that can detect all CPV-2 strains and uses a series of minor groove binding (MGB) probes to differentiate the CPV-2a, CPV-2b and CPV-2c variants (Decaro et al. 2005). The sensitivity of the real-time assay is 100 fg or 1 plaque-forming unit (PFU) of CPV, and the assay has been validated for the diagnosis of 203 field samples. The real-time PCR performed best in a study systematically comparing the following 5 diagnostic assays: ICA, HA,

virus isolation PCR and real-time PCR (Decaro et al. 2005; Decaro et al. 2006a; Decaro et al. 2006b; Desario et al. 2005; Desario et al. 2006).

Contamination of canine vaccines with non-vaccinal CPV is a significant problem. A PCR has been developed that can differentiate antigenically old-type virus from which vaccine strains are derived, from currently circulating strains (Costa et al. 2005; Senda et al. 1995). Variants arose from the old-type virus from the early 1980s (Parrish 1999). Internationally CPV-2b and CPV-2c have replaced CPV-2a as the circulating variant strains (Senda et al. 1995). In Australia the earliest CPV-2a strain is the only currently circulating strain (Meers et al. 2007).

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 for extraneous agent testing do not provide optimal growth conditions for CPV and will not be sufficient for primary isolation of CPV. The 9 CFR 113.47: *Detection of extraneous viruses by the fluorescent antibody technique* protocol includes FA for CPV. The FA for CPV is not sufficiently sensitive for extraneous agent testing.

The European Pharmacopoeia general monograph *Vaccines for veterinary use* and 5.2.4 *Cell cultures for the production of veterinary vaccines* describe generalised methods for extraneous agent testing using cell culture monolayers that are sensitive to the agent being tested, and detection using specific testing methods that are sensitive to the viral agent. The monographs do not specify details of a culture system or assay for CPV detection, simply that the methods should be sensitive.

Conclusion

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 are not acceptable.

Primary isolation of CPV in A72 or CRFK cells is recommended. Cell cultures should be freshly seeded and have a high mitotic index. Inoculation should be onto sub-confluent monolayers. Confirmation of CPE is by staining of the inclusions using an FA.

FA for CPV as described in 9 CFR 113.47: *Detection of extraneous viruses by the fluorescent antibody technique* is not sufficiently sensitive for extraneous agent testing.

Specific testing is required for CPV by real-time Taqman PCR using MGB probes to identify strain variants.

Caprine and ovine pox virus

Family *Poxviridae*, genus *Capripoxvirus*

Primary isolation of caprine and ovine pox virus is possible on primary cultures of bovine, ovine or caprine origin. Primary or secondary cultures of LT or lamb kidney (LK) cells are the most susceptible cell lines. Primary cultures derived from wool-breed sheep (principally Merino) yield virus titres 3 times greater than from other breeds (Binepal et al. 2001). Caprine and ovine pox virus can be adapted to growth on Vero cells; however, Vero cells are not recommended for primary isolation of virus due to strain variability in susceptibility. Caprine and ovine pox virus can be propagated on the chorioallantoic membrane of chicken embryos; however, as with Vero cell culture there is variability in strain susceptibility (Tantawi and Al Falluji 1979). Primary sheep embryo dermal cells are as susceptible (by some reports more susceptible) as LK cells for caprine and ovine pox virus isolation; however, the differentiation of CPE is problematic due to non-specific cell degeneration in dermal cells (Coackley and Capstick 1961; Koylu and Nada 1970). A

systematic study of titration of 12 isolates (both virulent and vaccine strains) of caprine and ovine pox virus in LT cells resulted in an average 1 log greater titres than in foetal bovine skin cells, 2 logs greater titres than in foetal bovine muscle cells, and 2–6 logs greater titres than in foetal bovine kidney and bovine thyroid cells (Binepal et al. 2001).

Recently the Canadian National Centre for Foreign Animal Disease and the Australian Animal Health Laboratory conducted a comparative study of *capripoxvirus* growth kinetics in the established ovine testis cell line (OA3.Ts), in primary LK cells, and in Vero cells. The objective of the study was to identify a cell line suitable for standardized diagnostic assays for *capripoxvirus* (Babiuk et al. 2007). The OA3.Ts cell line has been deposited in the American Type Culture Collection (ATCC) and is described by the ATCC as susceptible to orf virus infection. Isolates evaluated in this study were a Nigerian isolate, Indian goat pox, Kenyan sheep and goat pox, Yemen sheep and goat pox, and lumpy skin disease virus (LSDV, Neethling strain). The findings of the study were that the LK and OA3.Ts cell lines had equivalent growth kinetics and susceptibility to both low and high level MOI. The OA3.Ts cell line had the advantage of being a more homogenous cell line that forms tighter and more even cell monolayers in which plaque formation is more readily identified. The adoption of the OA3.Ts cell line for primary isolation of *capripoxvirus* is therefore highly recommended in place of the other cell lines considered in this review. It is to be expected that in the near future the use of this cell line will become the standard adopted for *capripoxvirus* primary isolation.

Caprine and ovine pox virus should be inoculated onto the monolayers in a small working volume and adsorption incubation for 1 hour at 37 °C is required before adding maintenance medium. Systematic studies of caprine and ovine pox virus entry into LT cells demonstrate that viral entry occurs with a minimum adsorption time of 1 minute; however, the latency period for detection of CPE is decreased with longer adsorption times, indicating that infectivity is greater with the longer adsorption times (Jassim and Keshavamurthy 1982).

Caprine and ovine pox virus infection has a highly variable latent period before the appearance of CPE and the growth kinetics of poxviruses are such that they can require up to 3 passages for virus to adapt to any particular culture situation and for CPE to become apparent. It is therefore critical that primary isolation follows the OIE recommendation of examination of the initial culture for 14 days, freeze-thawing 3 times, followed by re-inoculation onto fresh cell cultures. A minimum of 3 serial re-inoculations of fresh cultures should be performed to ensure adequate detection of virus.

Specific testing for caprine and ovine pox virus is not required as the virus can be readily identified by culture isolation and CPE detection.

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 will not meet the growth requirements of caprine and ovine pox virus and will not be sufficient for primary isolation of caprine and ovine pox virus. Sensitive cell lines required for primary isolation of caprine and ovine pox virus are not included in the 9 CFR protocol. Specific growth requirements of caprine and ovine pox virus will not be achieved using the 9 CFR protocol, as caprine and ovine pox virus infection has a highly variable latent period before the appearance of CPE and the growth kinetics of poxviruses are such that they can require up to 3 passages for virus to adapt to any particular culture situation and for CPE to become apparent.

The European Pharmacopoeia general monograph *Vaccines for veterinary use* and 5.2.4 *Cell cultures for the production of veterinary vaccines* describe generalised methods for extraneous agent testing using cell culture monolayers that are sensitive to the agent being tested, and detection using specific testing methods that are sensitive to the viral agent. The monographs do not specify

details of a culture system or assay for caprine and ovine pox virus detection, simply that the methods should be sensitive.

Conclusion

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 are not acceptable.

Primary isolation of caprine and ovine pox virus in the OA3.Ts cell line is recommended. Caprine and ovine pox virus infection requires adsorption incubation of 1 hour, cultures should be maintained for 14 days between passages, and 4 passages are required.

Specific testing for caprine and ovine pox virus is not required.

Caprine arthritis encephalitis virus and visna/maedi virus

Family *Retroviridae*, genus *Lentivirus*

Primary isolation of caprine arthritis encephalitis virus (CAEV) and visna/maedi (also referred to as maedi–visna) virus (MVV) by culture is not recommended for diagnostic purposes due to the low success rate and poor sensitivity. Primary isolation is by co-cultivation of infected monocytes/macrophages from tissues to sphingosylphosphorylcholine (SPC) matrix scaffolded cell monolayers. CPE from primary isolation is readily visible and can be confirmed by FA. This technique is not applicable to extraneous agent testing (OIE 2010).

A nested PCR test targeting the *gag* gene and a quantitative real-time PCR targeting the transmembrane domain have been developed for detection of CAEV proviral DNA and are most commonly used for CAEV and MVV detection. The real-time PCR of the *env* gene has 96% positive and 97% negative concordance with ELISA serology for detection of CAEV and MVV, and can detect as few as 10 copies of proviral DNA (Herrmann-Hoesing et al. 2007). Nested PCRs have poor specificity and it is recommended that positive results are checked by sequencing, restriction fragment length polymorphism (RFLP) or hybridization (de Andrés et al. 2005; Reddy et al. 1993; Wagter et al. 1996).

CAEV and MVV detection is recommended by a PERT assay (André et al. 2000; Graves et al. 1977; Reichert et al. 1992).

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 for extraneous agent testing will not be sufficient for primary isolation of CAEV and MVV. The 9 CFR guidelines do not include specific testing for CAEV and MVV.

The European Pharmacopoeia general monograph *Vaccines for veterinary use* and 5.2.4 *Cell cultures for the production of veterinary vaccines* describe generalised methods for extraneous agent testing using cell culture monolayers that are sensitive to the agent being tested, and detection using specific testing methods that are sensitive to the viral agent. The monographs do not specify details of a culture system or assay for CAEV and MVV detection, simply that the methods should be sensitive.

Conclusion

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 are not acceptable.

Primary isolation of CAEV and MVV is not recommended.

Specific testing is required. A PERT assay should be used as the initial diagnostic assay for CAEV and MVV detection in conjunction with PCR for detection of proviral DNA. Attempted isolation, confirmation and identification of retrovirus positives is required by sequencing of PCR products.

Classical swine fever virus

Family *Flaviviridae*, genus *Pestivirus*

Primary isolation of CSFV is in cell lines of porcine origin; in particular PK-15, NSK and NPTr cell lines are most sensitive. Growth of CSFV is dependent on the inoculation of rapidly dividing cell cultures rather than inoculation of established confluent monolayers. The sensitivity of culture isolation can be improved by seeding of PK-15 cells onto coverslips together with a 2% solution of porcine tonsil cells. Cultures should be maintained for 3 days and CPE should be confirmed by FA at 1–3 days.

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 will not meet the specific growth requirements of CSFV and will not be sufficient for primary isolation of CSFV. The generalised protocols do not meet the requirements of CSFV for susceptible cell lines or specific growth conditions for CSFV. The 9 CFR guidelines do not include specific testing for CSFV.

The European Pharmacopoeia general monograph *Vaccines for veterinary use* and 5.2.4 *Cell cultures for the production of veterinary vaccines* describe generalised methods for extraneous agent testing using cell culture monolayers that are sensitive to the agent being tested, and detection using specific testing methods that are sensitive to the viral agent. The monographs do not specify details of a culture system or assay for CSFV detection, simply that the methods should be sensitive.

Conclusion

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 are not acceptable.

Primary isolation of CSFV in PK-15, NSK or NPTr cell lines is recommended. PK-15 cells should be rapidly dividing and seeded onto coverslips together with a 2% solution of porcine tonsillar cells.

Specific testing is required for CSFV by FA at 1–3 days after inoculation.

Ectromelia virus

Family *Poxviridae*, genus *Orthopoxvirus*

Primary isolation of ectromelia virus (mouse pox virus) is recommended in primary Swiss mouse embryo (ME), BEL, CEF, HeLa, L929, monkey kidney (MARC-145), foetal rhesus monkey kidney (MA-104), primary mouse hepatocytes, lymphoma or hybridoma cell cultures (Barski and Cornefert 1960; Buller et al. 1987; Diefenthal and Habermehl 1959; Downie and McGaughey 1935; Habermehl and Diefenthal 1962; Karasek and Ronde 1969; Lees and Stephen 1985; Mahnel 1983; Mahnel 1987). Ectromelia virus is relatively easy to isolate and growth requirements are not exacting. Infected cultures are incubated for 3 days at 35 °C.

CPE is evident 48-72 hours after infection by early cell rounding and vacuolation, nuclear pyknosis, cellular fusion into giant cells, formation of eosinophilic, granular, cytoplasmic inclusions and elementary bodies (Downie and McGaughey 1935; Kitamoto et al. 1986). B-type inclusion bodies, sites of virus replication, and A-type inclusions are present (Lees and Stephen 1985). Infection can be detected by plaque formation on L929 monolayers under a carboxymethyl (CM)-cellulose overlay (Gendon and Chernos 1963). Pox virus particles may be demonstrated by electron microscopy (EM).

Some isolates do not show obvious CPE in culture even after 7 passages and specific testing is necessary (Barski and Cornefert 1960; Kitamoto et al. 1986). L929 cells infected at low, but not high MOI, will develop CPE (Lees and Stephen 1985).

Specific testing is possible by FA, PCR, real-time PCR, or microarray assays.

FA can be used to confirm infection using vaccinia-specific mAb (Kitamoto et al. 1986; Lees and Stephen 1985). Vaccinia and ectromelia virus are antigenically indistinguishable and vaccinia-specific antibody used in the FA cross-reacts with ectromelia virus and stains the type-B inclusion bodies. Typically only a proportion of the viral inclusion bodies present will fluoresce in the FA (Karasek and Ronde 1969). Serological assays such as FA are therefore limited to confirmation of infection with orthopoxvirus and cannot be used to differentiate ectromelia virus infection.

PCRs have been developed for detection of ectromelia virus targeting the haemagglutinin (H) and acidophilic-type inclusion body (ATI) genes of ectromelia virus (Meyer et al. 1997; Neubauer et al. 1997; Ropp et al. 1995). In one study a PCR targeting the ATI gene was developed that required differentiation of ectromelia virus from other members of the orthopox genus by size differentiation and RFLP (Meyer et al. 1997). A second PCR was developed targeting the ATI gene that was specific to ectromelia virus, highly sensitive and the detection limit of the assay was 5 fg DNA (Neubauer et al. 1997). PCRs targeting the H gene depend on multiplex assays for differentiating orthopoxviruses from other pox virus genera combined with RFLP analysis (Ropp et al. 1995). RFLP analysis of ectromelia virus has also been developed targeting the cytokine response modifier B (*crmB*) gene (Loparev et al. 2001).

A real-time PCR has been developed targeting a region of the orthopoxvirus F gene containing a single nucleotide polymorphism (SNP) (Bhatt et al. 1981; Olson et al. 2004; Trentin 1953; Trentin and Briody 1953). Ectromelia virus can be differentiated from other orthopoxviruses by melting curve analysis. The real-time PCR is highly specific to orthopoxviruses and the detection threshold is 1 fg of DNA. A real-time Taqman PCR has been developed targeting the H gene of orthopoxviruses (Bhatt et al. 1981; Ibrahim et al. 2003; Trentin 1953; Trentin and Briody 1953). The assay uses species specific dual labelled fluorescent probes and can differentiate ectromelia virus from other orthopoxviruses. The real-time Taqman PCR is reported to have a sensitivity of 96.1–99.5% (depending on the platform technology), specificity of 95.7–98.3% and a detection threshold of 100 fg DNA.

A microarray assay has been developed to detect and differentiate between all members of the Orthopoxvirus genus, including ectromelia virus (Ryabinin et al. 2006). The microarray assay targets the *C23L/B29R* and *B19R* gene regions. The microarray assay was reported to be a highly specific and sensitive assay for detection of ectromelia virus; however, the level of complexity required for analysis of the assay, and the lack of validation for identification of virus in extraneous agent testing, prohibits its recommendation at present.

Ectromelia virus is one of the agents targeted in the mouse antibody production (MAP) and IMPACT panel of tests. Use of the MAP test protocol for serological detection of ectromelia virus has limited specificity due to cross-reactivity with other orthopoxviruses. Use of IMPACT for PCR detection of ectromelia virus also has limited sensitivity and specificity, and the assay cannot differentiate ectromelia virus from other orthopoxviruses.

Ectromelia virus is a significant concern for extraneous agent testing as it has been implicated in several outbreaks of disease in the United States due to transmission of virus in biological products (Bhatt et al. 1981; Stang et al. 2005; Trentin 1953; Trentin and Briody 1953).

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 for extraneous agent testing will not be sufficient for primary isolation of ectromelia virus. The 9 CFR guidelines do not include specific testing for ectromelia virus.

The European Pharmacopoeia general monograph *Vaccines for veterinary use* and 5.2.4 *Cell cultures for the production of veterinary vaccines* describe generalised methods for extraneous agent testing using cell culture monolayers that are sensitive to the agent being tested, and detection using specific testing methods that are sensitive to the viral agent. The monographs do not specify details of a culture system or assay for ectromelia virus detection, simply that the methods should be sensitive.

Conclusion

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 are not acceptable.

Primary isolation of ectromelia virus in ME, BEL, CEF, HeLa, L929, MARC-145, MA-104 cells or primary mouse hepatocytes is recommended. Cultures should be incubated at 35 °C for 3 days and passaged 3–7 times. Growth should be confirmed by FA 48–72 hours after infection using vaccinia virus specific antibody.

Specific differentiation of ectromelia virus from other orthopoxviruses is required by molecular diagnosis by the real-time PCR targeting the *F* gene and melt curve analysis of the species specific SNP.

Epizootic haemorrhagic disease virus

Family *Reoviridae*, genus *Orbivirus*

There are 10 serotypes of epizootic haemorrhagic disease virus (EHDV). Primary isolation of EHDV is possible in BHK-21, Vero, CPAE and AA C3/36 cell lines, or by inoculation of ECE (McLaughlin et al. 2003). Direct inoculation of BHK-21 cells has been shown by several studies to be more sensitive than inoculation of ECE for the isolation of EHDV (Aradaib et al. 1995; Pearson et al. 1992). Cell lines created from white-tailed deer are being studied for EHDV culture isolation although published studies are not available at this stage.

Specific testing is required as culture isolation of EHDV is problematic and has low sensitivity for field and non-culture adapted isolates (Work et al. 1992). Specific testing is possible by AC-ELISA, EM (Tsai and Karstad 1970), FA (Jochim and Jones 1987), HA, AGID (Stott and Osburn 1983), VN, plaque reduction assay (Thompson et al. 1988), dot-blot hybridization (Venter et al. 1991), genome-electrophoretotyping, RT-PCR and PCR.

Assays based on immunological detection such as FA, AGID, AC-ELISA, VN and plaque-reduction assay have low sensitivity, and their usefulness is limited both by cross-reactivity with other orbiviruses and limited by specificity for selected serotypes of EHDV (Aradaib et al. 1994). An FA has been developed with improved specificity based on the use of mAbs; however, it is group specific and will not detect all EHDV serotypes (Jochim and Jones 1987). A sandwich AC-ELISA has been developed for EHDV based on detection of the inner coat protein VP7. The ELISA is specific to serogroup 7 and cannot detect all serotypes of EHDV. Poor repeatability of the assay has been addressed by expression of the coating antigen in a baculovirus system (Luo and Sabara 2005). The dot-blot hybridization assay has low sensitivity and is not suitable for extraneous agent testing as it is group-specific (Venter et al. 1991).

A range of molecular assays have been developed for detection of EHDV and are limited in specificity to either serotype 1 or 2 which are prevalent in the United States; RT-PCR targeting genome segment 6 of EHDV serotype 2 (Aradaib et al. 1994); nested RT-PCR assay targeting genome segment 7 of EHDV serotype 2 (Aradaib et al. 1995), RT-PCR assay targeting genome segment 3 of EHDV serotype 2 (Ohashi et al. 1999); and RT-PCR targeting genome segment 10 of EHDV serotype 1 (Aradaib et al. 1998b). The sensitivity of the RT-PCRs is low and adaptation of the assays to include chemiluminescent probe hybridization increased detection limits by 1000 times (Aradaib et al. 1994). In general, the nested RT-PCR and RT-PCR have an unacceptably high rate of false positives reportedly due to contamination, are limited to detection of single serotypes, and will not detect all EHDV serotypes. A Taqman real-time RT-PCR has been developed with comparable sensitivity to the nested RT-PCR that can detect all serotypes of EHDV (Wilson et al. 2009). The Taqman real-time RT-PCR does not suffer from contamination issues as the assay platform is a closed system.

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 for extraneous agent testing will not be sufficient for primary isolation of EHDV.

The European Pharmacopoeia general monograph *Vaccines for veterinary use* and 5.2.4 *Cell cultures for the production of veterinary vaccines* describe generalised methods for extraneous agent testing using cell culture monolayers that are sensitive to the agent being tested, and detection using specific testing methods that are sensitive to the viral agent. The monographs do not specify details of a culture system or assay for EHDV detection, simply that the methods should be sensitive.

Conclusion

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 are not acceptable.

Primary isolation of EHDV in BHK-21 cells is recommended.

Specific testing is required and should be undertaken by Taqman real-time RT-PCR.

Equid herpesvirus 1, 2, 3 and 4

Family *Herpesviridae*, genus *Varicellovirus* (Equid herpesvirus 1, 3 and 4) and *Rhadinovirus* (Equid herpesvirus 2)

Equid (equine) herpesvirus (EHV) can be isolated by culture in susceptible cells of equine origin. Susceptible cell lines are primary equine foetal kidney (EFK) or equine fibroblasts ((equine dermal cells (EDC) or lung)). EHV can be isolated on RK-13, BHK-21, MDBK, PK-15 and SEK but the

sensitivity is lower than for equine-derived lines. Adsorption incubation for 2 hours is required and cultures should be maintained for 14 days between passages. CPE is apparent by 7 days after inoculation and 2 passages are required. EHV has large and small plaque variants in tissue culture and culture detection has poor sensitivity (OIE 2010).

Specific testing in combination with cell culture is required to achieve an acceptable level of sensitivity for the detection of EHV. Specific testing is possible by FA or by nested, semi-nested or real-time PCR.

An FA has been developed for EHV using reference polyclonal antiserum prepared in swine against EHV-1 and conjugated to fluorescein isothiocyanate (FITC). The polyclonal serum is reported to have high affinity and avidity and is recommended for the FA test.

Specific testing by nested PCR is highly sensitive and specific for EHV-1, EHV-2 and EHV-4 detection. Semi-nested PCR assay detection of EHV-1 targeting the glycoprotein H (gH) gene and EHV-4 targeting the gB gene have a detection threshold of 12 and 8 genome copies respectively (Dynon et al. 2001; Kleiboeker and Chapman 2004; Varrasso et al. 2001). A single round PCR has been developed for the detection of EHV-3 and a semi-nested PCR for the detection of EHV-2 (Dynon et al. 2001). The assay has been adapted to a real-time PCR format. A PCR has been described targeting glycoprotein C (gC) of EHV-1 and EHV-4, and a nested PCR targeting gB (Borchers and Slater 1993; Lawrence et al. 1994). Direct comparison of the performance of these assays has not been reported. Molecular detection of EHV by PCR is the most sensitive and specific assay for detection of virus (Dynon et al. 2001).

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 for extraneous agent testing do not provide optimal growth requirements for EHV and are not suitable for primary isolation of EHV. The 9 CFR 113.47: *Detection of extraneous viruses by the fluorescent antibody technique* guidelines describes specific testing for EHV by FA.

The European Pharmacopoeia general monograph *Vaccines for veterinary use* and 5.2.4 *Cell cultures for the production of veterinary vaccines* describe generalised methods for extraneous agent testing using cell culture monolayers that are sensitive to the agent being tested, and detection using specific testing methods that are sensitive to the viral agent. The monographs do not specify details of a culture system or assay for EHV detection, simply that the methods should be sensitive.

Conclusion

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 are not acceptable.

Primary isolation of EHV in equine foetal kidney cells or equine fibroblasts is recommended. Cultures should be incubated for 14 days between passages and 3 passages are required. Adsorption incubation is required for 2 hours.

FA testing for EHV as described in 9 CFR 113.47: *Detection of extraneous viruses by the fluorescent antibody technique* is sufficient for EHV detection following culture isolation.

Specific testing is required in combination with culture isolation for EHV by PCR, nested PCR, semi-nested PCR or real-time PCR targeting the gH gene of EHV-1, EHV-2, EHV-3 and EHV-4.

Equine adenovirus

Family *Adenoviridae*, genus *Mastadenovirus*

Susceptible cell lines for primary isolation of equine adenovirus (EAdV) are foetal equine kidney (FEK), EDC, equine transitional cell carcinoma (ETCC), and equine kidney (EK 269) (Ardans et al. 1974; Harasawa and Higashi 1989; Shahrabadi et al. 1977; Studdert and Blackney 1982).

Eosinophilic inclusion bodies develop in the nucleus within 24 hours of inoculation and form dense crystalline arrays by 36–48 hours.

Different serotypes of EAdV vary in CPE observed in cell culture (Horner and Hunter 1982). EAdV serotype 2 (EAdV-2) is more difficult to isolate in cell culture than is EAdV serotype 1 (EAdV-1).

Specific testing is required for EAdV because of the difficulty of primary isolation for some serotypes of virus and the unreliability of CPE detection. Specific testing is possible by PCR or FA. PCRs targeting the hexon gene of EAdV-1 and EAdV-2 are highly sensitive and specific and are routinely used for the detection of EAdV (Dyndon et al. 2001; Dyndon et al. 2007).

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 for extraneous agent testing do not provide optimal growth requirements for EAdV and are not suitable for primary isolation of EAdV. The 9 CFR guidelines do not require specific testing for EAdV.

The European Pharmacopoeia general monograph *Vaccines for veterinary use* and 5.2.4 *Cell cultures for the production of veterinary vaccines* describe generalised methods for extraneous agent testing using cell culture monolayers that are sensitive to the agent being tested, and detection using specific testing methods that are sensitive to the viral agent. The monographs do not specify details of a culture system or assay for EAdV detection, simply that the methods should be sensitive.

Conclusion

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 are not acceptable.

Primary isolation of EAdV in FEK, EDC, ETCC or EK culture is recommended. Three culture passages are required and infected cultures should be examined for CPE 1–3 days after infection.

Specific testing by PCR targeting the hexon gene is required on both initial samples and on cultures after 3 passages.

Equine arteritis virus

Family *Arteriviridae*, genus *Arterivirus*

Susceptible cell cultures for the primary isolation of equine arteritis virus (EAV) are RK-13, rhesus monkey kidney (LLC-MK2), Vero, and primary rabbit, monkey or equine kidney. RK-13 cells (ATCC no. CCL-37) are most susceptible to infection with EAV (most RK-13 cells have BVDV contamination which increases detection sensitivity for EAV).

Primary isolation of EAV is unreliable and has poor repeatability. The sensitivity of isolation methods is enhanced by using monolayers that are 3–5 days old and RK-13 cells of higher passage

numbers, using a large inoculum size, and by incorporating carboxy methyl cellulose in the overlay medium. Virus is detected by CPE at 2–6 days. Two passages are required (OIE 2010).

The growth characteristics of EAV in RK-13 are different for highly virulent, moderately virulent and avirulent strains (Moore et al. 2002; Moore et al. 2003). CPE is more readily visible and plaque size is large for the more highly virulent strains of EAV.

Specific testing for EAV is required because culture isolation is unreliable and CPE detection is variable. Specific testing is possible by FA or RT-PCR.

An FA has been developed for EAV that uses polyclonal serum or mAbs directed against the nucleocapsid (N) protein and envelope (ENV) proteins.

RT-PCRs have been developed targeting the polymerase (*pol*), *N* and envelope (*env*) genes of EAV. The RT-PCR targeting the 3' end of the P gene is reported to have a detection limit of 2 TCID₅₀ and greater sensitivity than those targeting the *N* and *env* genes (St Laurent et al. 1994). RT-PCR amplification of the *pol* gene is routinely used for diagnosis with resulting high sensitivity and specificity (Dybon et al. 2001). A nested RT-PCR targeting the *pol* gene is reported to have sensitivity and specificity of 100% and 97% respectively (Gilbert et al. 1997b). A real-time Taqman RT-PCR targeting the *N* gene has been developed for EAV and is reported to have comparable sensitivity and specificity with the nested RT-PCR (Balasuriya et al. 2002; Gilbert et al. 1997b). The real-time Taqman RT-PCR can detect as little as 10 genomic copies of EAV (Balasuriya et al. 2002).

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 for extraneous agent testing do not provide optimal growth requirements for EAV and are not suitable for primary isolation of EAV. The 9 CFR 113.47: *Detection of extraneous viruses by the fluorescent antibody technique* guidelines describes specific testing for EAV by FA using either polyclonal serum or mAbs directed against the N protein and ENV proteins.

The European Pharmacopoeia general monograph *Vaccines for veterinary use* and 5.2.4 *Cell cultures for the production of veterinary vaccines* describe generalised methods for extraneous agent testing using cell culture monolayers that are sensitive to the agent being tested, and detection using specific testing methods that are sensitive to the viral agent. The monographs do not specify details of a culture system or assay for EAV detection, simply that the methods should be sensitive.

Conclusion

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 are not acceptable.

Primary isolation of EAV in RK-13 cells is recommended. Cultures should be of high passage numbers and test material should be inoculated onto established monolayers that are 3–5 days old. Three culture passages are required.

FA testing for EAV as described in 9 CFR 113.47: *Detection of extraneous viruses by the fluorescent antibody technique* is sufficient for EAV using either polyclonal serum or mAbs directed against the N protein and ENV proteins.

Specific testing is required by FA or RT-PCR targeting the P gene or real-time Taqman RT-PCR.

Equine encephalitis viruses

Family *Togaviridae*, genus *Alphavirus*

Primary isolation of equine encephalitis viruses (eastern equine encephalitis virus (EEEV), western equine encephalitis virus (WEEV) and Venezuelan equine encephalitis virus (VEEV)) is recommended in CEF, primary duck embryo fibroblasts (DEF), Vero, RK-13 or BHK-21 cells. The appearance of CPE is variable and unreliable and is more readily identified with infection of AA C3/C36 cells than in Vero cells. Culture adaptation of alphaviruses by initial isolation in Vero cells followed by passage in the indicator cell line AA C3/C36 can produce obvious CPE for those isolates that were plaque-negative in Vero cell passage. Confluent monolayers are inoculated with sample and incubated for 7 days. Two blind passages are required and adsorption incubation for 2 hours is required. Cultures demonstrating CPE are freeze-thawed and the cellular lysate is used for specific testing for virus (OIE 2010).

Specific testing for EEEV, WEEV, and VEEV is required due to the difficulty of detecting CPE in infected cultures. Specific testing for EEEV, WEEV and VEEV is possible by direct FA or EIA on infected monolayers or by testing of the cellular lysate by RT-PCR targeting the *ca* gene (Kramer et al. 2002).

FA is performed on EEEV, WEEV, and VEEV infected Vero cells using mAbs at 2 days after infection for EEEV, WEEV and VEEV.

FA will detect focus forming units (FFU) for non-cytolytic infections.

The EIA for EEEV, WEEV and VEEV has poor sensitivity and has a high incidence of false positives reported by surveillance programs. The sensitivity of EIA is greater for virus isolated by a 2-stage process on Vero cell cultures and AA C3/C36, than virus isolated only on AA C3/C36. Sensitivity is also increased by the inclusion of multiple culture passages. The antigen capture enzyme immunoassay (AC-EIA) is performed without culture isolation and has the lowest sensitivity.

There are a number of RT-PCRs that have been developed for detection of EEEV, WEEV and VEEV. A genus specific RT-PCR for detection of EEEV, WEEV and VEEV has been developed with a markedly higher sensitivity than primary isolation alone, and higher sensitivity than EIA or FA.

Single-species specific EEEV and WEEV real-time Taqman RT-PCRs targeting a region of the *NSP 1* gene are reported to have the highest sensitivity and specificity across a wide range of isolates (Huang et al. 2001; Lambert et al. 2003; Linssen et al. 2000; Strizki and Repik 1996).

Coupling of the RT-PCR with a colorimetric dot blot assay increased the sensitivity of the standard RT-PCR by 250 times (Armstrong et al. 1995).

A genus specific semi-nested RT-PCR has been developed that targets a region of the *NSP 1* gene conserved among all alphaviruses; however, this technique has lower sensitivity for detection than other species specific RT-PCRs (Pfeffer et al. 1997). This method has been adapted to a RT-PCR-ELISA format utilising species specific probes that have slightly higher detection sensitivity (Wang et al. 2006).

Multiplex RT-PCR for the simultaneous detection of a range of encephalitis viruses significantly reduces the sensitivity of detection relative to single RT-PCRs and is therefore not recommended.

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 for extraneous agent testing do not provide optimal growth requirements for equine encephalitis and are not suitable for primary isolation of WEEV, EEEV, and VEEV. The 9 CFR guidelines do not require specific testing for WEEV, EEEV, and VEEV.

The European Pharmacopoeia general monograph *Vaccines for veterinary use* and 5.2.4 *Cell cultures for the production of veterinary vaccines* describe generalised methods for extraneous agent testing using cell culture monolayers that are sensitive to the agent being tested, and detection using specific testing methods that are sensitive to the viral agent. The monographs do not specify details of a culture system or assay for WEEV, EEEV, and VEEV detection, simply that the methods should be sensitive.

Conclusion

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 are not acceptable.

A 2-stage primary isolation for WEEV, EEEV and VEEV is recommended. The first stage is inoculation and blind passage in Vero cells, followed by a second stage passage in the AA C3/C36 indicator cell line and detection by CPE. Adsorption incubation of 2 hours is required and cultures should be maintained for 7 days and passaged 3 times.

Specific testing for EEEV, WEEV and VEEV is required by the genus specific semi-nested RT-PCR targeting conserved sequence of the *NSP 1* gene, combined with single-species specific EEEV and WEEV real-time Taqman RT-PCRs targeting the *NSP 1* gene (a comparable assay for VEEV has not been published).

Equine infectious anaemia virus

Family *Retroviridae*, genus *Lentivirus*

Virus isolation is performed in primary equine leukocytes which is a difficult technique with a low success rate. Isolation of equine infectious anaemia virus (EIAV) on equine monocyte-derived macrophages (MDM) has a higher success rate than other culture systems (Raabe et al. 1998). CPE is evident within 12 days and 3 blind passages are required before assigning negative status to cultures. Infected cultures are detected by AC-ELISA, FA, PCR, RT-PCR, AGID or RT activity assay.

Virus isolation is not recommended for EIAV due to the difficulty of culturing the equine leukocytes and the poor success rate, therefore specific testing is required (OIE 2010).

EIAV is a significant biosecurity risk as there have been numerous outbreaks of equine infectious anaemia internationally resulting from contamination of biological materials. An example is the 2006 outbreak of equine infectious anaemia in Ireland originating from contaminated plasma from Italy (Quinlivan et al. 2007). The incidence of contamination is high due to the fact that EIAV infected horses establish life-long persistent viraemia.

Specific assays to detect EIAV include RT-PCR and PCR, RT activity assay, and AGID. A real-time Taqman RT-PCR has been developed targeting the highly conserved sequence of the core

protein of GAG polyprotein (Quinlivan et al. 2007). OIE recommended standard detection of low levels of genome is by a nested PCR for proviral DNA which is a rapid, sensitive and specific assay (Nagarajan and Simard 2001). The sensitivity of all molecular assays is limited by the high genetic variability of the EIAV genome. The most conserved regions of the *gag* gene have 80–91.5% sequence conservation between North American and Asian isolates (Quinlivan et al. 2007).

The AGID test detects the p29 GAG antigen in a microwell format (Nagarajan and Simard 2001) and the AC-ELISA detects the p26 CA protein (Shane et al. 1984).

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 for extraneous agent testing are not suitable for primary isolation of EIAV. The 9 CFR guidelines do not require specific testing for EIAV.

The European Pharmacopoeia general monograph *Vaccines for veterinary use* and 5.2.4 *Cell cultures for the production of veterinary vaccines* describe generalised methods for extraneous agent testing using cell culture monolayers that are sensitive to the agent being tested, and detection using specific testing methods that are sensitive to the viral agent. The monographs do not specify details of a culture system or assay for EIAV detection, simply that the methods should be sensitive.

Conclusion

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 are not acceptable.

Culture isolation for EIAV is not recommended.

Specific testing is required. A PERT assay is required as the initial diagnostic assay for EIAV, combined with nested PCR for detection of proviral DNA (André et al. 2000; Graves et al. 1977; Reichert et al. 1992).

Equine influenza virus

Family *Orthomyxoviridae*, genus *Influenzavirus A*

Primary isolation of equine influenza virus (EIV) is recommended in both MDCK cells and in ECE. Both culture systems are necessary because some variant H3N8 viruses will only grow in MDCK cells whilst other variants will only grow in eggs. Virus is isolated by allantoic inoculation of ECE that is 10–11 days old. Allantoic fluid is tested for HA activity using chicken or guinea pig red blood cells (RBCs). MDCK cultures are maintained for 7 days and passaged 5 times. Cultures are assessed for CPE daily. CPE is evident as cytolytic plaque formation. Serum-free medium is used for EIV isolation in MDCK (OIE 2010).

Specific testing is required because of the difficulty of culture isolation for some variant strains. Specific testing for EIV is by HA, virus neutralisation (VN), EIA, ELISA, FA, radioimmunoassay (RIA) or RT-PCR.

The Directigen FluA ELISA has poor performance and the sensitivity of the assay is variable (Quinlivan et al. 2004). The Directigen FluA kit detects viral antigen associated with cell membranes more readily than free virus in solution due to changes in viral structural proteins at viral attachment to cellular receptors (Cherian et al. 1994; Hermann et al. 2006; Ryan-Poirier et al. 1992).

Quinlivan (2004) conducted a systematic study comparing the sensitivity of virus isolation in MDCK and ECE, Directigen FluA ELISA, EIA and RT-PCR using nested primers targeting the *N* gene (Oxburgh and Hagström 1999) or a single RT-PCR targeting the *M* gene. RT-PCR targeting the *M* gene (Fouchier et al. 2000) combined with virus isolation in ECE was the most sensitive detection method. Directigen FluA ELISA had the lowest detection sensitivity. The RT-PCR targeting the *M* gene has been adapted to a SYBR green real-time RT-PCR with a 20% increase in detection sensitivity (Quinlivan et al. 2007). A second real-time Taqman RT-PCR targeting the HA2 domain has been developed (Sugita and Matsumura 2003). The threshold for detection is 10 median egg infective doses (EID₅₀) and the assay has greater sensitivity and specificity when directly compared to the RT-PCR and virus isolation in ECE. The 2 real-time RT-PCRs have not been compared directly for performance.

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 for extraneous agent testing do not provide the optimal growth requirements for EIV and are not suitable for primary isolation of EIV. The protocols 9 CFR 113.37: *Detection of pathogens by the chicken embryo inoculation test* and 9 CFR 113.34: *Detection of haemagglutinating viruses* describing the isolation of virus in ECE and detection by HA activity are suitable for EIV detection, but alone are not sufficient to detect all isolates of EIV.

The European Pharmacopoeia general monograph *Vaccines for veterinary use* and 5.2.4 *Cell cultures for the production of veterinary vaccines* describe generalised methods for extraneous agent testing using cell culture monolayers that are sensitive to the agent being tested, and detection using specific testing methods that are sensitive to the viral agent. The monographs do not specify details of a culture system or assay for EIV detection, simply that the methods should be sensitive.

Conclusion

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 are not acceptable.

The protocols 9 CFR 113.37: *Detection of pathogens by the chicken embryo inoculation test* and 9 CFR 113.34: *Detection of haemagglutinating viruses* describing the isolation of virus in ECE and detection by HA activity are suitable for detection of all isolates of EIV only if used in combination.

Primary isolation of EIV in both MDCK cells and by allantoic inoculation of ECE that is 10–11 days old is recommended. Allantoic fluid from infected ECE is tested for HA activity against chicken or guinea pig RBCs. MDCK cultures should be maintained for 7 days, passaged 5 times and observed daily for evidence of CPE. Serum supplements in the medium should be free of EIV reactivity.

Specific testing is required by either SYBR green real-time RT-PCR targeting the *M* gene or real-time Taqman RT-PCR targeting the HA2 domain.

Feline calicivirus

Family *Caliciviridae*, genus *Vesivirus*

Primary isolation of feline calicivirus (FCV) is possible in CRFK, FE and kitten kidney (KK) cells (Milek et al. 1976; Tham and Studdert 1986). Primary KK cultures are prepared from 1 week old kittens (Hara et al. 1976). The susceptibility of FE and KK cell cultures to FCV infection varies significantly between different preparations of primary cultures (Tham and Studdert 1986). Adsorption incubation for 1 hour is required for efficient infection with FCV. Attachment and viral

entry is most efficient for cells in suspension rather than monolayers, following trypsinization of cells, and at incubation temperatures of 4 °C rather than 37 °C (Kreutz et al. 1994). The replication cycle is short in CRFK cells and viral growth peaks at 8–12 hours after inoculation and thereafter titres decline. Three blind passages are required to demonstrate CPE for some isolates (Ossiboff et al. 2007). CPE is readily evident as cytolysis, rounding and detachment of cells, aggregation of cells, cytoplasmic paracrystalline arrays of virus, and plaque formation in monolayers. Infected cultures can be detected by plaque assay using agar overlays 3 days after infection of monolayers (Hara 1974; Ormerod and Jarrett 1978; Studdert and O'Shea 1975). FCV has 4 categories of plaque variants and the minute plaque variant is subject to inhibition by components of the agar overlay. FCV titres in culture are increased by co-infection with feline herpesvirus 1 (FHV-1) leading to a change in the sensitivity of primary isolation for detection of FCV (Wooley et al. 1976).

Specific testing is required as primary isolation in culture is unreliable.

CPE can be confirmed by FA using mAbs to FCV CA (Carter et al. 1989; Hara et al. 1974). A panel of mAbs raised against FCV antigen identified a single mAb that was reactive with a conserved antigenic determinant for all isolates and therefore has a broad range for detection of FCV (Tohya et al. 1991). A surface-enhanced Raman scattering (SERS) immunoassay has been developed for FCV detection. The SERS immunoassay uses a microchip platform coated with anti-FCV mAbs to capture viral antigen. The detection limit for the SERS immunoassay is 1 virus particle/μl (Driskell et al. 2005).

A RT-PCR has been developed targeting the hypervariable region of the *ca* gene and the assay has been further developed for strain typing using RT-PCR restriction endonuclease (RT-PCR-RE) (Sykes et al. 1998; Sykes et al. 2001). The RT-PCR has been validated for clinical and culture strains, and was found to be at least as sensitive as base-line culture methods of detection (Sykes et al. 1998). A nested RT-PCR has been developed targeting the *ca* gene (Marsilio et al. 2005; Radford et al. 1999). The nested RT-PCR was validated against 87 field samples and the relative sensitivity was greater than both virus isolation and RT-PCR; however, the assay is subject to false negatives due to cross contamination.

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 for extraneous agent testing do not provide optimal growth conditions for FCV and will not be sufficient for primary isolation of FCV. The 9 CFR guidelines do not include specific testing for FCV.

The European Pharmacopoeia general monograph *Vaccines for veterinary use* and 5.2.4 *Cell cultures for the production of veterinary vaccines* describe generalised methods for extraneous agent testing using cell culture monolayers that are sensitive to the agent being tested, and detection using specific testing methods that are sensitive to the viral agent. The monographs do not specify details of a culture system or assay for FCV detection, simply that the methods should be sensitive.

Conclusion

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 are not acceptable.

Primary isolation of FCV in CRFK is recommended. Cultures require adsorption incubation for 1 hour and cells should be inoculated in suspension rather than monolayers, following trypsinization of cells, and at incubation temperatures of 4 °C. Three culture passages are required followed by detection of virus by the appearance of CPE.

Specific testing is required for FCV by either SERS immunoassay or RT-PCR targeting the *ca* gene.

Felid herpesvirus 1

Family *Herpesviridae*, genus *Varicellovirus*

Primary isolation of felid herpesvirus 1 (FHV-1), also known as feline rhinotracheitis virus, is possible in CRFK, FEF, and primary feline kidney cells (Churchill 1982; Horimoto et al. 1992; Slater and York 1997). CPE may not be apparent for long periods of cultivation (may not appear for up to 64 days of cultivation). Feline kidney cell culture can establish persistent latent infections from which infectious virus cannot be recovered but can be induced to reactivate.

Specific testing is required for FHV-1 because of the difficulties of culture isolation. Specific testing is possible by ISH, HA, FA, VN, ELISA, RT-PCR for latency associated transcripts (LATs), PCR and real-time Taqman PCR.

Six different PCR and nested PCRs have been developed for detection of FHV-1 all targeting the *tk* gene sequence (Burgesser et al. 1999; Hara et al. 1996; Reubel et al. 1993; Stiles et al. 1997; Sykes et al. 1997; Vogtlin et al. 2002; Weigler et al. 1997). The *tk* PCR is reported to be more sensitive than virus isolation or FA (Burgesser et al. 1999; Maggs and Clarke 2005; Stiles et al. 1997; Sykes et al. 1997). The relative sensitivity of these 6 assays was evaluated concurrently for vaccine and clinical samples (Maggs and Clarke 2005). The study demonstrated marked variability in the detection sensitivities of the different assays and the range in detection limits was 3 logs dilution. The nested PCR was found to have the greatest detection sensitivity for both clinical and culture samples (Stiles et al. 1997).

A real-time Taqman PCR was developed targeting the gB gene sequence of FHV-1. The specificity of the assay was demonstrated for a range of herpesvirus isolates. The assay was able to detect European, American and vaccine isolates of FHV-1. The real-time Taqman PCR was directly compared with virus isolation in CRFK cell culture and conventional PCR. The detection limit for real-time Taqman PCR, virus isolation and PCR was 0.06 TCID₅₀/sample, with 100% detection for 0.06 TCID₅₀/sample, 60 TCID₅₀/sample and 6 TCID₅₀/sample respectively.

FHV produces LATs during persistent non-cytolytic infection in cell cultures (Townsend et al. 2004). Sensitive and specific detection of LATs by RT-PCR is recommended for detection of persistent and inapparent culture contamination.

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 for extraneous agent testing will not be sufficient for primary isolation of FHV-1. The 9 CFR guidelines do not include specific testing for FHV-1.

The European Pharmacopoeia general monograph *Vaccines for veterinary use* and 5.2.4 *Cell cultures for the production of veterinary vaccines* describe generalised methods for extraneous agent testing using cell culture monolayers that are sensitive to the agent being tested, and detection using specific testing methods that are sensitive to the viral agent. The monographs do not specify details of a culture system or assay for FHV-1 detection, simply that the methods should be sensitive.

Conclusion

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 are not acceptable.

Primary isolation of FHV-1 in CRFK cell culture is recommended.

Specific testing is required for FHV-1 by real-time Taqman PCR targeting the gB gene sequence. RT-PCR to detect LATs is recommended to detect inapparent latent culture infections with FHV-1.

Feline immunodeficiency virus

Family *Retroviridae*, genus *Lentivirus*

Primary isolation of feline immunodeficiency virus (FIV) in cell culture is problematic. FIV isolates are divisible into primary and cell culture adapted isolates. Adaptation of FIV to replicate in CRFK is accompanied by characteristic mutations in the ENV glycoprotein to allow binding of the virus to the host cell receptor CXCR4. Non-culture adapted FIV is difficult to isolate and difficult to identify. Culture adapted FIV can be propagated in CRFK, feline T-lymphoblastoid (LSA-1 and MYA-1) and peritoneal macrophages, (Brunner and Pedersen 1989; Miyazawa et al. 1989; Yamamoto et al. 1986). Co-cultivated feline dendritic cells (DC) and PBMC are reported to be more sensitive than other culture systems for FIV (Fletcher et al. 2006; Freer et al. 2007; van der Meer et al. 2007). The characteristics of the DC and PBMC culture system will vary between preparations and therefore the assay system is expected to have poor repeatability. The FIV LTR is a strong basal promoter and its activity as determined by reporter assays in different cell lines is an indication of infectivity by FIV in those cells (Mustafa et al. 2005). Based on LTR reporter assays feline cell lines have high levels of LTR activity; however, there are also basal levels of activity in simian CV-1 cell line transformed with SV40 (COS) indicating some susceptibility to infection. FIV infection of cell monolayers is enhanced by the use of a spinoculation procedure during the adsorption phase of the infection (Freer et al. 2007).

CRFK cell cultures can become contaminated with persistent non-cytolytic FIV infection (Stephens et al. 1991). Specific testing is required to ensure detection of persistent infections with FIV and to identify non-culturable FIV. Co-infection of cultures with FIV and feline leukaemia virus (FeLV) increases the quantity of FIV DNA by 10 times and therefore has a significant impact on the detection sensitivity of molecular assays (Torten et al. 1990).

Specific testing is required for FIV because culture isolation is problematic for FIV. Specific assays for FIV are possible by AC- ELISA, FA, ISH, ICA, PCR-Southern blot and real-time Taqman PCRs.

An FA has been developed using murine mAbs 3B7 and 1C11 directed against the *gag* gene products, and recognising the p24 CA protein expression in the cytoplasm of infected cells (Feveriere et al. 1991). Assays using monoclonal antibodies to target structural proteins of FIV will be limited in their application to detection of particular stages of the viral lifecycle as the GAG polyprotein undergoes a series of proteolytic and conformational changes in the period from translation to release and maturation of the virus (Egberink et al. 1990; Steinman et al. 1990; Talbott et al. 1989). This limitation will reduce the sensitivity of the assay to detection of the cleaved p24 protein, present only in the mature virion at the point of virus budding and release.

An ISH assay has been developed utilising a biotin-labelled RNA probe complementary to the *gag* gene sequence for detection of virus infected cultures (Ryan et al. 2006). The ISH assay is dependent on successful propagation of the virus in MYA-1 cells and for many test isolates cell cultivation is unlikely to be successful.

ELISA and ICA assays are reported to have lower sensitivity than molecular methods of detection.

A nested PCR combined with Southern blot has been used for detection of FIV provirus DNA in infected cell cultures (Jordan et al. 1995). The assay targets the FIV provirus p26 fragment and Southern blot analysis was used to confirm FIV detection. The assay was evaluated for FIV detection in semen and has not been validated for extraneous agent testing. A study examining the performance of PCR detection of FIV proviral DNA in laboratories across the United States and Canada reported assay sensitivities in the range of 41–93% and specificities in the range of 81–100% (Bienzle et al. 2004; Crawford et al. 2005; Mortola et al. 2004; Steinrigl and Klein 2004). The high frequency of false negatives and the poor repeatability of PCR methods for FIV detection is a significant issue. The high degree of genetic heterogeneity is considered to result in problems of primer mismatch and false negative PCR results for FIV (Kyaw-Tanner and Robinson 1996; Steinrigl and Klein 2004).

Real-time Taqman PCRs developed for detection of FIV proviral DNA target sequence encoding the GAG polyprotein (Leutenegger et al. 1999). The assay was determined to be 100% specific for FIV based on assaying 33 extraneous agents, and the sensitivity was evaluated for 55 FIV isolates. The detection limit was a single copy of template and the assay was highly repeatable. The real-time Taqman PCR for FIV has been evaluated using Bayesian statistical methods on results from 490 samples to establish a median sensitivity estimate of 0.92 and median specificity estimate of 0.99 (Leutenegger et al. 1999; Pinches et al. 2007; Sondgeroth et al. 2005).

Although there are no reports of its usage for FIV detection the PERT assay recommended for detection of other retroviruses could be applied for diagnosis in this case.

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 for extraneous agent testing will not be sufficient for primary isolation of FIV. The 9 CFR guidelines do not include specific testing for FIV.

The European Pharmacopoeia general monograph *Vaccines for veterinary use* and 5.2.4 *Cell cultures for the production of veterinary vaccines* describe generalised methods for extraneous agent testing using cell culture monolayers that are sensitive to the agent being tested, and detection using specific testing methods that are sensitive to the viral agent. The monographs do not specify details of a culture system or assay for FIV detection, simply that the methods should be sensitive.

Conclusion

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 are not acceptable.

Primary isolation of FIV is not recommended.

Specific testing is required. A PERT assay is required as the initial diagnostic assay for FIV detection in conjunction with a real-time Taqman PCR in parallel for detection of proviral DNA. Attempted isolation, confirmation and identification of retrovirus positives is then required by animal isolation or sequencing of PCR products.

Feline infectious peritonitis virus

Family *Coronaviridae*, genus *Coronavirus*

Primary isolation of feline infectious peritonitis virus (FIPV) is possible in CRFK, Vero, FE and feline kidney, lung and alveolar macrophages. CRFK is considered most susceptible to infection. Cytolytic plaques, giant cell formation and intra-nuclear and intra-cytoplasmic inclusions are seen

in CRFK cells 48 hours after infection. Infectivity of CRFK cells with FIPV is increased by low-speed centrifugation of culture plates at 400 g for 2 hours during the adsorption incubation in a small volume at 37 °C (spinoculation).

There are 2 serotypes of FIPV: serotype 1 being more difficult to cultivate than serotype 2. Culture isolation of FIPV has low sensitivity and most attempts to isolate clinical isolates have failed (Boyle et al. 1984; Hohdatsu et al. 1992; Pedersen et al. 1984). FIPV infections at low MOI typically result in persistent latent infection that is difficult to detect in cell culture.

Specific testing is therefore required for FIPV detection and is possible by RT-PCR and IP (Gunn-Moore et al. 1998).

RT-PCR has been developed targeting the 3' UTR for detection of the feline coronavirus (FCoV) genome (FCoV and FIPV) (Gunn-Moore et al. 1998; Lai and Cavanagh 1997; Li and Scott 1994). The assay sensitivity was increased by combining it with a hybridization probe.

A real-time SYBR green RT-PCR has been developed targeting the open reading frame (ORF) 1b for the generic detection of all coronaviruses, including FIPV (Escutenaire et al. 2007).

Specific testing by RT-PCR or IP assay for viral foci has been shown to improve the sensitivity of detection, in particular for FIPV serotype 1 and for persistent non-cytolytic infections.

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 for extraneous agent testing do not provide optimal growth conditions for FIPV and will not be sufficient for primary isolation of FIPV. The 9 CFR 113.47: *Detection of extraneous viruses by the fluorescent antibody technique* protocol includes FA testing for FIPV. The FA for FIPV is not sufficiently sensitive for extraneous agent testing.

The European Pharmacopoeia general monograph *Vaccines for veterinary use* and 5.2.4 *Cell cultures for the production of veterinary vaccines* describe generalised methods for extraneous agent testing using cell culture monolayers that are sensitive to the agent being tested, and detection using specific testing methods that are sensitive to the viral agent. The monographs do not specify details of a culture system or assay for FIPV detection, simply that the methods should be sensitive.

Conclusion

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 are not acceptable.

Primary isolation of FIPV in CRFK is recommended. Adsorption incubation using a spinoculation technique is required.

FA testing for FIPV as described in 9 CFR 113.47: *Detection of extraneous viruses by the fluorescent antibody technique* is not sufficiently sensitive for extraneous agent testing.

Specific testing is required for FIPV by RT-PCR targeting the 3' UTR of FCoV.

Feline leukaemia virus

Family *Retroviridae*, genus *Gammaretrovirus*

Primary isolation of FeLV is difficult and has a poor success rate. Primary isolation of FeLV can be attempted in the Moloney-murine sarcoma virus (M-MSV) transformed continuous line of cat cells (CCC), such as subclones 81, 89 and 8c. Super-infection of these cell lines with FeLV leads to lytic plaque formation. The resulting virus when passaged twice through FEF cells results in recovery of a M-MSV pseudo-typed virus that can be quantitatively titrated as an indicator assay, and there is complete restriction of CCC virus growth (Fischinger et al. 1974; Fujino et al. 2004). Lytic FFU for the pseudo-typed virus correlate with FFU for FeLV and are reported to be 2 times lower than the replicating FeLV virus titre. This method is highly suitable as an indicator assay for FeLV in test samples as it results in a form of endogenous retrovirus which can be propagated, harvested and quantified. Pseudo-typing of retroviruses as an indicator assay is widely used in an experimental setting but has not been evaluated for extraneous agent testing for retroviruses.

Because of the difficulty of primary isolation of FeLV specific testing is recommended.

Specific assays for FeLV include AC-ELISA, FA, ICA, PCR for proviral DNA, RT-PCR for viral RNA and real-time Taqman PCRs.

An AC-ELISA has been developed for detection of FeLV targeting the p27 GAG processing intermediate protein (Lutz et al. 1983). FAs developed using mAbs specific for GAG detection are used for FeLV detection, but show cross-reactivity with FIV antigens (Hardy, Jr. 1993). The AC-ELISA and FAs are dependent on the isolation of FeLV in cell culture and as culture isolation is problematic these assays are not recommended.

A real-time Taqman PCR has been developed for detection of all 3 serotypes FeLV-A, FeLV-B and FeLV-C targeting the unique short region of the LTR of FeLV (Tandon et al. 2005). The detection limit of the assay was 1 proviral copy of FeLV; the assay was highly precise and specific to FeLV. There was a high frequency of false negatives when the assay was evaluated for detection of FeLV in experimentally infected cats.

Although there are no reports of its usage for FeLV detection the PERT assay recommended for detection of other retroviruses could be applied for detection of FeLV.

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 for extraneous agent testing will not be sufficient for primary isolation of FeLV. The 9 CFR guidelines do not include specific testing for FeLV.

The European Pharmacopoeia general monograph *Vaccines for veterinary use* and 5.2.4 *Cell cultures for the production of veterinary vaccines* describe generalised methods for extraneous agent testing using cell culture monolayers that are sensitive to the agent being tested, and detection using specific testing methods that are sensitive to the viral agent. The monographs do not specify details of a culture system or assay for FeLV detection, simply that the methods should be sensitive.

Conclusion

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 are not acceptable.

Primary isolation of FeLV is not recommended.

Specific testing is required. A PERT assay is required as the initial diagnostic assay for FeLV detection in conjunction with a real-time Taqman PCR for detection of proviral DNA. Attempted isolation, confirmation and identification of retrovirus positives is then required by animal isolation or sequencing of PCR products.

Feline panleukopaemia virus

Family *Parvoviridae*, genus *Parvovirus*

Primary isolation of feline panleukopaemia virus (FPV) is possible in CRFK, feline tongue cell line (Fc3Tg (ATCC no. CCL-176)), feline lung cell line (AK-D (ATCC no. CCL 150)), or in mitogen-stimulated feline PBL (Parrish and Carmichael 1986). CRFK is the most susceptible cell line for isolation of FPV and yields the highest viral titres. FPV and CPV are considered host range variants, as FPV replicates efficiently only in feline cell lines but not canine cell lines and CPV replicates in both feline and canine cell lines (Flagstad 1973; Flagstad 1975; Spitzer et al. 1997; Truyen and Parrish 1992).

Freshly seeded cells with a high mitotic index are more sensitive to FPV infection than established monolayers (Truyen and Parrish 1992). Seeding cell monolayers at low concentrations and using optimal MOI is important to achieving sensitive detection. CPE is evident as transient intranuclear inclusions at around 3 days after inoculation. Diagnosis of FPV by detection of CPE is unreliable because of the transient nature of inclusions and therefore specific testing is required.

Specific testing is possible by FA, ICA, HA, ISH, PCR and real-time Taqman PCR.

FPV and CPV share 98% sequence homology at the nucleotide level and a generic PCR has been developed for carnivore parvoviruses (Liu et al. 2001; Mochizuki 2006). The FA, HA, ISH and PCR cannot differentiate between CPV and FPV (Decaro et al. 2008). HA using FPV-specific conformational monoclonal antibodies can differentiate FPV from CPV; however, the assay sensitivity is too low to be useful for extraneous agent testing.

A real-time Taqman PCR has been developed for detection of FPV and CPV and uses a MGB probe targeting a single polymorphism in the VP2 gene to differentiate between FPV and CPV (Decaro et al. 2008). The assay has been shown to be 100% specific to FPV, the detection sensitivity was 10 copies of DNA, and the assay has very low inter- and intra-assay variability and is highly reproducible. The assay was validated against 75 field samples, samples from experimental infections and vaccine strains.

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 for extraneous agent testing do not provide optimal growth conditions for FPV and will not be sufficient for primary isolation of FPV. The 9 CFR 113.47: *Detection of extraneous viruses by the fluorescent antibody technique* protocol includes FA testing for FPV. The FA for FPV is not sufficiently sensitive for extraneous agent testing.

The European Pharmacopoeia general monograph *Vaccines for veterinary use* and 5.2.4 *Cell cultures for the production of veterinary vaccines* describe generalised methods for extraneous agent testing using cell culture monolayers that are sensitive to the agent being tested, and detection using specific testing methods that are sensitive to the viral agent. The monographs do not specify details of a culture system or assay for FPV detection, simply that the methods should be sensitive.

Conclusion

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 are not acceptable.

Primary isolation of FPV in the CRFK cell line is recommended. Cell cultures should be freshly seeded and have a high mitotic index. Inoculation should be onto sub-confluent monolayers. Confirmation of CPE is by staining of the inclusions using an FA.

FA testing for FPV as described in 9 CFR 113.47: *Detection of extraneous viruses by the fluorescent antibody technique* is not sufficiently sensitive for extraneous agent testing.

Specific testing is required for FPV by real-time Taqman PCR using an FPV-specific MGB probe targeting the VP2 gene.

Foot-and-mouth disease virus

Family *Picornaviridae*, genus *Aphthovirus*

Sensitive cell culture systems for the primary isolation of foot-and-mouth disease virus (FMDV) include primary bovine calf thyroid cells, primary pig, calf or lamb kidney cells, and unweaned mice that are 2–7 days old. Established cell lines, such as BHK-21 and porcine kidney (IB-RS-2) may be used but are generally less sensitive than primary cells for detecting low amounts of infectivity. Primary bovine calf thyroid cells are recommended as the most sensitive means of detection of FMDV; however, there are several problems associated with the use of these cell lines. Monolayers of primary bovine calf thyroid cells are mixtures of epithelial cells which are highly susceptible to FMDV and fibroblasts which are not susceptible. For isolation of samples with a low titre of FMDV this could be problematic due to the tendency for the fibroblasts to overgrow the epithelial cells with each successive passage. CPE and plaque formation can also be difficult to detect due to the heterogeneous and irregular monolayer formation of the primary cell cultures. These problems can be mitigated by the use of monolayers of primary bovine calf thyroid cells with minimal passaging to reduce the likelihood of overgrowth of epithelial cells by fibroblasts and to increase the uniformity of the monolayer so plaque formations is more readily identified. Concurrent FMDV isolation in IB-RS-2 is required to detect highly pig-adapted strains of the virus.

There are several established cell lines suitable for FMDV culture: BHK-21, IB-RS-2, (HmLu-1) and hamster embryo (NIL-2 or IFFA-3) (Barteling 2002; Clarke and Spier 1980; Radlett 1987). Vero cells are not susceptible to FMDV infectivity. The OIE recommendations for FMDV isolation include the BHK-21 and IB-RS-2 cell lines for FMD primary isolation with the caveat that they are “*generally less sensitive* than primary cells for detecting low amounts of infectivity”. This caveat is included as a consequence of the numerous reports of working stocks of BHK-21 cell lines sourced from different laboratories with pleomorphic culture characteristics and varying susceptibility to FMDV infection across the range of FMDV subtypes.

Clarke and Spier (1980) published a systematic study of BHK-21 cell lines for susceptibility to FMDV isolates of O, C and Asia serotypes. In this study the BHK-21 cell lines were sourced from a range of different laboratories and had varying passage numbers. All 3 subtypes O, C and Asia could be isolated from at least 1 BHK-21 cell culture source. BHK-21 cultures of higher passage number also demonstrated a decline in susceptibility to FMDV infection. In this study BHK-21 cell lines designated Brescia (Italy), ICRF (London), Lelystad (Netherlands), Wellcome foundation (Pirbright), Wellcome foundation (Spain), Wellcome foundation (West Germany) had 0% sensitivity for C and Asia subtypes, whereas BHK-21 cell lines designated AVRI (Pirbright), Padua

(Italy), Pan American FMD Centre (Brazil), Razi Institute (Iran) and Nong Serai (Thailand) had 100% detection (n=10) for O, C and Asia subtypes. Relative numbers of susceptible and unsuspensible cells in the BHK-21 population determine the overall culture susceptibility. Heterogeneity of the BHK-21 cell line stocks could be demonstrated by the capacity to clonally select for susceptibility to FMDV infection. It is important to note that BHK-21 cells sourced from ATCC (ATCC no. CCL-10) do not specify FMDV susceptibility.

There are reports of local differences in media and handling of the BHK-21 cell line resulting in changes in the cell line characteristics, and it is considered that no source of BHK-21 cell line is universally more susceptible or productive than another and susceptibility to FMDV infection is best managed by instituting culture guidelines and testing (Radlett 1987). The BHK-21 cell line is used internationally for the propagation of 50% of FMDV vaccines (~1000 million doses annually) since the 1960's, when there was a shift away from FMDV propagation in primary cultures due to biosafety concerns. Therefore considerable knowledge exists regarding FMDV susceptibility of BHK-21 stocks within the vaccine industry and there are numerous characterized BHK-21 stocks held by pharmaceutical companies. Pirbright FMDV laboratories, Coopers Animal Health, Ash Road, Woking, Surrey, England maintains BHK-21 seed stocks with broad and well-defined FMDV susceptibility. The unpredictable nature of FMDV-susceptibility has been managed in these situations by verification of susceptibility of BHK-21 stocks using appropriate FMDV subtype standards. Working cell banks have been established for BHK-21 cells of known susceptibility so as to minimise variability in the cell lines introduced through passaging. The establishment of such working cell banks is a priority of the OIE *Global Initiative for FMD*.

BHK-21 cell lines with defined specifications would be considered to be highly sensitive for FMDV isolation. Specifications for suitable BHK-21 cell lines would be:

- (i) BHK-21 cell sourced from a physical containment level 4 (PC4) licensed facility with the capacity for FMDV infectivity assays
- (ii) BHK-21 cell lines that have been tested at this facility for FMDV infectivity for subtypes O, A, C, Asia and SAT-1, SAT-2 and SAT-3
- (iii) maintenance of stable susceptibility of the BHK-21 cell line can be demonstrated by the source facility after 5 passages
- (iv) BHK-21 cells used in the assay are no more than 5 passages beyond the passage with confirmed FMDV susceptibility testing
- (v) the culture conditions (medium, serum, culture volume and culture vessels) used by the source facility are adopted by the testing facility to minimise culture-induced changes in the cell line, and
- (vi) sensitivity of FMDV detection in the BHK-21 clonal line is at least $10^{2.3}$ TCID₅₀/ml for all subtypes (Amaral-Doel et al. 1993).

Specified clones of BHK-21 sensitive to FMDV may be difficult for testing laboratories to access from the working cell banks established by *The Global Initiative for FMD* program. On this basis the specified BHK-21 cell lines cannot be included as a general testing requirement for FMDV at this point in time.

FMDV infection should be performed on monolayer cell cultures as Clarke and Spier (1980) showed that suspension cultures were less sensitive than monolayers. Inoculation should be of primary monolayers of calf thyroid cells established from the initial plating of cells. FMDV is rapidly cytolitic and in general viral plaques are evident after overnight culture. Inoculated monolayers are incubated for 48 hours between passages and examined for CPE after 48 hours.

Cells are then freeze-thawed and used to inoculate fresh culture and examined for CPE after a further 48 hours. Cultures should be maintained for at least 3 passages to detect low levels of virus.

Specific testing for FMD virus is recommended as detection of virus by CPE may be ambiguous for non-culture adapted isolates of FMDV, or may not appear within the first 3 passages for low titre virus contamination.

Specific testing for FMD virus is recommended by either AC-ELISA or molecular testing by RT-PCR or real-time fluorogenic RT-PCR (Reid et al. 2000; Reid et al. 2001; Reid et al. 2003). The AC-ELISA and RT-PCR are reported to be of equivalent sensitivity. Specific testing should be performed on the supernatant from the final passage together with an earlier passage (either 1st or 2nd passage). The detection sensitivity of AC-ELISA is improved by testing at later passages, as a high proportion of positives are detected on the second and third passages that on the first passage were AC-ELISA negative and without evidence of CPE (Ferris and Dawson 1988). From the second passage 100% AC-ELISA positives were detected for CPE positives and 92.3% AC-ELISA positives for overall samples as detection by AC-ELISA gave greater sensitivity than culture alone at second passage. Reid et al (2000) reported a significant increase in sensitivity for AC-ELISA detection following 2 culture passages when compared to AC-ELISA without culture isolation.

Specific testing for FMDV by the real-time fluorogenic RT-PCR was found to be more sensitive than culture isolation, with a reported detection limit of $10^{-2.3}$ TCID₅₀/ml as compared to a culture detection limit of $10^{2.3}$ TCID₅₀/ml (four logs lower than RT-PCR detection limit) (Amaral-Doel et al. 1993). The RT-PCR is not suitable for application without primary culture isolation due to the potential occurrence of PCR inhibition by blood-derived components and other components of virus seed stocks. The recommended RT-PCR utilizes universal primer sets capable of detecting all serotypes as described by Reid et al (2000; 2001; 2003).

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 will not meet the growth requirements of FMDV and will not be sufficient for the primary isolation of FMDV. Vero cells are not suitable for culture isolation of FMDV. The 9 CFR guidelines specify the maintenance of infected monolayers for at least 14 days during which time the culture should be passaged at least once. FMDV is rapidly cytolytic and in general viral plaques are evident after overnight culture. The 2 passages required by the 9 CFR guidelines will not be sufficient for detection of low titre virus. The 9 CFR guidelines do not include specific testing for FMDV.

The European Pharmacopoeia general monograph *Vaccines for veterinary use* and 5.2.4 *Cell cultures for the production of veterinary vaccines* describe generalised methods for extraneous agent testing using cell culture monolayers that are sensitive to the agent being tested, and detection using specific testing methods that are sensitive to the viral agent. The monographs do not specify details of a culture system or assay for FMDV detection, simply that the methods should be sensitive.

Conclusion

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 are not acceptable.

Primary isolation of FMDV in both primary bovine thyroid cells and IB-RS-2 cells is recommended. Inoculation should be of primary monolayers of calf thyroid cells established from the initial plating of cells. Inoculated monolayers are incubated for 48 hours between passages, passaged at least 3 times and examined for CPE after 48 hours.

Specific testing is required for FMDV by either ELISA or by molecular testing (RT-PCR or real-time fluorogenic RT-PCR). Specific testing is required for both the final passage and an earlier passage (1st or 2nd).

Hantaan virus

Family *Bunyaviridae*, genus *Hantavirus*

Primary isolation of Hantaan virus (HTNV), also known as Korean haemorrhagic fever virus, is possible in Vero, Vero-E6, BHK-21 or A-549 cells (Rang et al. 2006). Culture of HTNV requires 30 minutes adsorption prior to addition of maintenance medium. Cultures should be maintained for 12 days and passaged 6 times (McCormick et al. 1982). Infectious HTNV must be purified from supernatant only as the virions are not cell-associated. Culture isolation and passaging of HTNV is difficult because the virus is non-cytolytic and cannot be readily detected in culture. Passaging of virus is facilitated by using agar overlays on infected monolayers. The agar overlays are removed and the cell monolayers developed using FA to detect viral plaques. Virus is then picked from the corresponding plaques in the agar overlay, and re-inoculated onto fresh monolayers. HTNV grows to low titres in culture (maximum of 5 logs) but viral titres can be amplified by 3 logs by using the described plaque purification method. Many isolates of HTNV require culture adaptation and at least 6 passages are recommended. Vero or Vero-E6 cells of low passage number have greater susceptibility to infection and should be used for primary isolation of HTNV (Rang et al. 2006). Defective interfering (DI) particles are reported to occur during primary isolation of some isolates of HTNV and to interfere with growth of the virus in culture (Patel and Elliott 1992; Prescott et al. 2007). Characteristic virus morphology can be identified by EM of negative-contrast stained and gradient-purified HTNV (McCormick et al. 1982; White et al. 1982).

Specific testing is required for HTNV due to the difficulties of culture isolation and detection of the non-cytolytic virus.

FA tests should be performed with polyclonal antiserum due to the high frequency of quasispecies, and the genetic and antigenic heterogeneity of HTNV (McCormick et al. 1982; Rang et al. 2006). Fluorescent staining of viral antigen is confined to the cytoplasm and has a diffuse granular pattern. Immunoglobulin M (IgM) indirect immunofluorescence assay (IFA) gives a higher frequency of false positives than Immunoglobulin G (IgG) IFA (Vaheri et al. 2008). A focus chemiluminescent assay has been developed for HTNV that improves the detection sensitivity by 500 times relative to FA (Heider et al. 2001). HA, ISH and VN assays have been developed for HTNV. In general, serological assays for HTNV have low sensitivity due to antigenic variability. Serological assays also have low specificity as the 4 hantaviruses responsible for the haemorrhagic and fever syndrome in field mice, HTNV, Puumala virus (PUUV), Seoul virus (SEOV) and Dobrava virus (DOBV), are serologically cross-reactive and these viruses are closely related (Vapalahti et al. 1996). In general, serological detection of viral antigen and culture isolation are reported to have low sensitivity and specificity (Garin et al. 2001; Schilling et al. 2007). Many isolates of HTNV that do not grow in culture and are not detected serologically by AC-ELISA have been diagnosed by molecular methods (Horling et al. 1995).

RT-PCRs have been developed for detection of HTNV targeting the N gene within the small viral RNA segment (S-RNA) (Rang et al. 2006). The S-RNA segment is present during active virus infection at higher levels than the large viral RNA segment (L-RNA) or medium viral RNA segment (M-RNA) and is therefore more readily detected. The RT-PCR has high sensitivity and specificity and the detection threshold is reported to be 10⁻⁵ FFU or 1 genomic copy (Garin et al. 2001; Horling et al. 1995). The RT-PCR can detect all serotypes of *Hantavirus* and can detect isolates which are

negative by AC-ELISA and culture negative (Horling et al. 1995). A touchdown nested RT-PCR has been developed using genus-specific degenerate primers for *Hantavirus* targeting the S-RNA fragment (Scharninghausen et al. 1999). The nested RT-PCR was adapted to a real-time Taqman RT-PCR utilising a DOBV-specific dual-labelled fluorescent probe (Weidmann et al. 2005). The real-time Taqman RT-PCR does not detect serotypes of *Hantavirus* other than DOBV and has a detection threshold of 10 genomic copies (Weidmann et al. 2005). Relative to the real-time Taqman RT-PCR, serological assays had very low sensitivity of 46.5% and 15.6% respectively for IFA and ELISA (Weidmann et al. 2005). A real-time Taqman RT-PCR has similarly been developed targeting the S-RNA fragment and specific to PUUV serotypes (Garin et al. 2001). The detection threshold for this assay is 10 TCID₅₀/ml and it was found to have 30 times greater sensitivity than the nested RT-PCR. A multi-target, one-step, real-time Taqman RT-PCR has been developed targeting the S-RNA fragment and utilising dual-labelled fluorescent probes specific to the serotypes DOBV, HTNV, PUUV and SEOV (Aitichou et al. 2005). The detection limits of the DOBV, HTNV, PUUV and SEOV assays were 25, 25, 25, and 12.5 PFU respectively, the sensitivity of the assays were 98%, 96%, 92% and 94% respectively, and the specificities were 100%, 100%, 98% and 100% respectively.

Hantaviruses are considered to be emerging and re-emerging pathogens as reported outbreaks have been associated with new genotypic variants that tend to then remain temporally and geographically stable across localised host populations and geographic regions. Ongoing review and validation of the sensitivity of diagnostic assays in use is necessary due to this pattern of constant genotypic drift (Chen et al. 2004; Chu et al. 2001; Jiang et al. 2005; Johansson et al. 2008; Kang et al. 2001; Liang et al. 2000; Miyamoto et al. 2003; Muranyi et al. 2004; Run et al. 2007; Tao et al. 2007; Wu et al. 2005; Zhang et al. 2007a; Zhang et al. 2007c; Zou et al. 2006; Zou et al. 2008).

HTNV is one of the agents targeted in the IMPACT panel of tests. The IMPACT panel tests for HTNV using the highly specific and sensitive multi-target, one-step, real-time Taqman RT-PCR targeting the S-RNA fragment and utilising dual-labelled fluorescent probes specific to the serotypes DOBV, HTNV, PUUV and SEOV (Aitichou et al. 2005).

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 for extraneous agent testing will not be sufficient for primary isolation of HTNV. The 9 CFR guidelines do not include specific testing for HTNV.

The European Pharmacopoeia general monograph *Vaccines for veterinary use* and 5.2.4 *Cell cultures for the production of veterinary vaccines* describe generalised methods for extraneous agent testing using cell culture monolayers that are sensitive to the agent being tested, and detection using specific testing methods that are sensitive to the viral agent. The monographs do not specify details of a culture system or assay for HTNV detection, simply that the methods should be sensitive.

Conclusion

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 are not acceptable.

Primary isolation of HTNV in either Vero or Vero-E6 cells is recommended. Cultures should be maintained for 12 days between passages, adsorption incubation is required for 30 minutes and cultures should be passaged 6 times. Vero or Vero-E6 cells should be of low passage number.

Specific testing is required for HTNV by the multi-target, one-step, real-time Taqman RT-PCR targeting the S-RNA fragment and utilising dual-labelled fluorescent probes specific to the serotypes DOBV, HTNV, PUUV and SEOV.

Horse pox virus

Family *Poxviridae*, genus *Orthopoxvirus*

Horse pox virus (HPV) is now known to be vaccinia virus. Primary isolation of HPV is in primary SEK, BK, CTC or calf skin, or on the chorioallantoic membrane of ECE.

CPE detection of HPV in culture or on the chorioallantoic membrane of ECE has low sensitivity and specific testing is recommended. Specific testing is possible by PCR or FA (Davies and Otema 1981; Tulman et al. 2006).

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 for extraneous agent testing are not suitable for primary isolation of HPV. The 9 CFR guidelines do not require specific testing for HPV.

The European Pharmacopoeia general monograph *Vaccines for veterinary use* and 5.2.4 *Cell cultures for the production of veterinary vaccines* describe generalised methods for extraneous agent testing using cell culture monolayers that are sensitive to the agent being tested, and detection using specific testing methods that are sensitive to the viral agent. The monographs do not specify details of a culture system or assay for HPV detection, simply that the methods should be sensitive.

Conclusion

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 are not acceptable.

Primary isolation of HPV in primary SEK, BK, CTC, calf skin or ECE is recommended.

Specific testing is required for HPV by PCR or FA.

Jaagsiekte sheep retrovirus

Family *Retroviridae*, genus *Betaretrovirus*

Jaagsiekte sheep retrovirus (JSRV) is also known as ovine pulmonary adenocarcinoma virus or pulmonary adenomatosis virus. A suitable culture system has not been developed for the primary isolation of JSRV.

Specific testing for JSRV is required as culture isolation is not possible. There are no serological diagnostic assays available for JSRV. Diagnostic molecular assays have been developed to detect the JSRV LTR and can detect both proviral DNA and RNA transcripts. The hemi-nested RT-PCR (González et al. 2001) has good sensitivity and can detect a single copy of JSRV genome in 500 ng DNA, but has very poor specificity and has a high number of false positives. A 1 step PCR for detection of proviral DNA had lower sensitivity but higher specificity (De Las Heras et al. 2005).

The PCR diagnostic assays for JSRV that have been developed have not been evaluated for use in extraneous agent testing of biological products and there is published data only for their application to the diagnosis of JSRV in live sheep flocks. Therefore it must be considered that any

recommendation to adopt these PCRs for this purpose would not be supported by scientific validation at this time. Issues such as the possible inhibition of the PCR by media or blood-derived components of the test material are possible factors in the use of this assay.

These considerations would have to be weighed against the significant risk from retrovirus contamination of biologicals. Historically it is recognised that intravenous inoculation of retroviruses as contaminants in biologicals is effectively a process of host passaging of the viruses that will permit host adaptation and could permit evolution of the virus towards virulence and therefore is an important factor in the control of ovine pulmonary adenomatosis.

PERT is widely used for human therapeutics and vaccines as a general testing method to identify all sources of retroviral contamination and could find application for detection for JSRV.

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 will not be sufficient for the primary isolation of JSRV. The 9 CFR guidelines do not include specific testing for JSRV.

The European Pharmacopoeia general monograph *Vaccines for veterinary use* and 5.2.4 *Cell cultures for the production of veterinary vaccines* describe generalised methods for extraneous agent testing using cell culture monolayers that are sensitive to the agent being tested, and detection using specific testing methods that are sensitive to the viral agent. The monographs do not specify details of a culture system or assay for JSRV detection, simply that the methods should be sensitive.

Conclusion

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 are not acceptable.

Primary isolation of JSRV is not recommended.

Specific testing is required. A PERT assay is required as the initial diagnostic assay for JSRV detection in conjunction with PCR for detection of proviral DNA. Attempted isolation, confirmation and identification of retrovirus positives should then be undertaken by animal isolation, or sequencing of PCR products.

Japanese encephalitis virus and West Nile virus

Family *Flaviviridae*, genus *Flavivirus*

Primary isolation of Japanese encephalitis virus (JEV) can be performed in PK-15, BHK-21, Vero, MDBK, or AA C3/36 cells. Primary isolation of West Nile virus (WNV) is recommended in RK-13, Vero cells or ECE. Adsorption incubation for 2 hours is required and cultures should be maintained for 7 days and three culture passages are required for CPE detection. CPE in infected cultures is typically difficult to identify.

Specific testing for JEV and WNV is required due to the difficulty of detecting CPE in infected cultures.

FA is performed on JEV or WNV infected Vero cells using mAbs at 3 days after infection for JEV and WNV (Payne et al. 2006).

FA will detect FFU for both cytolytic and non-cytolytic infections.

A number of RT-PCRs have been developed to detect the JEV and WNV *flavivirus* group. RT-PCRs have been developed targeting the *E*, *NS3* and *NS5* genes; however, problems occur with the detection of the range of phenotypic variants. A number of approaches have been taken to overcome this problem of inadequate phenotypic range. Assays have been designed based on conserved universal sequences in the *NS5* gene, degenerate primers have been used, or multiplex assays targeting different genotypes have been developed. A RT-PCR based on universal primers Flav100F and Flav200R targeting the *NS5* gene and 3'UTR is reported to have the greatest range for JEV and WNV genotypic variants (Maher-Sturgess et al. 2008).

Real-time RT-PCRs for detection of JEV or WNV have been shown to be more sensitive, specific and accurate than RT-PCRs. A real-time SYBR Green I assay targeting the *NS3* gene of JEV is reported to have 100% specificity and the detection sensitivity was reported to be 2 times greater than standard RT-PCR and 4 times greater than virus isolation (Santhosh et al. 2007). A real-time RT-LAMP assay targeting the *E* gene of JEV is reported to have a detection limit of 0.1 PFU and a sensitivity and specificity of 100% and 86% respectively (Parida et al. 2006).

WNV isolates cluster into 2 clades; western and eastern clusters. The SYBR Green I and real-time RT-PCR and real-time RT-LAMP assays cannot detect all WNV isolates from the eastern cluster of viruses. A real-time Taqman RT-PCR targeting the *E* gene has been developed utilising universal degenerate primers for WNV and JEV and a universal probe for WNV and JEV, together with WNV specific and JEV specific fluorogenic probes (Shirato et al. 2005). In contrast to the other reported real-time format assays the real-time Taqman assay is reported to detect both clades of WNV. The overall detection limit for this assay is reported to be ~0.1 PFU or 1 µg DNA. A second real-time Taqman RT-PCR has been developed for detection of JEV based on the *NS5* gene (Pyke et al. 2004). This assay has the advantage of targeting the *NS5* gene that is known to have the potential to detect a wider phenotypic range of flaviviruses with the greater sensitivity of a real-time assay format. This assay is therefore considered optimal for detection of JEV but a WNV specific probe has not been incorporated into the assay format.

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 for extraneous agent testing do not provide the optimal growth requirements for JEV and WNV and are not suitable for primary isolation of JEV and WNV. The 9 CFR guidelines do not require specific testing for JEV and WNV.

The European Pharmacopoeia general monograph *Vaccines for veterinary use* and 5.2.4 *Cell cultures for the production of veterinary vaccines* describe generalised methods for extraneous agent testing using cell culture monolayers that are sensitive to the agent being tested, and detection using specific testing methods that are sensitive to the viral agent. The monographs do not specify details of a culture system or assay for JEV and WNV detection, simply that the methods should be sensitive.

Conclusion

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 are not acceptable.

Primary isolation of JEV in PK-15, BHK-21, Vero, MDBK, or AA C3/36 cells, and of WNV in RK-13, Vero cells or ECE is recommended. Adsorption incubation for 2 hours is required and cultures should be maintained for 7 days and passaged 3 times.

Specific testing is required for WNV and JEV by FA or by the real-time Taqman RT-PCR utilising universal degenerate primers for WNV and JEV and a universal probe for WNV and JEV, together with WNV specific and JEV specific fluorogenic probes (Shirato et al. 2005).

Louping ill virus

Family *Flaviviridae*, genus *Flavivirus*

Susceptible culture systems for isolation of louping ill virus (LIV) are SK, SEK, yolk sac of ECE, BHK, BHK-89, AA, HeLa, human nasopharyngeal carcinoma (KB) and human bone marrow carcinoma (Detroit-6) cells (Brotherston and Boyce 1970; Edward 1947; Karpovich and Levkovich 1959; Marriott et al. 2006; Reháček 1965; Venugopal and Gould 1992; Von Zeipel and Svedmyr 1958; Warren and Cutchins 1957; Williams 1958; Xiao et al. 1986). Field strains of LIV require several passages to establish culture adaptation before infected cultures develop readily identifiable CPE (Venugopal and Gould 1992). For culture adapted isolates cytolytic CPE and plaque formation are evident 5 days after inoculation. Cell monolayers recover rapidly from cytolytic infection and establish persistent non-cytolytic infections with LIV. Low MOI may also result in persistent infection without evidence of CPE. Persistent infection of cultures can prevent super-infection or co-infection with non-culture adapted strains.

Because culture isolation can be problematic specific testing is required for LIV detection. Specific testing is possible by FA or RT-PCR.

An FA has been developed using mAbs specific to LIV. The FA is sensitive and specific. Studies with isolate specific mAbs and dual fluorescence have shown that FA can identify super-infection with multiple arboviruses even in persistently infected cultures in which there is no evidence of CPE.

A number of RT-PCRs have been developed, including a 1 stage RT-PCR, nested RT-PCR and real-time RT-PCR. The RT-PCR and nested RT-PCRs have the disadvantage of requiring sequencing of the product to confirm LIV infection (Gaunt et al. 1997). The real-time RT-PCR targets the *env* gene (Marriott et al. 2006). This assay is reported to have equivalent sensitivity to plaque titration in cell culture and can detect between 1-10 PFU/ml. The real-time RT-PCR has the advantage of detecting isolates that are not culture adapted and are difficult to culture.

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 for extraneous agent testing will not be sufficient for primary isolation of LIV. The 9 CFR guidelines do not include specific testing for LIV.

The European Pharmacopoeia general monographs *Vaccines for veterinary use* and 5.2.4 *Cell cultures for the production of veterinary vaccines* describe generalised methods for extraneous agent testing using cell culture monolayers that are sensitive to the agent being tested, and detection using specific testing methods that are sensitive to the viral agent. The monographs do not specify details of a culture system or assay for LIV detection, simply that the methods should be sensitive.

Conclusion

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 are not acceptable.

Culture isolation of LIV in SK, SEK, yolk sac of ECE, BHK, BHK-89, AA, HeLa, KB or Detroit-6 cells is recommended.

Specific testing is required for LIV by FA or real-time RT-PCR.

Lumpy skin disease virus

Family *Poxviridae*, genus *Capripoxvirus*

Primary isolation of LSDV is possible on LT; cells derived from breeds of wool sheep are most sensitive. Susceptible cell lines are primary bovine, ovine or caprine cells, Vero cell lines and the chorioallantoic membrane of ECE. CPE is evident within 14 days and 2 passages are required. Confirmation of LSDV as the aetiological agent of CPE is by FA directed against group specific antigen (OIE 2010).

Recently the Canadian National Centre for Foreign Animal Disease and the Australian Animal Health Laboratory conducted a comparative study of capripoxvirus growth kinetics in the established OA3.Ts, in primary lamb kidney cells, and in Vero cells, with the objective of identifying a cell line suitable for standardized diagnostic assays for capripoxvirus (Babiuk et al. 2007). The OA3.Ts cell line has been deposited in the American type culture collection (ATCC) and is described by the ATCC as susceptible to orf virus infection. Isolates evaluated in this study were a Nigerian isolate, Indian goat pox, Kenyan sheep and goat pox, Yemen sheep and goat pox, and LSDV (Neethling strain). The findings of the study were that the LT and OA3.Ts cell lines had equivalent growth kinetics and susceptibility to both low and high level MOI. The OA3.Ts cell line had the advantage of being a more homogenous cell line that forms tighter and more even cell monolayers in which plaque formation is more readily identified. The OA3.Ts cell line is recommended for the isolation of LSDV pending further validation.

The generalised culture methods described by 9 CFR 113.52, 113.53 and 113.55 are suitable for the primary isolation of LSDV provided LT cells or OA3.Ts cells are used. The 9 CFR guidelines do not require specific testing for LSDV.

The European Pharmacopoeia general monograph *Vaccines for veterinary use* and 5.2.4 *Cell cultures for the production of veterinary vaccines* describe generalised methods for extraneous agent testing using cell culture monolayers that are sensitive to the agent being tested, and detection using specific testing methods that are sensitive to the viral agent. The monographs do not specify details of a culture system or assay for LSDV detection, simply that the methods should be sensitive.

Conclusion

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 are acceptable provided LT cells or OA3.Ts cells are used.

Primary isolation of LSDV on LT cells from wool breed sheep or in the OA3.Ts cell line is recommended.

Specific testing is not required for detection.

Lymphocytic choriomeningitis virus

Family *Arenaviridae*, genus *Arenavirus*

Primary isolation of lymphocytic choriomeningitis virus (LCMV) is possible in Vero, BHK-21, C3H mouse fibroblasts (L), baby hamster kidney cell line transformed with simian virus 40 (SV40) (C13/SV28), ME and CEF cell cultures. LCMV can infect and propagate in a wide range of mammalian cell lines (Asper et al. 2001; Lehmann-Grube et al. 1975). Intracranial inoculation of mice is the most sensitive means of detection. CPE is evident in infected cultures after 48–72 hours (Rehman and Wagner 1972). The virions remain cell-associated and bud from the cell membrane in large vesicular blebs that are readily identified by EM. LCMV grows to high titres in susceptible L cell cultures, but titres decline with subsequent passages and establishment of persistent infection.

In culture systems LCMV exhibits cyclical growth patterns between acute and persistent infection (Hotchin et al. 1975). LCMV infection is most readily detected during the acute phase of virus growth. LCMV multiplies as a quasi-species with varying cytolytic and infectious properties (Hotchin et al. 1975). Plaque assays have been developed for LCMV detection using BHK-21 monolayers with agarose overlays. There are typically 2 plaque variants; the turbid and clear plaques. Clear plaques are associated with acute infection and turbid plaques with persistent infection.

LCMV represents a significant biosecurity concern as it is a frequent contaminant of established cell lines and is problematic to detect (van der Zeijst et al. 1983b; van der Zeijst et al. 1983a). Most mammalian cell lines cultivated *in vitro* can be infected with LCMV with no apparent effects on cell morphology or propagation, whilst infection is detected by FA and the budding of viral particles is evident by EM. In some cases viral persistence in cell lines is associated with intracellular antigen accumulation, but extracellular virus is not produced and viral antigen is absent from the cell membrane (van der Zeijst et al. 1983b; van der Zeijst et al. 1983a).

LCMV establishes persistent non-cytolytic, inapparent infections of cell lines (Stanwick and Kirk 1976). Persistent infection has been associated with the production of DI particles which may inhibit super-infection and the primary isolation of LCMV in these cell lines (Stanwick and Kirk 1976). There are published reports of organ donors having tested negative for LCMV by culture, EM, serology, molecular analysis and ISH, that subsequently transmitted the virus to a cluster of organ recipients (Fischer et al. 2006). These reports are indicative of the difficulty that exists in detecting latent infection with LCMV.

Specific testing has been developed by FA, immunolabelling electron microscopy (IEM), ELISA, RT-PCR and fluorogenic nuclease real-time RT-PCR (fnRT-PCR).

Serological assays target both a complement-fixing, soluble viral antigen and an intracellular antigen (Lehmann-Grube et al. 1975). FA and IEM have been developed to detect the intracellular antigen of LCMV infection. FA uses polyclonal hyperimmune antisera and the fluorescent staining typically has a granular intra-cytoplasmic pattern (Hotchin et al. 1975). ELISAs have been developed targeting the soluble antigen and are reportedly 64 times more sensitive than CF or FA (Ivanov et al. 1981). The described serological assays have lower sensitivity than molecular assays.

RT-PCR and semi-nested RT-PCRs have been developed for LCMV targeting the *N* gene encoded on the S-RNA fragment (Asper et al. 2001). An fnRT-PCR has been developed targeting the *N* gene of LCMV (Besselsen et al. 2003). The detection threshold of the assay was 1 pg of RNA and it is reported to be 100% specific when tested with a panel of mouse pathogens (Besselsen et al. 2003).

A SYBR green real-time quantitative RT-PCR (qRT-PCR) has been developed targeting both the *N* and glycoprotein genes encoded on the S-RNA fragment of LCMV (McCausland and Crotty 2008). This assay is limited in its application due to low specificity and the amplification of non-specific products. This problem can be addressed through identification of false negatives by melt curve analysis; however, the amplification of non-specific products in turn lowers the efficiency of detection of true positives and reduces the assay sensitivity (McCausland and Crotty 2008).

LCMV is one of the agents targeted in the IMPACT panel of tests. The PCR used for LCMV detection is the SYBR green real-time qRT-PCR, and as noted previously this assay is problematic due to a high frequency of false negative results (McCausland and Crotty 2008).

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 for extraneous agent testing will not be sufficient for primary isolation of LCMV. The 9 CFR guidelines do not include specific testing for LCMV.

The European Pharmacopoeia general monograph *Vaccines for veterinary use* and 5.2.4 *Cell cultures for the production of veterinary vaccines* describe generalised methods for extraneous agent testing using cell culture monolayers that are sensitive to the agent being tested, and detection using specific testing methods that are sensitive to the viral agent. The monographs do not specify details of a culture system or assay for LCMV detection, simply that the methods should be sensitive.

Conclusion

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 are not acceptable.

Primary isolation of LCMV in Vero, BHK-21, L, C13/SV28, ME or CEF cell cultures is recommended.

Specific testing is required for LCMV by the real-time fnRT-PCR targeting the *N* gene encoded on the S-RNA fragment.

Murine adenovirus

Family *Adenoviridae*, genus *Mastadenovirus*

Murine adenovirus (MuAdV) can be isolated in Vero, L, ME cells and PBL (Cepko et al. 1983; Pirofski et al. 1991). Virus growth is evident by CPE in culture, which consists of cellular rounding and clumping and refractile intracellular inclusions. CPE is confirmed by FA for the hexon protein or common group antigen (common to adenoviruses from humans, simians, canines, porcines, murines, bovines, avians, and amphibians), or by using hyperimmune murine ascitic fluid. Fluorescent staining is evident in the nucleus of MuAdV infected cells 24–48 hours after infection (Cepko et al. 1983; Pirofski et al. 1991; Sambrook et al. 1980; Wadell et al. 1980).

PCR identification of MuAdV is possible by *Mastadenovirus* group specific PCRs targeting the hexon gene (Sambrook et al. 1980; Wadell et al. 1980). There are no published studies reporting on the sensitivity or specificity of this group specific PCR and it has not been validated for the purposes of detection of MuAdV. Positives from the group specific PCR assay can be identified as MuAdV by serotyping, sequencing or restriction endonuclease digestion of PCR product (PCR-RE). A random PCR or particle associated nucleic acid PCR (PAN-PCR) method for generalised detection of viruses has detected 6 MuAdV-1 isolates from extraneous materials (Stang et al. 2005).

The MAP test and IMPACT are test protocols that have been widely adopted for screening of laboratory colonies. The MAP and IMPACT tests target panels of 15–19 extraneous agents, including MuAdV. The sensitivity and specificity of these assays is only equivalent to that of the individual component assays.

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 for extraneous agent testing are sufficient for primary isolation of MuAdV. The 9 CFR guidelines do not include specific testing for MuAdV.

The European Pharmacopoeia general monograph *Vaccines for veterinary use* and 5.2.4 *Cell cultures for the production of veterinary vaccines* describe generalised methods for extraneous agent testing using cell culture monolayers that are sensitive to the agent being tested, and detection using specific testing methods that are sensitive to the viral agent. The monographs do not specify details of a culture system or assay for MuAdV detection, simply that the methods should be sensitive.

Conclusion

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 are acceptable.

Primary isolation of MuAdV in Vero, L, ME or PBL cells is recommended.

Detection is by CPE and specific testing for MuAdV by FA targeting the common group antigen is required.

Orf virus

Family *Poxviridae*, genus *Parapoxvirus*

Primary isolation of orf virus is recommended in primary lamb keratinocytes (PLK), SEK, BEK, BHK, CEF, LT, BEL, lamb fibroblasts, sheep thyroid cells and ovine embryonic lung cells (Balassu and Robinson 1987; Burgu and Toker 1984; Dobric 1995a; Dobric 1995b; Hessami et al. 1979; Hussain and Burger 1989; Liebermann 1967; Mayr et al. 1981; Mazur and Machado 1990; Ogiso et al. 2002; Onwuka et al. 1995; Perez et al. 1981; Pospischil and Bachmann 1980; Rao and Malik 1982; Saddour 1989; Scagliarini et al. 2005; Schmidt 1967a; Schmidt 1967b; Scott et al. 1981; Torfason and Guonadottir 2002; Traykova and Argirova 1986). Orf virus grows to titres 1 log higher in CEF than in SEK.

Orf virus can be isolated in PLK organotypic cultures prepared from the foreskin of 12 month old lambs although this technique is highly specialised and time consuming (Scagliarini et al. 2005). The PLK cells are prepared as collagen rafts using fibroblast feeder cells. CPE is evident in infected cultures 2–3 days post infection as ballooning degeneration of the superficial layers of the PLK. Orf virus is slow growing and virus isolation is problematic and unreliable for field strains that have not been laboratory adapted.

Specific testing is required for orf virus because of the difficulty of primary isolation.

A real-time Taqman PCR has been developed targeting the orf virus *B2L* gene that encodes the major ENV protein (Gallina et al. 2006). This assay is highly reproducible, efficient and specific to

the orf virus. The inter-assay and intra-assay variability is within $\pm 0.25 \log_{10}$ S.D. and the detection threshold is 10 TCID₅₀/ml.

Conventional PCR targeting the *B2L* and *A24R* genes are not specific to the orf virus as they cross-amplify a range of different pox viruses that can then be differentiated by sequencing (Inoshima et al. 2000; Torfason and Guonadottir 2002).

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 for extraneous agent testing will not be sufficient for primary isolation of orf virus. The 9 CFR guidelines do not include specific testing for orf virus.

The European Pharmacopoeia general monograph *Vaccines for veterinary use* and 5.2.4 *Cell cultures for the production of veterinary vaccines* describe generalised methods for extraneous agent testing using cell culture monolayers that are sensitive to the agent being tested, and detection using specific testing methods that are sensitive to the viral agent. The monographs do not specify details of a culture system or assay for orf virus detection, simply that the methods should be sensitive.

Conclusion

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 are not acceptable.

Primary isolation of orf virus in CEF is recommended.

Specific testing is required for orf virus by real-time Taqman PCR.

Ovine adenovirus

Family *Adenoviridae*, genus *Mastadenovirus*

Adenoviruses originating from ovines include both BAdV and OAdV. BAdV group 1 belongs to the genus *Mastadenovirus* whereas group 2 together with OAdV belong to the genus *Atadenovirus*. There are 6 serotypes of OAdV which includes atypical OAdV (Adair et al. 1982; Adair et al. 1983; Adair et al. 1985; Both 2004; Kumin et al. 2004; Lehmkuhl and Hobbs 2008). Two adenovirus serotypes are recognised in caprine hosts; goat adenovirus 1 (GAdV-1) classified within OAdV D, and OAdV-5 (Lehmkuhl and Cutlip 1999; Lehmkuhl and Hobbs 2008).

Primary isolation of ovine adenovirus is possible in SEK, LT, SK and ovine foetal turbinate (OFT) cell cultures. OFT cells have been used for isolation of both ovine and caprine adenovirus isolates, although there have not been any published comparative studies of isolation sensitivity for these isolates in different cell lines (Lehmkuhl and Hobbs 2008). CPE is apparent within 2–3 days after inoculation. At low MOI CPE may occur as single small foci that take at least 7 days to spread across 50% of the monolayer. Three passages are required to ensure culture adaptation of isolates, and cultures should be incubated for 7 days between passages.

Specific testing is required to ensure detection of all serotypes. BAdV, OAdV and GAdV-1 can be detected by FA using a mAb against a common, mammalian group antigen.

PCRs targeting the hexon gene have not been sufficiently evaluated to establish the detection sensitivity.

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 for extraneous agent testing are suitable for primary isolation of OAdV provided SEK, LT, SK or OFT cells are used. The generic protocol 9 CFR 113.47: *Detection of extraneous viruses by the fluorescent antibody technique* stipulates testing of ovine lines for bovine adenovirus and does not include testing specific to OAdV.

The European Pharmacopoeia general monograph *Vaccines for veterinary use* and 5.2.4 *Cell cultures for the production of veterinary vaccines* describe generalised methods for extraneous agent testing using cell culture monolayers that are sensitive to the agent being tested, and detection using specific testing methods that are sensitive to the viral agent. The monographs do not specify details of a culture system or assay for OAdV detection, simply that the methods should be sensitive.

Conclusion

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 are acceptable provided SEK, LT, SK or OFT cells are used.

Primary isolation of OAdV in SEK, LT, SK or OFT cells is recommended.

The generic protocol 9 CFR 113.47: *Detection of extraneous viruses by the fluorescent antibody technique* stipulates testing of ovine lines for bovine adenovirus and does not include testing specific to OAdV.

Specific testing is required for OAdV by FA using a common mammalian group antigen.

Peste-des-petits-ruminants virus

Family *Paramyxoviridae*, genus *Morbillivirus*

Primary isolation of peste-des-petits-ruminants virus (PPRV) is possible in primary lamb kidney cells and Vero cells. CPE will appear within 5 days on Vero cells. At least 2 further blind passages are required as in some cases CPE does not appear until later passages. CPE due to PPRV infection consists of microscopic syncytia that require skilled evaluation for detection.

Specific testing for PPRV is required because of the difficulty of detecting the subtle CPE in culture isolated virus. Specific testing is possible by RT-PCR, VN, HA, ELISA and FA. However, a study comparing the detection sensitivity of assays for PPRV recorded no improvement from visual detection of CPE in Vero cell cultures with the use of specific VN and HA (Raj et al. 2000).

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 will meet the growth requirements of PPRV and will be sufficient for primary isolation of PPRV. The 9 CFR protocols do not include specific testing for PPRV.

The European Pharmacopoeia general monograph *Vaccines for veterinary use* and 5.2.4 *Cell cultures for the production of veterinary vaccines* describe generalised methods for extraneous agent testing using cell culture monolayers that are sensitive to the agent being tested, and detection using specific testing methods that are sensitive to the viral agent. The monographs do not specify details of a culture system or assay for PPRV detection, simply that the methods should be sensitive.

Conclusion

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 are acceptable.

Primary isolation of PPRV in primary lamb kidney cells or Vero cells is recommended. Cultures should be maintained for 3 passages.

Specific testing is required for the final passage of PRRV by RT-PCR, ELISA or FA.

Porcine adenovirus

Family *Adenoviridae*, genus *Mastadenovirus*

Primary isolation of porcine adenovirus (PAdV) is recommended in SK, PK-15, swine kidney (SK-6), primary porcine thyroid, tracheal organ cultures (TOC) and ST (Dea and Elazhary 1984; Derbyshire et al. 1968; Granzow et al. 1988; Hirahara et al. 1990; Kapp 1983; Koestner et al. 1968; Kwon and Spradbrow 1971; Shadduck et al. 1967; Shadduck et al. 1969; Tischer et al. 1968; Tischer and Kohler 1968). Porcine thyroid cells are reported to be most susceptible to PAdV infection (Dea and Elazhary 1984). Vero cells can be transduced with PAdV but some PAdV strains do not complete a replication cycle due to failure of translation of late stage proteins (Reddy et al. 1999).

Culture isolation is not sufficiently sensitive alone to detect PAdV and specific testing is required. An FA has been described using antibody directed against a common group antigen that will detect all 5 serotypes of PAdV. RT-PCRs have been developed targeting the *E4* ORF; however, there are no comprehensive reports of their application for diagnostic purposes (Li et al. 2007; Xing and Tikoo 2004).

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 for extraneous agent testing will be sufficient for primary isolation of PAdV provided culture is in porcine thyroid cells. The guideline 9 CFR 113.47: *Detection of extraneous viruses by the fluorescent antibody technique* describing the FA on infected cell monolayers is sufficient for PAdV detection.

The European Pharmacopoeia general monograph *Vaccines for veterinary use* and 5.2.4 *Cell cultures for the production of veterinary vaccines* describe generalised methods for extraneous agent testing using cell culture monolayers that are sensitive to the agent being tested, and detection using specific testing methods that are sensitive to the viral agent. The monographs do not specify details of a culture system or assay for PAdV detection, simply that the methods should be sensitive.

Conclusion

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 are acceptable provided culture is in porcine thyroid cells.

Primary isolation of PAdV in porcine thyroid cells is recommended.

The guideline 9 CFR 113.47: *Detection of extraneous viruses by the fluorescent antibody technique* describing the FA on infected cell monolayers is sufficient for PAdV detection.

Specific testing for PAdV is required by FA using antibody against a common group antigen.

Porcine circovirus 2

Family *Circoviridae*, genus *Circovirus*

Primary isolation of porcine circovirus 2 (PCV-2) is recommended in PK-15 cells sourced from gnotobiotic pigs and validated as not contaminated with PCV. Culture of PCV can be problematic to detect. Persistent low-level infection with PCV occurs frequently and there can be minimal detectable CPE.

PCV is a significant biosecurity issue as there are many reports of porcine circovirus 1 (PCV-1) and PCV-2 contamination detected in vaccines, xenotransplants, cell lines and biological products of porcine origin, and these have been associated with transmission to susceptible populations. PCV-2 was first identified as a persistent, low-titre, non-cytopathic infection of the PK-15 cell line (Allan et al. 1995; Jung et al. 2006; Katayama et al. 1998; Quintana et al. 2006; Tischer et al. 1986).

Specific testing is required for PCV because of the difficulty of detection of the virus in cell culture.

A number of molecular detection assays have been developed for PCV-2 (Cao et al. 2005; Caprioli et al. 2006; Choi and Chae 1999; Meehan et al. 1998; Todd 2000). A PCR has been developed targeting the ORF 1 of PCV-1 and another targeting ORF 1 of PCV-2. The threshold for detection by the PCR was found to be $10^{1.4}$ TCID₅₀/ ml and specificity was found to be 100% (Fenaux et al. 2004; Lee et al. 2007). A molecular beacon real-time PCR targeting the same region had approximately equivalent sensitivity to the conventional PCR (McKillen et al. 2007).

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 for extraneous agent testing will not be sufficient for primary isolation of PCV. The 9 CFR guidelines do not include specific testing for PCV.

The European Pharmacopoeia general monograph *Vaccines for veterinary use* and 5.2.4 *Cell cultures for the production of veterinary vaccines* describe generalised methods for extraneous agent testing using cell culture monolayers that are sensitive to the agent being tested, and detection using specific testing methods that are sensitive to the viral agent. The monographs do not specify details of a culture system or assay for PCV detection, simply that the methods should be sensitive.

Conclusion

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 are not acceptable.

Primary isolation of PCV in PK-15 cells sourced from gnotobiotic pigs is recommended.

Specific testing is required for PCV by PCR or real-time PCR.

Porcine epidemic diarrhoea virus

Family *Coronaviridae*, genus *Coronavirus*

Primary isolation of porcine epidemic diarrhoea virus (PEDV) is possible in Vero cells, primary porcine bladder cells and primary porcine kidney cells on cellulose coated tissue culture plates (Kim et al. 2007b; Shibata et al. 2000). Trypsin supplementation of the medium is required for successful culture of PEDV and to release the highly cell-associated virus (Hofmann and Wyler 1988). PEDV

is unable to grow in porcine cell cultures permissive to transmissible gastroenteritis virus (TGEV) (Kim and Chae 1999). Attempted primary isolation of PEDV in 6 types of primary foetal porcine cells and 10 established cell lines is reported to have failed and it is believed this is due to the inability of the cell cultures to tolerate the addition of trypsin required for virus growth (Kusanagi et al. 1992). PEDV cannot be cultured in Vero cells without adaptation by several passages (Kim and Chae 1999). Cultures require adsorption incubation for 2 hours, cultures should be maintained for 5–7 days between passages and 5 blind passages are required (Shibata et al. 2000). Plaque assay is confirmed by FA or by focus forming assay (FFA) using agar overlays (Cruz and Shin 2007; Hofmann and Wyler 1988). Isolates of PEDV adapted to cell culture growth by passage in Vero cell lines can then be readily adapted to growth in swine kidney epithelial (KSE6), IB-RS-2, MA104, swine embryo kidney (ESK) and multiple myeloma (MM) cell lines (Kadoi et al. 2002; Kusanagi et al. 1992). The culture adapted isolates show complete and marked CPE in KSE6 and IB-RS-2 cells that is not dependent on the addition of trypsin (Kadoi et al. 2002). Primary isolation of PEDV is difficult and not all strains have been successfully isolated (Hofmann and Wyler 1988; Kadoi et al. 2002; Kweon et al. 1999).

Specific testing is required for PEDV because of the difficulty of isolation for some wildtype strains and also because cell culture adapted strains may establish inapparent infections in culture without obvious CPE. Specific testing is possible by FA, IEM, ISH, ELISA, RT-PCR and real-time RT-PCR (Callebaut et al. 1982; Jung et al. 2003; Jung and Chae 2005; Kim and Chae 2001; Kim et al. 2000b; Kim and Chae 1999; Kim and Chae 2000; Kim and Chae 2002; Pensaert 1999; Rodak et al. 1999; Shibata et al. 2000; Sueyoshi et al. 1995; van Nieuwstadt et al. 1988). FA, ISH, ELISA, and IEM are based on the use of mAbs and in general are of low specificity and sensitivity (Carvajal et al. 1995; van Nieuwstadt and Zetstra 1991) (Kim and Chae 2001).

A RT-PCR has been developed for detection of PEDV for culture-isolated virus targeting the *M* gene sequence (Ishikawa et al. 1997). The sensitivity and specificity of the RT-PCR for PEDV is too low to be suitable for extraneous agent testing and was found to be comparable to the sensitivity of antigen-based tests; FA, ISH and ELISA (Paton et al. 1997). The RT-PCR has the advantage over antigen-based tests in that it can detect PEDV in passages prior to cell culture adaptation and before the appearance of CPE (Kim and Chae 1999). When adapted to a real-time Taqman RT-PCR format the assay sensitivity is improved by 10–100 times (Kim et al. 2007b). RT-PCRs have also been developed targeting ORF 3 and the *S* gene (Kim et al. 2000b; Song et al. 2003). ORF 3 sequence undergoes nucleotide changes associated with culture adaptation that can be differentiated by RFLP analysis using *HindIII* restriction endonuclease digestion (Song et al. 2003). Multiplex and duplex RT-PCRs have been developed based on this assay for simultaneous detection of PEDV, TGEV and group A rotaviruses (Kim et al. 2000b; Song et al. 2006a; Song et al. 2006b). There have been numerous studies focusing on the use of molecular assays for diagnosis of PEDV in faeces. The results of these studies cannot necessarily be translated to culture adapted virus.

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 for extraneous agent testing will not be sufficient for primary isolation of PEDV. The 9 CFR guidelines do not include specific testing for PEDV.

The European Pharmacopoeia general monograph *Vaccines for veterinary use* and 5.2.4 *Cell cultures for the production of veterinary vaccines* describe generalised methods for extraneous agent testing using cell culture monolayers that are sensitive to the agent being tested, and detection using specific testing methods that are sensitive to the viral agent. The monographs do not specify details of a culture system or assay for PEDV detection, simply that the methods should be sensitive.

Conclusion

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 are not acceptable.

Primary isolation of PEDV in Vero cells using medium supplemented with trypsin is recommended. Cultures require adsorption incubation for 2 hours, cultures should be maintained for 5–7 days between passages, and 5 blind passages are required.

Specific testing is required for PEDV by real-time Taqman RT-PCR targeting the *M* gene.

Porcine haemagglutinating encephalomyelitis virus

Family *Coronaviridae*, genus *Coronavirus*

Primary isolation of porcine haemagglutinating encephalomyelitis virus (HEV) is recommended in human rectal adenocarcinoma (HRT-18), SK, ESK, ST, porcine kidney epithelial (FS-L3), swine kidney (SK-K), PK-15 and SK-6 cell lines, and secondary porcine thyroid cells (Greig 1969; Mengeling et al. 1972). SK-K and secondary porcine thyroid cells are most susceptible to HEV infection (Hirano et al. 1990). SK-K cells infected with HEV demonstrate clear CPE whereas in ESK cells addition of diethylaminoethyl-dextran (DEAE-dextran) to the medium is required for CPE detection (Hirano et al. 1990). Cultures are maintained in serum-free medium as serum inhibits the adsorption of virus onto the cell membrane (Schultze et al. 1990). Two blind passages are required and CPE is evident at 2 days after infection as detached and rounded, fused cells.

Co-infecting viral agents can interfere with infection and isolation of HEV (Mengeling 1973). Presence of co-infecting coronaviruses interferes with cultivation and results in defective virus particles. Growth profiles for HEV are extremely variable and culture isolation of HEV has poor repeatability.

Specific testing is required for HEV because of the difficulties of culture isolation.

An FA has been developed for HEV using a polyclonal antiserum raised in rabbits against whole virus that has been passaged in mice. The FA is highly sensitive (Yagami et al. 1986).

Detection of HEV by nested RT-PCR targeting the spike gene is highly sensitive and specific (Sekiguchi et al. 2004). A comparison of RT-PCRs developed to target the *pol*, spike and structural genes identified the assay targeting the spike gene as the most sensitive. This assay had a detection limit of 0.1 TCID₅₀/50 µl and therefore could detect dilutions of virus that were negative by infection assays.

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 for extraneous agent testing do not provide optimal growth conditions for HEV and will not be sufficient for primary isolation of HEV. The guideline 9 CFR 113.47: *Detection of extraneous viruses by the fluorescent antibody technique* describing the FA on infected cell monolayers is sufficient for HEV detection.

The European Pharmacopoeia general monograph *Vaccines for veterinary use* and 5.2.4 *Cell cultures for the production of veterinary vaccines* describe generalised methods for extraneous agent testing using cell culture monolayers that are sensitive to the agent being tested, and detection using specific testing methods that are sensitive to the viral agent. The monographs do not specify details of a culture system or assay for HEV detection, simply that the methods should be sensitive.

Conclusion

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 are not acceptable.

Primary isolation of HEV in SK-K and secondary porcine thyroid cells is recommended. Cultures should be maintained in serum-free medium and should be passaged 3 times.

FA for HEV as described in 9 CFR 113.47: *Detection of extraneous viruses by the fluorescent antibody technique* is sufficient for HEV detection.

Specific testing is required for HEV by FA or nested RT-PCR targeting the spike gene.

Porcine parvovirus

Family *Parvoviridae*, genus *Parvovirus*

Susceptible cell cultures for primary isolation of porcine parvovirus (PPV) are A-72 cells, Madin-Darby canine kidney (MDCK), PK-15, Vero, ESK, NSK, NPTr, subclone of Chinese hamster fibroblast V79 cells (SB), or ST cells. Primary or secondary SK cells are the most susceptible to PPV infection.

PPV should be inoculated into cell culture as a suspension and this is followed by plating of cells and monolayer formation over the next 4 days. The cell monolayers are passaged for 28 days. Cell cultures must be of low-passage number and must be rapidly dividing cells as PPV is dependent on host polymerases present during the interphase synthesis (S) phase of the cell cycle for the completion of its replication cycle. PPV growth is improved by the use of roller bottles or microcarrier beads. Serum supplements are used at minimal concentrations and should have minimal haemagglutination inhibitory activity for PPV. The presence of antibody specific for PPV in the serum used to supplement the maintenance medium can interfere with primary isolation of PPV.

Infection with high titres of PPV is readily evident as a marked cytolytic effect 2–5 days after infection. CPE is confirmed by FA which is highly sensitive and specific. Confirmation of PPV is also possible by HA.

PPV false positives originating from contamination in the laboratory are common due to the extreme stability of PPV and its resistance to disinfection procedures. Contamination can also originate from infected tissues used to prepare the primary porcine kidney cells. In order to prevent false positive results during primary isolation of PPV, cell cultures should be prepared from gnotobiotic pigs or specific pathogen free (SPF) pigs.

Empty and defective virus particles occur at high frequency when PPV growth conditions are suboptimal. Defective virus particles interfere with productive infection and reduce the detection sensitivity. PPV has a ratio of 10^6 virus particles/PFU (Heldt et al. 2006). Therefore the threshold for detection by CPE and FA will be titres greater than 7–8 logs of virus particles. For extraneous agent testing it is important to be able to detect very low titres and a more sensitive method is required such as PCR.

Specific testing for PPV is required because of the low detection sensitivity of primary isolation of PPV.

PCRs have been developed for PPV with high detection sensitivity (Baylis et al. 2007; Huang et al. 2004; Molitor et al. 1991; Soares et al. 1999). A PCR targeting the (*NSP*)-1 gene sequence is reported to have improved the detection sensitivity by a 6 log dilution of virus relative to culture isolation and HA (Soares et al. 1999). A PCR targeting the viral protein (*VP*) 2 gene sequence is also highly sensitive and specific (Molitor et al. 1991; Prikhod'ko et al. 2003). The detection threshold for this PCR is 100 fg of replicative form DNA or 1 PFU. A real-time SYBR green nested PCR has been developed with a detection threshold of 100 genome copies or 0.2 logs TCID₅₀/ ml (Prikhod'ko et al. 2003).

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 for extraneous agent testing will not be sufficient for primary isolation of PPV. The guideline 9 CFR 113.47: *Detection of extraneous viruses by the fluorescent antibody technique* describing the FA is not sufficiently sensitive for detection of PPV.

The European Pharmacopoeia general monograph *Vaccines for veterinary use* and 5.2.4 *Cell cultures for the production of veterinary vaccines* describe generalised methods for extraneous agent testing using cell culture monolayers that are sensitive to the agent being tested, and detection using specific testing methods that are sensitive to the viral agent. The monographs do not specify details of a culture system or assay for PPV detection, simply that the methods should be sensitive.

Conclusion

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 are not acceptable.

Primary isolation of PPV in primary or secondary SK cells is recommended. Culture isolation should be in cells that are of low passage number and are rapidly dividing. Cultures should be prepared from gnotobiotic or SPF pigs. PPV should be inoculated into suspension cultures with subsequent plating of monolayers or grown in roller bottles or on microcarrier beads. The serum supplement percentages should be minimal and should be tested as free of PPV contamination or reactivity. Cultures are required to be maintained for 28 days.

The FA for PPV as described in 9 CFR 113.47: *Detection of extraneous viruses by the fluorescent antibody technique* is not sufficiently sensitive for detection of PPV.

Specific testing is required for PPV by PCR or real-time SYBR green nested PCR.

Porcine reproductive and respiratory syndrome virus

Family *Arteriviridae*, genus *Arterivirus*

Primary isolation of porcine reproductive and respiratory syndrome virus (PRRSV) is difficult. Alveolar macrophages harvested from pigs less than 6–8 weeks old are most susceptible to infection and preparations of macrophages differ markedly in their susceptibility to infection. Macrophage batches should be prepared from SPF pigs and must be validated with control virus for susceptibility to PRRSV infection. Primary isolation of PRRSV is possible in MARC-145 and MA-104 but the sensitivity is lower than for alveolar macrophages and some strains of the European subtype cannot be isolated in these cell lines (OIE 2010).

CPE is evident 1–2 days after inoculation and is confirmed by FA or ISH. ISH uses a digoxigenin-labelled cDNA probe targeting the *N* sequence (Laroche et al. 1996). Some isolates of PRRSV do not produce CPE or the CPE is barely discernable and may only become evident after multiple

passages. Therefore 2 blind passages are required to ensure CPE is readily detected. CPE alone is not considered to be sufficient for diagnosis of PRRSV and specific testing is required.

Specific testing is required for PRRSV as culture isolation is unreliable.

A number of RT-PCRs and nested RT-PCRs have been developed targeting the PRRSV N protein (ORF 7) sequence (Christopher-Hennings et al. 1995; Drolet et al. 2003; Magar and Larochelle 2004; Mardassi et al. 1994; Suarez et al. 1994; Van Woensel et al. 1994). The detection limit for the RT-PCR is reported to be $10^{2.5}$ TCID₅₀/ml (Van Woensel et al. 1994). The nested RT-PCR can detect 10 TCID₅₀/ml and is therefore 3 times more sensitive than the RT-PCR (Christopher-Hennings et al. 1995). The nested RT-PCR has been combined with Southern blotting to differentiate between European and American isolates (Mardassi et al. 1994). This method uses a specific IAF-exp91 N probe targeting the 3' end of the genome in which there is a deletion present in European strains but not American strains (Mardassi et al. 1994). A real-time Taqman RT-PCR has been developed targeting ORF 7 and using dual-labelled probes specific to the American and European strains (Kleiboeker et al. 2005). The detection limit for the real-time Taqman assay is 1 TCID₅₀/ml and the sensitivity was comparable when the assay was multiplexed. A multiplex nested RT-PCR has been developed targeting the *pol* gene (ORF 1b) (Gilbert et al. 1997a). The multiplex assay has the advantage of typing the American and European antigenic types of PRRSV. The detection limit for the nested form of this assay was 10 TCID₅₀/ml and the detection limit was 10^3 TCID₅₀/ml for the multiplex assay.

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 for extraneous agent testing will not be sufficient for primary isolation of PRRSV. The 9 CFR guidelines do not include specific testing for PRRSV.

The European Pharmacopoeia general monograph *Vaccines for veterinary use* and 5.2.4 *Cell cultures for the production of veterinary vaccines* describe generalised methods for extraneous agent testing using cell culture monolayers that are sensitive to the agent being tested, and detection using specific testing methods that are sensitive to the viral agent. The monographs do not specify details of a culture system or assay for PRRSV detection, simply that the methods should be sensitive.

Conclusion

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 are not acceptable.

Primary isolation of PRRSV in cell culture is not recommended.

Specific testing for PRRSV is required by real-time Taqman RT-PCR targeting ORF 7.

Porcine respiratory coronavirus

Family *Coronaviridae*, genus *Coronavirus*

PRCV is a deletion mutant of TGEV (refer to entry for TGEV).

Porcine teschovirus 1

Family *Picornaviridae*, genus *Teschovirus*

Porcine teschovirus 1 is also known as polioencephalomyelitis virus or porcine enterovirus. Primary isolation of polioencephalomyelitis virus (PEV) is recommended in SK cells. Inocula should be added to the culture monolayers and incubated for 1 hour at 37 °C to allow adsorption before the addition of maintenance medium. At least 3 passages are required and CPE is evident as foci of pyknotic refractile cells 3–4 days after infection. Confirmation of culture isolation is by FA on monolayers of infected SK cells using hyperimmune porcine serum (OIE 2010).

A group-specific nested RT-PCR has been designed targeting a region of the 5' UTR of PEV conserved in all 13 serotypes (Zell et al. 2000). A real-time Taqman RT-PCR has been developed targeting the 5' UTR of PEV and utilising serotype-specific dual-labelled probes (Krumbholz et al. 2003). The major advantage of the real-time Taqman RT-PCR is enhanced specificity and sensitivity relative to the nested RT-PCR format. The assay has a detection threshold of 1000 copies for CPE group I and 100 copies for CPE groups II and III (Krumbholz et al. 2003; Zell et al. 2000).

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 for extraneous agent testing will not be sufficient for primary isolation of PEV. The 9 CFR guidelines do not include specific testing for PEV.

The European Pharmacopoeia general monograph *Vaccines for veterinary use* and 5.2.4 *Cell cultures for the production of veterinary vaccines* describe generalised methods for extraneous agent testing using cell culture monolayers that are sensitive to the agent being tested, and detection using specific testing methods that are sensitive to the viral agent. The monographs do not specify details of a culture system or assay for PEV detection, simply that the methods should be sensitive.

Conclusion

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 are not acceptable.

Primary isolation of PEV in SK cells is recommended. Three culture passages are required and adsorption incubation for 1 hour is required.

Specific testing is required for PEV either by FA or real-time Taqman RT-PCR.

Pseudorabies virus

Family *Herpesviridae*, genus *Varicellovirus*

Pseudorabies virus is also known as Aujeszky's disease virus or suid herpesvirus 1. Primary isolation of pseudorabies virus (PRV) is in myeloblastic leukaemia (ML), ST, PK-15, SK-6, and SK cell lines (Onyekaba et al. 1987). A comparative study of the susceptibility of cell lines to PRV found higher titres resulted from infection of ML cells than SK or ST cells. A second study reports that PRV was isolated most frequently in SK-15 cells (Tahir and Goyal 1995). Cultures should be incubated for 7 days and passaged at least once. CPE is evident by 3 days after infection as cell detachment, formation of syncytia, and acidophilic intranuclear inclusions with margined chromatin. The success of isolation is variable, as low levels of virus can be inhibited by the presence of other agents, and PRV can establish persistent, non-cytolytic infections.

Specific testing is recommended for PRV because of inconsistent results for virus isolation and low sensitivity of detection of CPE for non-cytolytic or persistent culture adapted isolates.

Specific testing for PRV can be done by PCR or by FA. The FA utilises polyclonal or monoclonal antibodies against intracellular antigens (Stewart et al. 1967; Tahir and Goyal 1995; Tsai et al. 1989). FA is dependent on virus growth in culture and therefore will not detect PRV isolates that cannot be cultivated or persistent latent infections.

A number of different PCR techniques have been applied to the detection of PRV. Conventional PCRs targeting the gp50, gE and the gI genes has been widely applied for the detection of PRV (Belák et al. 1989; Hasebe et al. 1993; Jacobs et al. 1999). A loop-mediated isothermal amplification (LAMP) assay has been described that targets the DNA-binding gene of PRV (En et al. 2008). The LAMP assay has a detection limit of 10 fg of DNA and is reported to be 1000 times more sensitive than comparable PCR detection. Real-time Taqman PCRs have been developed targeting the gE, gB and gG genes (Yoon et al. 2006; Zhang et al. 2007b). The real-time assays targeting the gB and gE genes have detection thresholds of 10^2 and $10^{2.8}$ copies respectively. The real-time PCRs have the advantage of being sufficiently sensitive to detect very low levels of virus present in latent or persistent cellular infection. PCRs for PRV have been adapted to microarray assays, digitonin-labelled probe detection in microwell format, and multiplex PCR (Boutin et al. 1994; Cao et al. 2005; Huang et al. 2004). A fluorescence ISH (FISH) PCR was found to have lower sensitivity than PCR (Liao et al. 2001).

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 for extraneous agent testing will not be sufficient for primary isolation of PRV. The 9 CFR guidelines do not include specific testing for PRV.

The European Pharmacopoeia general monograph *Vaccines for veterinary use* and 5.2.4 *Cell cultures for the production of veterinary vaccines* describe generalised methods for extraneous agent testing using cell culture monolayers that are sensitive to the agent being tested, and detection using specific testing methods that are sensitive to the viral agent. The monographs do not specify details of a culture system or assay for PRV detection, simply that the methods should be sensitive.

Conclusion

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 are not acceptable.

Primary isolation of PRV in ML or SK-15 cells is recommended.

Specific testing for PRV is required by LAMP or real-time Taqman PCR.

Rabbit fibroma virus

Family *Poxviridae*, genus *Leporipoxvirus*

Rabbit fibroma virus is also known as Shope fibroma virus (SFV). Primary isolation of SFV is possible in rabbit kidney, rabbit testes, monkey kidney, rabbit embryonic cell cultures, human amniotic culture, chorioallantoic membrane (CAM), Statens Seruminstitut rabbit cornea (SIRC), rabbit kidney (LLC-RK1), RK-13 and HeLa cell lines (Hodes and Chang 1968; Pogo and Dales 1971; Willer et al. 1999). Rabbit embryonic kidney cell cultures are most susceptible to SFV infection.

Tissues from newborn and embryonic rabbits are more susceptible than tissue cultures from adult rabbits to SFV infection and the virus replicates to higher titres. Cellular proliferation and aggregation and the formation of pocks in SFV-infected rabbit kidney or rabbit testes cells is only reliably seen at low MOI in mixed populations rather than clonal cultures and in embryonic cell lines (Israeli 2008).

The culture yield of myxoma viruses can be increased markedly by the use of spinoculation in shell vials or roller bottle cultivation methods instead of stationary methods (Hughes 1993; Kassner et al. 1991). Rolling of cultures before infection stimulates mitotic rates and RNA transcription and therefore enhances virus growth. The spinoculation shell vial culture method is reported to increase the sensitivity of detection of myxoma viruses by 50%. In this method the virus is added to the rabbit kidney embryonic cell monolayers and centrifuged at 1270 times gravity ($\times g$) for 20 minutes to increase adsorption. The cultures are then incubated for 8 hours and then assessed by FA (Padgett et al. 1962).

SFV infection is not cytolytic as the virus stimulates cellular proliferation and virus buds from the intact plasma membrane. SFV infection can be detected 20 hours after infection of rabbit kidney cells as nuclear vacuolation and eosinophilic, cytoplasmic inclusion bodies when stained with Giemsa. Inclusion body formation and nuclear vacuolation is restricted to SFV infection of cell lines of epithelial origin and infection of fibroblast-type cells results in the formation of large stellate cells. Inclusion bodies can be confirmed by FA using hyperimmune SFV specific rabbit antiserum (Hodes and Chang 1968).

Specific testing is possible by PCR amplification of the *env* gene based on the Lausanne strain; however, there are no reports of validation of this assay for diagnostic purposes (Farsang et al. 2003).

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 for extraneous agent testing will not be sufficient for primary isolation of SFV. The 9 CFR guidelines do not include specific testing for SFV.

The European Pharmacopoeia general monograph *Vaccines for veterinary use* and 5.2.4 *Cell cultures for the production of veterinary vaccines* describe generalised methods for extraneous agent testing using cell culture monolayers that are sensitive to the agent being tested, and detection using specific testing methods that are sensitive to the viral agent. The monographs do not specify details of a culture system or assay for SFV detection, simply that the methods should be sensitive.

Conclusion

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 are not acceptable.

Primary isolation of SFV in rabbit embryonic kidney cell cultures using a spinoculation, shell vial technique is recommended.

Specific testing is recommended by FA to detect inclusion body formation.

Rabbit haemorrhagic disease virus

Family *Caliciviridae*, genus *Lagovirus*

Rabbit haemorrhagic disease virus is also known as rabbit calicivirus. There are no known culture systems that can support the growth of rabbit calicivirus (RCV). RCV can be isolated by inoculation of rabbits and harvest from liver tissue (Capucci et al. 1990; OIE 2010).

Defective virus particles are generated during chronic infection of rabbits and are characterised by truncated, non-haemagglutinating, capsid structural units called smooth RCV (s-RCV) (Capucci et al. 1991). Defective s-RCV particles interfere with detection by diagnostic assays based on viral protein recognition such as HA and ELISA. The s-RCV virions are temperature sensitive and do not react in HAs at room temperature; however, their function is restored at 4 °C (Capucci et al. 1996).

Specific testing is required for diagnosis of RCV and primary culture isolation is not recommended. Specific testing is possible by HA, EM, IEM, ELISA and PCR.

The IEM method is recommended by the OIE as the most sensitive diagnostic assay for RCV. This assay uses a convalescent hyperimmune RCV-specific rabbit antiserum to stain the virus sample, which is then concentrated by ultracentrifugation, negatively stained and examined by EM. The RCV specific antiserum immunoprecipitates the virus and increases the virus recovery. RCV is evident as non-enveloped, icosahedral viral particles of 32–35nm. The IEM assay has been adapted to use immunogold-conjugated hyperimmune RCV specific rabbit antiserum to enhance detection sensitivity.

The HA requires human blood group O red blood cells, freshly collected, stored overnight in Alsever's solution and washed in 0.85% PBS (pH6.5) (Liu et al. 1984). The efficacy of the assay is reduced at pH above 6.5 (Capucci et al. 1991). The HA is reported to generate on average 10% false negative results relative to ELISA or EM. The HA is not recommended due to poor repeatability.

Antigenic variants of RCV do not react with the mAb 1H8 specific to RCV which is a virus neutralising antibody that can protect rabbits against infection (Capucci et al. 1998; Grazioli et al. 2000). Diagnostic assays using mAb 1H8 will not detect antigenic variants and are therefore not suitable for extraneous agent testing.

A RT-PCR has been developed targeting the sequence of the VP60 coat protein of RCV. RT-PCR detection is reported to be 4 logs more sensitive than ELISA detection for RCV (Gould et al. 1997). The RT-PCR has been adapted to a real-time Taqman RT-PCR with a reported sensitivity of 100%, the threshold for detection is 10 genomic copies and virus detection is reported to be possible in an experimental system up until 15 weeks after infection when detection by ELISA, FA and experimental transmission were no longer positive (Gall et al. 2007). However, the real-time Taqman RT-PCR has not undergone sufficient validation in a field or diagnostic setting.

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 for extraneous agent testing will not be sufficient for primary isolation of RCV. The 9 CFR guidelines do not include specific testing for RCV.

The European Pharmacopoeia general monograph *Vaccines for veterinary use* and 5.2.4 *Cell cultures for the production of veterinary vaccines* describe generalised methods for extraneous agent testing using cell culture monolayers that are sensitive to the agent being tested, and detection

using specific testing methods that are sensitive to the viral agent. The monographs do not specify details of a culture system or assay for RCV detection, simply that the methods should be sensitive.

Conclusion

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 are not acceptable.

Primary isolation of RCV by inoculation of rabbits and harvesting of RCV from infected liver is recommended.

Specific testing is required by IEM using immunogold-conjugated hyperimmune RCV specific rabbit antiserum.

Rabies virus

Family *Rhabdoviridae*, genus *Lyssavirus*

The standard for rabies virus diagnosis is primary isolation in murine neuroblastoma cell line (Neuro-2a (ATCC no. CCL-131)) followed by the standardised FA (OIE 2010).

BHK-21 cells are suitable for isolation of some laboratory adapted strains of rabies virus but are not permissive for all street strains of the virus. The permissiveness of BHK-21 to rabies virus is increased by treatment with DEAE-dextran. However, titration of rabies virus in Neuro-2a cells demonstrates greater sensitivity for detection of rabies than treated BHK-21. Rabies virus isolation requires an adsorption step for 2 hours prior to culture and infected cultures should be maintained for 4 days (Hanlon et al. 2005; Rudd et al. 1980; Rudd and Trimarchi 1987).

FA will give good sensitivity combined with primary isolation in Neuro-2a cells. FA positive cells appear within 2 hours of cell infection; however, the FA is performed 48 hours after infection in the standardised protocol. Cell staining is done using fluorescent conjugated antibodies. Specific antibodies may be polyclonal serum directed against viral protein, polyclonal serum against N protein, or a mix of mAbs. The FA when performed with polyclonal serum has been shown to have efficacy against all 7 serogroups of lyssaviruses.

Other specific testing methods developed for rabies virus are RT-PCRs and nucleic acid hybridization probes and AC-ELISA.

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 for extraneous agent testing do not provide optimal growth conditions for rabies virus and are not suitable for primary isolation of rabies virus. The 9 CFR 113.47: *Detection of extraneous viruses by the fluorescent antibody technique* protocol for rabies virus FA will give good sensitivity when combined with primary isolation in Neuro-2a cells and provided the optimal growth conditions outlined above are used.

The European Pharmacopoeia general monograph *Vaccines for veterinary use* and 5.2.4 *Cell cultures for the production of veterinary vaccines* describe generalised methods for extraneous agent testing using cell culture monolayers that are sensitive to the agent being tested, and detection using specific testing methods that are sensitive to the viral agent. The monographs do not specify details of a culture system or assay for rabies virus detection, simply that the methods should be sensitive.

Conclusion

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 are not acceptable.

Primary isolation of rabies virus in Neuro-2a cells is recommended. Rabies virus isolation requires an adsorption step for 1 hour prior to culture and infected cultures should be maintained for 4 days.

FA testing for rabies virus as described in 9 CFR 113.47: *Detection of extraneous viruses by the fluorescent antibody technique* will give good sensitivity combined with primary isolation in Neuro-2a cells.

Specific testing is required by FA, 48 hours after infection of Neuro-2a cells.

Rift Valley fever virus

Family *Bunyaviridae*, genus *Phlebovirus*

Susceptible cell lines for Rift Valley fever virus (RVFV) are Vero, BHK-21, CER, foetal rhesus monkey diploid lung (DBS-FR_hL-2), and AAC3/36. BHK-21 cells are reported to be sensitive for detection of even low levels of RVFV. Traditionally the standard isolation method for RVFV has been IC inoculation of suckling mice. Anderson *et al* (1989) report that isolation in Vero, AA C3/C6 and DBS-FR_hL-2 cell lines are at least as sensitive as IC inoculation of suckling mice or hamsters. In this study viral titres produced from infection of AA C6/C3 were higher for some isolates than in Vero or DBS-FR_hL-2. By 18 hours post-infection CPE was evident for 1/11 isolates, by 72 hours for 9/11 isolates and by day 6 for 10/11 isolates. CPE is evident within 12–24 hours after infection as intra-nuclear and cytoplasmic rod-shaped inclusions, followed by complete cytolysis of the cellular monolayer (Anderson, Jr. *et al.* 1989; Ellis *et al.* 1988).

High MOI can be inhibitory for virus growth due to the formation of defective particles. This can be circumvented by inoculation with serial dilutions of test material. Incubation for 1 hour to allow adsorption is important to attachment and entry of virus into host cells. Serum supplements in medium should be free of RVFV reactivity. Cultures should be maintained for 6 days to ensure detection of CPE (Garcia *et al.* 2001; Sall *et al.* 1997; Sall *et al.* 1999; Sall *et al.* 2001).

Primary isolation in AA C3/C6 cells is adequate for RVFV detection due to the profound cytolytic effect of RVFV in cell culture and readily identifiable CPE.

Potential methods of specific testing are the FA or RT-PCR/real-time RT-PCR targeting the *NSs* sequence. The FA using polyclonal serum against RVFV resulted in similar detection sensitivity as detection by CPE alone.

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 for extraneous agent testing do not provide optimal growth requirements of RVFV and are not suitable for primary isolation of RVFV. The 9 CFR guidelines do not include specific testing for RVFV.

The European Pharmacopoeia general monograph *Vaccines for veterinary use* and 5.2.4 *Cell cultures for the production of veterinary vaccines* describe generalised methods for extraneous agent testing using cell culture monolayers that are sensitive to the agent being tested, and detection using specific testing methods that are sensitive to the viral agent. The monographs do not specify details of a culture system or assay for RVFV detection, simply that the methods should be sensitive.

Conclusion

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 are not acceptable.

Primary isolation of RVFV in AA C3/C6 cells is recommended. Cultures should be maintained for 6 days, 1 hour adsorption incubation is required, and inoculation should be with serial dilutions of the test material. Serum supplements must be tested to confirm freedom from RVFV reactivity.

Specific testing for RVFV is not required due to the profound cytolytic effect of RVFV in cell culture and readily identifiable CPE.

Rinderpest virus

Family *Paramyxoviridae*, genus *Morbillivirus*

Rinderpest virus can be readily detected by primary isolation in Vero cell lines. The incubation period required for detection of CPE will vary according to the culture conditions and virus strains. CPE appears within 7 days but can take up to 3 weeks. A blind passage must be performed before declaring a sample negative. The OIE guidelines for rinderpest virus primary isolation require the test material in a working volume of 1–2 ml to be inoculated directly into the medium of cultured cell monolayers.

Additional specific testing for rinderpest virus is not required as the virus is readily identified by culture isolation and detection of CPE.

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 will meet the growth requirements of rinderpest virus and will be sufficient for primary isolation of rinderpest virus. The generalised 9 CFR protocol is for inoculation of a 15% solution of the test material in culture medium onto Vero cell monolayers, and 14 days maintenance period for examination for CPE.

The European Pharmacopoeia general monograph *Vaccines for veterinary use* and 5.2.4 *Cell cultures for the production of veterinary vaccines* describe generalised methods for extraneous agent testing using cell culture monolayers that are sensitive to the agent being tested, and detection using specific testing methods that are sensitive to the viral agent. The monographs do not specify details of a culture system or assay for rinderpest virus detection, simply that the methods should be sensitive.

Conclusion

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 are acceptable.

Primary isolation of rinderpest virus in Vero cells is recommended. Cultures should be maintained for 3 weeks and at least 1 blind passage is required.

Specific testing is not required for rinderpest virus.

Rotavirus (bovine and porcine)

Family *Reoviridae*, genus *Rotavirus*

Seven serotypes of rotaviruses (RVs) are recognised; domestic animals are hosts for serotypes 3, 4, 5, 6 and 7. Serotype 3 has been isolated from equines and canines, serotype 4 from porcines, and serotype 5 from porcines and equines. Bovine RV isolates are serotype 6, and avian isolates are serotype 7. Common culture isolation methods are required for all RV serotypes and discussion of the factors influencing these methods applies to all serotypes. Primary isolation of RV is problematic and generally has a low success rate for non-culture adapted strains. MA104 cells are the most sensitive culture system for primary isolation of RV. Other susceptible cell cultures are African green monkey kidney (AGMK), MDBK, rhesus monkey kidney (LLC-MK2), HeLa, subclone of African green monkey kidney epithelial (BSC-1), BEK, BK, GBK and chicken kidney (CKC) (Hirano et al. 1987; Murakami et al. 1983; Tsunemitsu et al. 1991).

Virus should be pre-treated with trypsin and cultivated in roller bottles with trypsin incorporated in the maintenance medium. Infected cultures should be propagated for 6 serial passages. RV particles when released from the host cell are non-infective and undergo a process of maturation that is calcium dependent. Calcium supplementation of the medium is therefore necessary to ensure the production of infectious virus. Productive infection results in CPE within 48 hours. CPE is characterised by vacuolation, small rounded cells, and intracytoplasmic eosinophilic inclusion bodies (Babiuk et al. 1977; Barnett et al. 1979; Ruiz et al. 1996).

Specific testing methods are recommended due to the problems associated with culture isolation. RT-PCR has the advantage of detection of virus in the event that primary isolation is unsuccessful. Because of the large number of groups and serotypes of rotavirus, genotyping by RT-PCR is generally preferred to serotyping or immune assays for rotavirus detection. A nested RT-PCR has been developed targeting the *N* gene of RV. The threshold for detection has been determined to be 2×10^2 TCID₅₀/0.1ml (Alfieri et al. 2003; Beck et al. 2007; Falcone et al. 1999; Hardy et al. 1992; Sunil-Chandra and Mahalingam 1996; Zheng et al. 1989).

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 for extraneous agent testing do not provide optimal growth requirements of RV and are not suitable for primary isolation of RV. The 9 CFR guidelines do not include specific testing for RV.

The European Pharmacopoeia general monograph *Vaccines for veterinary use* and 5.2.4 *Cell cultures for the production of veterinary vaccines* describe generalised methods for extraneous agent testing using cell culture monolayers that are sensitive to the agent being tested, and detection using specific testing methods that are sensitive to the viral agent. The monographs do not specify details of a culture system or assay for RV detection, simply that the methods should be sensitive.

Conclusion

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 are not acceptable.

Primary isolation of RV in MA-104 cells is recommended. Cultures should be pre-treated with trypsin, cultivation is required in roller bottles and calcium supplementation is required for the culture medium.

Specific testing for RV is required by nested RT-PCR targeting the *N* gene.

Sendai virus

Family *Paramyxoviridae*, genus *Respirovirus*

Sendai virus is also known as murine parainfluenza virus 1. Primary isolation of Sendai virus (SeV) is possible in Vero, monkey kidney (MK), SK, clone of swine kidney (PS-Y15), LLC-MK2, BEK, BHK-21, CKC, chicken embryo lung, chicken embryo skin and muscle cells, or by allantoic inoculation into ECE that are 11 days old (Darlington et al. 1970; Homma 1971; Ito 1976; Nagata et al. 1965; Parker and Reynolds 1968; Shibuta 1972; Shigeta 1964; Sugita et al. 1974). Allantoic inoculation of ECE and inoculation of PS-Y15 are the most sensitive methods of primary isolation of SeV. SeV is isolated in ECE that is 11 days old by allantoic inoculation, incubation at 34 °C for 48 hours, and then the virus is harvested from clarified allantoic fluid (Agungpriyono et al. 1999). CPE is evident by the formation of fused giant cells and intra-cytoplasmic and intra-nuclear inclusion bodies (Faisca and Desmecht 2007).

SeV establishes persistent, inapparent, non-cytopathic infections of cell cultures. These cultures are reported to enhance the cytopathic phenotype empirically observed from superinfection with some pathogens and to inhibit growth of other pathogens (Maeno et al. 1966; Sugita 1981).

Specific testing is required to ensure detection of inapparent non-cytopathic culture contaminants. Specific testing is possible by FA, HA, plaque assay, RT-PCR, and real-time fnRT-PCR.

HA for SeV is performed on guinea pig or chicken RBC at 4 °C for 4 hours (Parker and Reynolds 1968).

Plaque assay requires trypsin cleavage of the F protein and then infection of LLC-MK2 monolayers with agarose overlays (Homma 1971; Sugita et al. 1974). There are large plaque, cytopathic (RL) and small plaque, non-cytopathic (RS) variants of SeV (Sugita 1981). The RL and RS variants have equivalent growth kinetics when grown in isolation, but co-infection or superinfection of the RL variant with the RS variant inhibits the growth of the RL variant. From these findings it could be deduced that inadvertent undetected contamination of cell cultures with the RS variant would interfere with detection of the RL variants in test samples.

A real-time fnRT-PCR has been developed targeting the *N* and pneumonia virus of mice (PVM) attachment (*G*) genes for detection of SeV and utilising dual-labelled fluorescent probes (Maeno et al. 1966; Wagner et al. 2003). The real-time fnRT-PCR had a detection limit of 10 fg RNA, was reported to have 100% sensitivity when tested for a range of other RNA viruses and paramyxoviruses, and the detection sensitivity was comparable to the MAP assay (Wagner et al. 2003).

SeV is one of the agents targeted in the MAP and IMPACT panel of tests. The IMPACT panel tests for SeV using the highly specific and highly sensitive real-time fnRT-PCR targeting the *N* and *G* genes.

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 for extraneous agent testing will not be sufficient for primary isolation of SeV. The 9 CFR guidelines do not include specific testing for SeV.

The European Pharmacopoeia general monograph *Vaccines for veterinary use* and 5.2.4 *Cell cultures for the production of veterinary vaccines* describe generalised methods for extraneous agent testing using cell culture monolayers that are sensitive to the agent being tested, and detection

using specific testing methods that are sensitive to the viral agent. The monographs do not specify details of a culture system or assay for SeV detection, simply that the methods should be sensitive.

Conclusion

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 are not acceptable.

Primary isolation of SeV in either PS-Y15 cells or by allantoic inoculation of ECE is recommended.

Specific testing is required by real-time fnRT-PCR targeting the *N* and *G* genes of SeV.

Swine influenza virus

Family *Orthomyxoviridae*, genus *Influenzavirus A*

Swine influenza virus (SIV) isolation is possible in ECE, ESK, MDCK, Vero, BHK, St Jude porcine lung epithelial (SJPL) and MRC-5 cell lines (Hermann et al. 2006; Karasin et al. 2000; Karasin et al. 2002; Ma et al. 2006; Shin et al. 2006). There are marked strain differences in growth properties for different isolates of SIV. The MDCK cell line is reported to have greater detection sensitivity than the Vero or MRC-5 cell lines and lower detection sensitivity than the ESK cell line (Gaush and Smith 1968; Murakami et al. 1988). Some SIV strains do not grow well in ECE and likewise other strains of SIV do not grow well in MDCK (Carman et al. 1999; Zhou et al. 1999; Zhou et al. 2000). The SJPL cell line has been reported to have greater sensitivity for SIV isolation than MDCK and to support the growth of a wide spectrum of isolates due to a higher density of Sia2-3Gal and Sia2-6Gal receptors (Gaush and Smith 1968; Seo et al. 2001). Because of differences in growth for different strains, primary virus isolation is recommended in both ECE as well as in either ESK or SJPL cell lines as the most sensitive detection method (Clavijo et al. 2002).

Supplementation of culture medium with trypsin enhances CPE in infected cultures (Meguro et al. 1979). Culture medium is removed and the monolayers are washed 3 times prior to inoculation. The inoculum is then incubated on the monolayer at 37 °C for 1 hour to allow adsorption of the virus. Plates are observed for CPE and the medium tested by HA and HI at 3–6 days. Cultures should be maintained for 2 passages (Clavijo et al. 2002). Specificity of the HA is low; however, this is improved when followed by a HI assay using specific antiserum. In general, anti-sera against the H1N1 or H3N2 strains of SIV do not cross-react (Webby et al. 2000). There are reports of some antigenic variant isolates of SIV not reacting in the HI assay using standard reference serum.

Culture isolation of SIV is problematic due to viral strain variations in culture infectivity and due to the inherent low detection sensitivity. The frequency of false negative results for SIV by culture isolation and FA is reported to be significantly greater than for influenza viruses from other host species due to greater genotypic and phenotypic heterogeneity in SIV (Fouchier et al. 2000). The sensitivity of the Directigen FluA ELISA is variable (Quinlivan et al. 2004). The Directigen FluA kit detects viral antigen associated with cell membranes more readily than free virus in solution due to changes in viral structural proteins at viral attachment to cellular receptors (Cherian et al. 1994; Hermann et al. 2006; Ryan-Poirier et al. 1992).

Specific RT-PCR, nested RT-PCR, real-time RT-PCR, or biosensor assays have been developed for SIV. RT-PCRs have been developed targeting the *M* gene (Fouchier et al. 2000). A real-time RT-PCR targeting the *M* gene of SIV is reported to have a detection threshold of 2 genomic copies or 0.5 TCID₅₀/ml and a sensitivity of 94 % and specificity of 85 % relative to culture isolation (Hermann et al. 2006; Richt et al. 2004). Detection sensitivity can be markedly improved by culture

isolation followed by real-time RT-PCR. Ethanol fixation of test material is reported to significantly enhance the sensitivity of real-time RT-PCRs for diagnosis of influenza viruses whilst the presence of mucin in test material inhibits the assay (Clavijo et al. 2002; Hermann et al. 2006; Krafft et al. 2005). The results from these studies indicate the efficacy of the real-time RT-PCR for SIV may be significantly influenced by components of the test material, and therefore the positive controls for the assay should include test inocula spiked with reference virus. A method for typing of SIV strains has been developed by combining the RT-PCR targeting the *M* gene of SIV with a heteroduplex mobility assay (Ellis and Zambon 2001). The RT-PCR for SIV has been combined with an enzyme immunoassay detection system resulting in 2 and 3 fold increases in detection sensitivity relative to RT-PCR alone and culture isolation respectively (Cherian et al. 1994; Hermann et al. 2006).

A number of biosensor assays have been developed to detect influenza viruses and to simultaneously identify HA/NA subtypes and epidemiological markers. These systems have the advantage of a rapid diagnosis, sensitive detection and the capacity for a broad range of subtyping. Microarray assays for influenza viruses have been developed that use a number of different technologies: oligonucleotide arrays (GreeneChipResp), Cy3/Cy5-conjugated dCTP incorporated into RT-PCR amplified targets for microarray detection, RT-PCR amplified target sequences incorporating biotinylated tags and detected by microarray, high density arrays using on-chip amplification, and low-density arrays for detection of RNA sequences (Fluchip-55). All these biosensor assays suffer from poor specificity and the rate of false positives is reported to be as high as 50% in some studies (Mehlmann et al. 2006; Quan et al. 2007; Townsend et al. 2006).

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 for extraneous agent testing will not be sufficient for primary isolation of SIV. The 9 CFR guidelines do not include specific testing for SIV.

The European Pharmacopoeia general monograph *Vaccines for veterinary use* and 5.2.4 *Cell cultures for the production of veterinary vaccines* describe generalised methods for extraneous agent testing using cell culture monolayers that are sensitive to the agent being tested, and detection using specific testing methods that are sensitive to the viral agent. The monographs do not specify details of a culture system or assay for SIV detection, simply that the methods should be sensitive.

Conclusion

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 are not acceptable.

Primary isolation of SIV both in ECE and in either ESK or SJPL cell lines is recommended. Culture medium should be supplemented with trypsin, 1 hour adsorption incubation is required, and cultures should be passaged at least twice.

Specific testing is required for SIV by real-time RT-PCR targeting the *M* gene. Because of the potential for inhibition of the real-time RT-PCR by components of the sample, the sample should be ethanol purified and test samples spiked with reference virus used as positive controls for the assay.

Swine pox virus

Family *Poxviridae*, genus *Suipoxvirus*

Swine pox virus (SPV) can be isolated in SK, ST, PK-15 and IB-RS-2 cell lines (Bina and Pandey 2002; de Boer 1975; Kasza et al. 1960; Kubin 1972; Lodetti et al. 1969; Mayr 1959; Mohanty et al. 1989). Primary isolation in ECE is unreliable and is not recommended (Kubin 1972). One published

study has reported the successful culture adaptation of a recombinant SPV isolate to porcine, human, monkey, hamster, rabbit and bovine cells (Bárcena and Blasco 1998). However, in general, SPV is very host-cell specific in its growth requirements and attempts at adaptation of the virus to growth in MDBK, BHK-21, BEK and HeLa cell lines or to growth in hosts other than swine are mostly reported to be unsuccessful (Garg and Meyer 1972; Garg and Meyer 1973; Kasza et al. 1960; Lodetti et al. 1969; Mayr 1959; Meyer and Conroy 1972).

CPE is evident 36–48 hours after seeding of SPV-infected PK-15 cells. CPE is characterised by granulation and vacuolation of the cytoplasm, and formation of cellular aggregates (Kasza et al. 1960; Mayr 1959). For some isolates of SPV, CPE is not evident until culture passage 4–7 (Gunenkov and Syurin 1966; Kasza et al. 1960; Kubin 1972; Mayr 1959). Detection by primary isolation and CPE is not sufficiently sensitive without specific testing.

Specific testing for SPV is possible by FA or IP assay (Cheville 1966; de Boer 1975; Garg and Meyer 1973; Mohanty et al. 1989). IP assay can detect viral inclusions in the cytoplasm but not the nucleus of infected cells from 6 hours and optimal detection is at 24–48 hours after infection.

PCR detection of SPV has been reported; however, there are no published reports of PCRs for SPV that are sufficiently validated for recommendation.

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 for extraneous agent testing will not be sufficient for primary isolation of SPV. The 9 CFR guidelines do not include specific testing for SPV.

The European Pharmacopoeia general monograph *Vaccines for veterinary use* and 5.2.4 *Cell cultures for the production of veterinary vaccines* describe generalised methods for extraneous agent testing using cell culture monolayers that are sensitive to the agent being tested, and detection using specific testing methods that are sensitive to the viral agent. The monographs do not specify details of a culture system or assay for SPV detection, simply that the methods should be sensitive.

Conclusion

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 are not acceptable.

Primary isolation of SPV in SK, ST, PK-15 or IB-RS-2 cell lines is recommended. Cultures should be maintained for 7 passages and FA performed 48 hours after culture inoculation.

Specific testing is required for SPV by FA or IP assay.

Swine vesicular disease virus

Family *Picornaviridae*, genus *Enterovirus*

Primary isolation of swine vesicular disease virus (SVDV) is on IB-RS-2 or other susceptible primary porcine cells. In general SVDV will grow in cells of porcine origin. Ferrari *et al* (2003) reported the development of 2 porcine cell lines, NSK and NPTr, immortalised by the activity of endogenous retroviruses. These cell lines had equivalent susceptibility and titres to that of reference primary porcine culture systems for the primary isolation of swine vesicular disease virus, pseudorabies virus, porcine parvovirus, classical swine fever virus, transmissible gastroenteritis virus, encephalomyocarditis virus and other swine enteroviruses. These cell lines would be highly suitable for the detection of extraneous agents in biological products of porcine origin. Although

there are reports of growth in primary calf thyroid cells and calf kidney cells, overall the results for SVDV isolation are variable in these lines. Ferris *et al* (2002) report the isolation of SVDV in primary calf thyroid, primary calf kidney and primary piglet kidney cell lines that were immortalised by oncogene transformation. SVDV could be isolated only from several of the immortalized piglet kidney cell lines; however, the sensitivity was far lower than for the standard IB-RS-2 cell line.

The rate of growth of SVDV in different culture systems reflects the efficiency of the 2A protease activity (Inoue *et al.* 2005). Diagnostic systems for detection of SVDV must also be suitable for detection of the attenuated strains that have mutations at Arg20 of the 2A protease. Therefore only highly sensitive cell culture systems such as IB-RS-2, NSK or NPTr cell lines are suitable.

Serum free medium is required for the isolation of SVDV due to serum inhibition of receptor binding by the virus.

CPE is readily identified in SVDV infected cultures after 48 hours, but may require 2–3 blind passages to become evident. Attenuated strains of SVDV produce CPE that is difficult to identify and therefore specific testing is recommended for the supernatant from the infected primary culture isolation.

Specific testing for SVDV is required to ensure detection of all isolates of SVDV. Specific testing for SVDV is recommended by either RT-PCR or the AC-ELISA.

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 will not meet the specific growth requirements of SVDV and will not be sufficient for primary isolation of SVDV. The generalised protocols do not meet the requirements of SVDV for susceptible cell lines, the number of culture passages and the supplementation of growth medium with serum will inhibit SVDV isolation. The 9 CFR guidelines do not include specific testing for SVDV.

The European Pharmacopoeia general monograph *Vaccines for veterinary use* and 5.2.4 *Cell cultures for the production of veterinary vaccines* describe generalised methods for extraneous agent testing using cell culture monolayers that are sensitive to the agent being tested, and detection using specific testing methods that are sensitive to the viral agent. The monographs do not specify details of a culture system or assay for SVDV detection, simply that the methods should be sensitive.

Conclusion

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 are not acceptable.

Primary isolation of SVDV in IB-RS-2, NSK or NPTr cell lines is recommended. Serum-free medium should be used to prevent serum inhibition of receptor binding of the virus. Cultures should be incubated for 3 days between passages, and cultures should be maintained for at least 3 passages.

Specific testing is required for SVDV by either RT-PCR or the AC-ELISA.

Theiler's murine encephalomyelitis virus

Family *Picornaviridae*, genus *Cardiovirus*

Primary isolation of Theiler's murine encephalomyelitis virus (TMEV) is possible in ME, BHK-21 or murine macrophage (J774) cells (Lipton et al. 1984; Obuchi et al. 1997; Oleszak et al. 1988). Virus should be incubated on the monolayer for 90 minutes to allow adsorption prior to the addition of maintenance medium. Infected cultures are frozen and thawed 3 times in succession, and then used to reinoculate fresh culture monolayers. CPE is evident within 24 hours. TMEV can be identified by plaque formation on BHK-21 cell monolayers (Rodrigues et al. 2005). Approximately 25–30% of cardiovirus isolates fail to grow in cell culture (Rotbart 1991).

There are 2 subgroups or plaque size variants of TMEV; subgroup 1 or large plaque size variant is highly cytolytic, non-persistent, and produces readily identified CPE in culture; and subgroup 2 or small plaque size variant is non-cytolytic, establishes persistent infection, and is difficult to detect by CPE alone (Lipton 1980; Lipton et al. 1984; Lipton and Friedmann 1980; Oleszak et al. 1988). The small TMEV isolates from the cytolytic subgroup 1 (large plaque size variants) do not replicate in J774 cells whereas subgroup 2 (small plaque size variants) infect J774 cells, but at lower sensitivity than BHK-21 cells (Obuchi et al. 1997). The subgroup 2 (small plaque size variants) are temperature sensitive and their growth rate is 500 times greater at 37 °C than at 39 °C. Primary isolation of TMEV is recommended in BHK-21 or ME cells rather than J774 cells to ensure detection of both small and large plaque size variants.

Specific testing is required for TMEV because detection of CPE is problematic for small plaque size variants and because persistent infections in culture may not be detectable. Specific testing is possible by FA, RT-PCR, real-time Taqman RT-PCR, nucleic acid hybridization, microarray and MAP assay.

RT-PCRs have been developed for TMEV targeting the cardiovirus internal ribosomal entry site (IRES) (Rodrigues et al. 2005). There are no published reports detailing the sensitivity and specificity of this assay or validating its use for diagnosis of TMEV.

A real-time Taqman RT-PCR has been developed targeting the TMEV IRES (Trottier et al. 2002). The real-time Taqman assay has a detection threshold of 20–30 genomic copies per µg total RNA, high specificity, and can detect persistent infections.

Nucleic acid hybridization techniques have been developed for detection of cardioviruses; however, sensitivity, specificity and ease of application are recognised as issues for diagnostic applications (Rotbart 1991).

TMEV is one of the agents targeted in the MAP and IMPACT panel of tests. Serological detection of TMEV with the MAP panel of tests has good cross-reactivity between the 4 serotypes of TMEV: TMEV in mice; rat encephalomyelitis virus, Vilyuisk human encephalomyelitis virus (a human isolate of TMEV); and Saffold virus (a second human isolate of TMEV) (Chiu et al. 2008; Clifford and Watson 2008; Jones et al. 2007). The IMPACT panel of tests uses the real-time Taqman RT-PCR targeting the TMEV IRES described above.

TMEV isolates have been identified from screening of samples using a pan-viral DNA microarray (Virochip; University of California, San Francisco) (Chiu et al. 2008).

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 for extraneous agent testing will not be sufficient for primary isolation of TMEV. The 9 CFR guidelines do not include specific testing for TMEV.

The European Pharmacopoeia general monograph *Vaccines for veterinary use* and 5.2.4 *Cell cultures for the production of veterinary vaccines* describe generalised methods for extraneous agent testing using cell culture monolayers that are sensitive to the agent being tested, and detection using specific testing methods that are sensitive to the viral agent. The monographs do not specify details of a culture system or assay for TMEV detection, simply that the methods should be sensitive.

Conclusion

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 are not acceptable.

Primary isolation of TMEV in BHK-21 or ME cells is recommended. Virus should be incubated on the monolayer for 90 minutes to allow adsorption prior to the addition of maintenance medium. Infected cultures should be frozen and thawed 3 times in succession, and then used to reinoculate fresh culture monolayers. For isolates of cardiovirus that can be isolated in cell culture CPE should be evident within 24 hours.

Specific testing should be undertaken by real-time Taqman RT-PCR targeting the TMEV IRES.

Transmissible gastroenteritis virus

Family *Coronaviridae*, genus *Coronavirus*

TGEV does not grow well in cell culture and primary isolation is unreliable. TGEV can be isolated in primary or secondary pig kidney cell monolayers, porcine thyroid, or ST that are 3–4 days old. Primary isolation in SK cells is most sensitive. Adsorption for 1 hour is required for virus attachment and entry followed by addition of culture medium. CPE is evident at 3–7 days as cell rounding, detachment and syncytia formation. Plaque assays using an agar overlay increase the sensitivity of detection of CPE and CPE can be confirmed by FA (OIE 2010).

Specific testing for TGEV is required due to the low success rate for primary isolation.

Specific testing by nested RT-PCR is the most sensitive means of detection and does not rely on culture isolation. A dual-nested RT-PCR has been developed that can detect and differentiate TGEV and PRCV (a deletion mutant of TGEV) (Kim et al. 2000a; Paton et al. 1997). The dual-nested RT-PCR targets a common sequence of the ORF 1b and differentiates between TGEV and PRCV by targeting the *S* gene deletion sequences. A SYBR green RT-PCR has been developed targeting the same region of ORF 1b (Escutenaire et al. 2007). This assay uses generic coronavirus primers and differentiates viruses by melting curve analysis. The detection threshold is 10 genomic copies.

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 for extraneous agent testing will not be sufficient for primary isolation of TGEV. The guideline 9 CFR 113.47: *Detection of extraneous viruses by the fluorescent antibody technique* describing the FA is not sufficiently sensitive for detection of TGEV.

The European Pharmacopoeia general monograph *Vaccines for veterinary use* and 5.2.4 *Cell cultures for the production of veterinary vaccines* describe generalised methods for extraneous

agent testing using cell culture monolayers that are sensitive to the agent being tested, and detection using specific testing methods that are sensitive to the viral agent. The monographs do not specify details of a culture system or assay for TGEV detection, simply that the methods should be sensitive.

Conclusion

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 are not acceptable.

Primary isolation of TGEV in culture is not recommended.

The FA for TGEV as described in 9 CFR 113.47: *Detection of extraneous viruses by the fluorescent antibody technique* is not sufficiently sensitive for detection of TGEV.

Specific testing is required for coronaviruses TGEV and PRCV by either the dual-nested RT-PCR method targeting the *S* gene deletion sequences of ORF 1b, or by the generic coronavirus RT-PCR and the use of melting curve analysis to differentiate between TGEV and PRCV.

Vesicular stomatitis virus

Family *Rhabdoviridae*, genus *Vesiculovirus*

Vesicular stomatitis virus (VSV) can be detected by primary isolation in Vero, BHK-21, IB-RS-2, BK and LK cells. VSV is readily detected by profound cytopathic CPE within 7 days after inoculation. Three passages are recommended (OIE 2010).

Specific testing developed for VSV are immunoassays using the supernatant from infected cultures and FA for the cell culture using VS-specific fluorescent antibody conjugate. Immunoassays developed are the ELISA, the complement fixation assay (CFA) and FA. The most commonly used immune assay is the indirect ELISA. The ELISA has better sensitivity and specificity than the CFA or FA but has lower reactivity for some serotypes. A hemi-nested PCR has been developed targeting the *L* gene of VSV and a second assay targeting the *P* gene (Ferris and Donaldson 1988; Höfner et al. 1994; Rodriguez et al. 1993). There are no published studies comparing the performance of the 2 assays.

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 for extraneous agent testing are adequate for detection of VSV. The 9 CFR guidelines do not include specific testing for VSV.

The European Pharmacopoeia general monograph *Vaccines for veterinary use* and 5.2.4 *Cell cultures for the production of veterinary vaccines* describe generalised methods for extraneous agent testing using cell culture monolayers that are sensitive to the agent being tested, and detection using specific testing methods that are sensitive to the viral agent. The monographs do not specify details of a culture system or assay for VSV detection, simply that the methods should be sensitive.

Conclusion

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 are acceptable.

Primary isolation of VSV in Vero, BHK-21, IB-RS-2, BK and LK cells is recommended. Cultures should be maintained for 7 days and 3 passages are required.

Specific testing is not required because of the reliability of detection by primary isolation and evidence of CPE.

Viruses — avian

Anatid herpesvirus 1

Family *Herpesviridae* (Unassigned to a genus)

Anatid herpesvirus 1 is also known as duck enteritis virus or duck plague herpesvirus. Primary isolation of duck enteritis virus (DEV) is possible in DEF cells sourced from 19–20 day old embryonic Pekin, Muscovy, wood ducks, pintails, lesser scaup, black and red-head ducks, Pekin duck embryo fibroblast (ATCC no. CCL-141) cells, embryonated duck eggs (EDE) or ducklings. Primary DEF cell culture is more susceptible to DEV isolation than EDE, duckling or Pekin duck embryo fibroblast cell culture. Greatest sensitivity, highest viral titres and most obvious plaque formation are seen in DEF cells sourced from Muscovy and wood duck cells. DEF cells sourced from Pekin, black and red-head ducks have intermediate sensitivity, and poor results are seen for DEF sourced from pintail and lesser scaup. Whilst DEF culture is reported to be the most sensitive isolation method there are reports of DEV field isolates that could not be isolated in DEF but were isolated by inoculation of ducklings and SN assay (Hanson and Willis 1976). The Pekin duck embryo fibroblast cell line has moderate sensitivity but plaque formation is indistinct (Barr et al. 1992; Kocan 1976; Lam and Lin 1986; OIE 2010; Shawky 2000; Shawky and Schat 2002; Shawky and Sandhu 1997).

Primary DEF cultures should be passaged 3 times prior to virus inoculation. Virus is adsorbed for 1 hour and the infected cultures are incubated for 3 days at 39.5–41.5 °C. CPE is evident as rounded, clumped cells that enlarge and become necrotic, formation of syncytia, intranuclear inclusions and granulation of the cytoplasm 48–96 hours after infection. Plaque formation is evident after 3 days for infected cell monolayers with agar overlays. Three passages are necessary to ensure detection of low levels of virus. Variability in growth characteristics is seen for different virus isolates and influences the efficacy of isolation, as well as the tendency for low levels of virus to establish persistent latent infection. Specific testing is recommended because of the difficulty of primary isolation of virus in cell culture.

Specific assays for DEV are FA, ISH, EM, SN, AC-ELISA, PCR, and real-time PCR.

A FA has been developed to confirm CPE in DEV-infected cultures using a fluorophore-conjugated mAb specific to DEV (Erickson et al. 1975; Proctor 1975). A SN assay has been developed that utilises either DEV-infected DEF monolayers or EDE. These assays have low sensitivity and are limited by the lack of an available standard positive anti-DEV serum.

A PCR has been developed for detection of DEV targeting the *pol* gene (Plummer et al. 1998). The PCR is combined with RFLP to confirm diagnosis. The PCR restriction fragment length polymorphism (PCR-RFLP) assay has a history of having been applied successfully to the diagnosis of DEV across wide geographic regions (Hansen et al. 1999; Hansen et al. 2000; Plummer et al. 1998; Pritchard et al. 1999). The PCR is reported to be highly specific for DEV and did not react with herpesviruses of other species. The PCR is reported to have a detection limit of 1 fg of genomic DNA and to be 20 times more sensitive than culture isolation alone (Hansen et al. 1999; Plummer et al. 1998). The PCR has been adapted to a real-time Taqman PCR targeting the *pol* gene of DEV (Plummer et al. 1998; Qi et al. 2008a; Qi et al. 2008b; Yang et al. 2005). The Taqman assay has greater sensitivity and specificity than the PCR alone and the detection limit for the assay

is 23 genomic copies. Molecular assays have the advantage of detection of latent inapparent infections.

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 for extraneous agent testing will not be sufficient for primary isolation of DEV. The 9 CFR guidelines do not include specific testing for DEV.

The European Pharmacopoeia 2.6.24 *Avian viral vaccines: tests for extraneous agents in seed lots* do not provide guidelines for testing for antibodies in ducklings, but does specify SN assay for DEV. This method has been used for detection of clinical cases in numerous studies. The virus rapidly causes significant clinical disease within 3 days and seroconversion detectable by SN assay; however, the technique is problematic due to the failure to isolate all strains of DEV. The efficacy of this method has not been established for the detection of low levels of contaminating virus in biological products. A DEV-specific antiserum standard for the SN test and SPF ducklings can be difficult to obtain.

Conclusion

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 are not acceptable.

Primary isolation of DEV in DEF sourced from Muscovy or wood duck is recommended. Primary DEF cultures should be passaged 3 times prior to virus inoculation. Virus is adsorbed for 1 hour, the infected cultures are incubated for 3 days at 39.5–41.5 °C, and 3 passages are required.

Specific testing is required for DEV by real-time Taqman PCR targeting the *pol* gene of DEV.

The method described by European Pharmacopoeia 2.6.24 is unreliable due to the failure to isolate all strains of DEV.

Avian adenoviruses

Family *Adenoviridae*, genus *Aviadenovirus* and *Atadenovirus*

Avian isolates of adenoviruses comprise 2 genera: the genus *Aviadenovirus* encompasses serotypes 1, 2, 3, 4, 5, 6, 7, 8a, 8b, 9, 10 and 11, and the genus *Atadenovirus* encompasses the group III avian adenovirus, duck adenovirus A (egg drop syndrome (EDS) virus). Two parallel and different systems of nomenclature are in use by Europe and the United States to classify the serotypes of avian adenoviruses, complicating the correlation of serotype specific data. A single system of nomenclature was adopted by the International Committee on Taxonomy of Viruses (ICTV) in 2006 and strain classification under this system has been cross referenced to the European and the United States nomenclature by (Büchen-Osmond 2006; Steer et al. 2009). Primary isolation of all serotypes of avian adenoviruses (AAdV) is possible in chicken embryo liver (CELi), CEF, CKC and chicken hepatoma (CH-SAH) cells. CH-SAH has been shown to be more sensitive than the conventional culture systems of CELi or CKC cells, and viral titres obtained are 2 logs higher than in other culture systems. Susceptibility of CELi and chicken embryo kidney (CEK) cells is inconsistent between preparations and some isolates of AAdV have failed to grow in these cells. CEF is poorly susceptible to AAdV. Primary isolation of all viruses from mixed infections with more than 1 AAdV serotype have been reported to be successful from inoculation of ECE that were not able to be isolated from CH-SAH cell cultures (Ojkic et al. 2008). AAdV can form latent infections in culture and culture adaptation occurs. Primary isolation of AAdV may not be associated with gross evidence of CPE or pathology, therefore specific testing is required.

Specific testing is required for AAdV as some isolates establish inapparent infections in primary cultures. Specific testing is possible by agar gel precipitation (AGP), IFA, EIA, ELISA, RT-PCR or real-time RT-PCR.

When comparing serological methods the greatest sensitivity is achieved with the ELISA that targets the group specific antigen (GSA). The GSA is conserved within all 12 serotypes of AAdV group I (AAdV-I). ELISA for AAdV-I GSA has the advantages of high sensitivity (can detect 10^2 TCID₅₀ virus) and broad-spectrum reactivity (Calnek et al. 1982; Saifuddin and Wilks 1990). The ELISA has significantly higher sensitivity than the AGP assay for detection of AAdV. Monreal and Dorn (1981) found in a parallel study that the ELISA was able to detect 47/55 samples as positive whereas the AGP could only detect 23/55 positives. Hess M (2000) reports ELISA detection of all 32 positive test samples of AAdV whereas AGP detected only 14 samples. Dawson et al (1982) report a significantly greater sensitivity for ELISA detection of AAdV than for EIA detection.

PCR has been developed for the detection of AAdV-I and AAdV group II (AAdV-II) based on the *pol* gene (Hanson et al. 2006; Jiang et al. 1999; Kumar et al. 2003; Moscoso et al. 2007; Pantin-Jackwood et al. 2007; Pantin-Jackwood et al. 2008; Persia et al. 2002; Toro et al. 1999; Wang et al. 2008; Xie and Khan 2000). The PCR is highly sensitive and specific, and has a threshold for detection of 1-10 fg of DNA.

The group III avian adenovirus, duck adenovirus A (EDS) is serologically unrelated to AAdV-I and AAdV-II and therefore specific testing for these viruses will not detect EDS. Specific testing for EDS is possible by HI, VN, HA, AC-ELISA or PCR. An AC-ELISA has been developed using F8 mAb specific to the hexon protein to capture EDS virus and polyclonal serum for detection (Dhinakar Raj et al. 2003). The sensitivity of the AC-ELISA for culture isolated EDS was 98% and specificity was 96%. A PCR assay targeting the J fragment of the EDS genome was shown to have equivalent sensitivity and specificity to the AC-ELISA, and both tests had 15 fold greater detection sensitivity when compared to HA test for 5 logs of virus (Dhinakar Raj et al. 2003).

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 for extraneous agent testing will not be sufficient for primary isolation of AAdV-I. The 9 CFR guidelines do not include specific testing for AAdV.

The European Pharmacopoeia 2.6.24 refers to 3 methods for AAdV detection: primary isolation by inoculation of embryonic chickens 9-11 days old by the chorioallantoic, allantoic and yolk sac routes and examination after 7-12 days; inoculation of CKC and examination for CPE after culturing for 21 days and passaging at 4-7 day intervals; and inoculation of 2 week old chickens twice, at 2 week intervals and collection of serum. The serum is then tested for AAdV-I antibodies using the SN, EIA or AGP tests, and tested for egg drop syndrome virus using either haemagglutination inhibition (HI) or EIA. The sensitivity of these methods is significantly lower than culture isolation and specific testing by ELISA or PCR for AAdV-I.

Conclusion

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 are not acceptable.

Primary isolation of AAdV-I in CH-SAH is recommended.

Specific testing is required for AAdV-I by either ELISA or PCR.

Specific testing is required for EDS by either AC-ELISA or PCR.

The methods described by European Pharmacopoeia 2.6.24 are not acceptable.

Avian encephalomyelitis virus

Family *Picornaviridae*, genus *Hepatovirus*

Primary isolation of avian encephalomyelitis virus (AEV) is possible in CEF, baby grivet monkey kidney cells subclone (BGM-70), embryonic neuroglial cells, or by intracranial or allantoic inoculation of ECE. Growth is slow in allantoic fluid and better for intracranial inoculation of ECE. AEV will grow to only very low titres in CEF, and without evidence of CPE. AEV can be grown in embryonic neuroglial cell cultures without evidence of CPE, but viral detection is possible using FA. AEV can also be propagated in the cell line BGM-70, but growth requires multiple passages before culture adaptation occurs. AEV that has been culture-adapted by initial passages in ECE has been shown to grow to relatively high titres in embryonic neuroglial cell lines. Isolation of AEV in chickens 2 weeks old followed by serological detection is of low sensitivity.

In general primary isolation combined with serological assays has poor specificity due to non-specific reactions and cross-reactions. Therefore specific testing is recommended. Specific testing for AEV is possible by AGP, EIA, AC-ELISA or RT-PCR.

The AGP test has the advantage of rapid detection of AEV antigen at 4 days after infection, but has very low sensitivity, and its specificity can be reduced by antigenic variability amongst AEV isolates. The sensitivity of the AC-ELISA test is better than that of the AGP, but is still too low for recommendation.

RT-PCR for AEV *VP2* gene is highly sensitive and specific and can detect as little as 10 pg of viral RNA.

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 for extraneous agent testing will not be sufficient for primary isolation of AEV. The 9 CFR guidelines do not include specific testing for AEV.

The European Pharmacopoeia 2.6.24 refers to general methods for detection of extraneous agents and specific methods for detection of AEV. The general methods are gross pathology and histopathology following inoculation of test material into the allantoic cavity, CAM and yolk sac of embryonated eggs, and by CPE detection in CKC monolayers. Specific isolation of AEV is by inoculation of chickens 2 weeks old and testing of serum for AEV by AGP or EIA. The sensitivity of both the general and specific methods is very low and they are not acceptable.

Conclusion

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 are not acceptable.

Primary isolation of AEV by intracranial inoculation of ECE is recommended.

Specific testing is required for AEV by RT-PCR targeting the *VP2* gene sequence.

The sensitivity of the methods described by European Pharmacopoeia 2.6.24 is very low and not acceptable.

Avian influenza virus

Family *Orthomyxoviridae*, genus *Influenzavirus A*

Primary isolation of avian influenza virus (AIV) is by allantoic inoculation of 9–11 day old embryonated SPF fowl eggs. The recommendation is for incubation of inoculated eggs for 4–7 days between passages. Allantoic fluid is harvested and tested for AIV. Allantoic fluid testing negative is passaged into fresh eggs at least 3 more times.

Specific testing is required to detect AIV in the allantoic fluid and HA and RT-PCR is recommended. Samples from the final passage together with an earlier passage should be tested.

The generalised culture method described in 9 CFR 113.37: *Detection of pathogens by the chicken embryo inoculation test* will not provide optimal growth conditions for AIV and will not be sufficient for isolation. The protocol 9 CFR 113.37 has a single passage in embryonated eggs, which is insufficient for isolation of low titre viruses. The 9 CFR guidelines do not include specific testing for AIV.

The European Pharmacopoeia 2.6.24 specifies isolation of AIV by inoculation of chickens 2 weeks old and testing of serum for AIV by AGP or EIA. The European Pharmacopoeia 2.6.24 also includes requirements for generic detection of extraneous agents by gross pathology and histopathology following inoculation of test material into the allantoic cavity, CAM and yolk sac of embryonated eggs, and by CPE detection in CKC monolayers. The sensitivity of both the generic and specific methods is very low and they are not acceptable.

Conclusion

The generalised culture method of 9 CFR 113.37: *Detection of pathogens by the chicken embryo inoculation test* is not acceptable.

Primary isolation of AIV by allantoic inoculation of 9–11 day old embryonated SPF fowl eggs is recommended. Inoculated eggs should be incubated for 4–7 days between passages, and a total of 4 passages in eggs are required.

The sensitivity of the method described by European Pharmacopoeia 2.6.24 is very low and is not acceptable.

Specific testing for AIV is required for the harvested allantoic fluid by HA and RT-PCR.

Avian leukosis virus

Family *Retroviridae* genus *Alpharetrovirus*

Avian leukosis virus (ALV) comprises 6 subgroups on the basis of antigenic typing of the surface glycoprotein p27; subgroups A, B, C, D and J are transmitted as infectious viral particles and are classified as exogenous ALVs; subgroup E is non-pathogenic, it is integrated into the genome of a high proportion of chickens and is classified as endogenous ALV. Subgroups A and B are highly prevalent, pathogenic exogenous viruses and are aetiological agents for clinical disease and oncogenesis. Subgroups C and D are rare exogenous viruses and subgroup J is a highly prevalent, severely pathogenic exogenous virus with a high transmission rate. Both endogenous and exogenous ALV can be transmitted vertically in biological materials originating from chickens.

ALV can be isolated by culture in CEF. CEF should be harvested from 9–11 day old embryos that are genetically susceptible to ALV subgroups A, B and J. CEF from C/E strain chickens are resistant to infection with endogenous ALV and support the growth of exogenous ALV. Super-infection of CEF with virus of a particular subgroup interferes with isolation of virus of that subgroup and CEF infected with ALV are not suitable for ALV culture isolation.

ALV is thermolabile and samples are required to be stored at -70°C prior to inoculation into culture. Confluent monolayers of CEF of a susceptible phenotype should be inoculated with virus and adsorption incubation for 1 hour is required, followed by incubation for at least 21 days at $35\text{--}37^{\circ}\text{C}$. Efficient propagation of ALV is highly dependent on optimal cell culture conditions, results can be unreliable and isolation requires several subcultures over at least 21 days. In one report an unusual isolate of ALV subgroup A was obtained as a contaminant from commercial vaccines by culture isolation in CEF (Silva et al. 2007). The isolate was unusual in that virus growth was very, very slow in CEF and this was proposed to be due to the virus having an LTR sequence homologous with subgroup E viruses rather than subgroup A viruses. This report illustrates the potential difficulty of using the growth properties of exogenous viruses and detection methods developed for isolation of field strains for detecting vaccine contaminants which are atypical ALV isolates.

Specific testing for ALV is required because of the unreliability of culture isolation and because of the lack of discernable CPE for ALV-infected CEF. Most strains of ALV produce no visible morphological changes in cell culture assays (except after prolonged passage (Calnek 1968). Propagation of ALV is unreliable due to culture variables, CEF phenotypic resistance can inhibit virus growth, and super-infection of CEF with virus of the same subgroup can interfere with virus isolation. Specific testing for ALV is possible by methods that detect the GAG, POL or ENV viral proteins, or detect proviral DNA or viral RNA, or by enzyme bioassays.

Available assays based on detection of group specific viral protein are the Complement Fixation Assay for Avian Leukosis (COFAL (Sarma et al. 1964), ELISA (Crittenden et al. 1987; Fadly and Witter 1998; Smith et al. 1979), phenotypic mixing assay (PMA (Okazaki et al. 1979), Resistance-Inducing Factor (RIF) assay (Rubin 1960), Non-producer cell activation (NPA) (Rispen et al. 1970), direct and indirect FA and flow cytometry assays, and RIA (Estola et al. 1974; Sandelin et al. 1974). A limitation of assays based on detection of viral protein is that in general they do not distinguish between endogenous and exogenous ALV, because group-specific antigens can be produced by both types of virus. This limitation can be overcome in part by specialised culture techniques involving the use of combinations of CEF with phenotypes that are both sensitive and resistant to the different subgroups of ALV.

The COFAL and ELISA are the most commonly used assays. The COFAL test uses hamster serum (Sarma et al. 1964). Mammalian antiserum used in ELISA assays will detect all exogenous and endogenous strains of ALV whereas avian antiserum will be subgroup specific (Hunt et al. 2000; Hunt et al. 1999; Kelloff and Vogt 1966; Payne et al. 1966). Studies comparing the COFAL and ELISA assays have found that the sensitivity of the ELISA assay is greater than that of the COFAL assay. This is further supported by the finding that contamination of commercial vaccines with ALV has been detected by ELISA assay for samples that were found to be negative by COFAL assay.

The basis of the RIF assay is interference by virus of the same subgroup to result in at least a 10 times reduction in foci of the indicator infection with stock Rous sarcoma virus (RSV) (Rovigatti and Astrin 1983; Vogt and Ishizaki 1966). The usefulness of the RIF assay is limited to ALV

isolates that are not rapidly cytopathic. The appearance of viral foci in the RIF test is very dependent on optimal cell culture conditions and results can be unreliable.

Non-producer cells are produced from CEF transformed with *env*-defective RSV (BH-RSV), then superinfection with leukosis helper virus results in detectable virus production. A variation of the test is infection of CEF cells with genotype susceptible to infection with endogenous and exogenous avian leukosis virus (C/O), with subgroup E RSV then superinfection with exogenous virus to produce detectable virus. The NPA test can also be performed using a Japanese quail cell line transformed by RSV that is high titre and ENV defective (R(-)Q) for assay of endogenous and exogenous ALV (Crittenden et al. 1979). The NPA test is a specialised technique and its use is limited by the availability of required phenotypes of CEF transformed with the defective BH-RSV. There are reports of false positive results when non-producer cells are coinfecting with mixtures of complementing retroviruses (Zhang et al. 2008).

PCR and RT-PCR assays have been developed for ALV that mostly target sequence within the *env* locus or the LTR sequence. A subgroup-specific PCR assay has been developed for ALV subgroup A targeting the proviral DNA (Silva et al. 2007; van Woensel et al. 1992), and generic nested PCR and RT-PCR assays have been developed for detection of exogenous but not endogenous strains of ALV targeting the proviral conserved LTR and U5 sequences (Fadly and Witter 1998; García et al. 2003; Häuptli et al. 1997; Lupiani et al. 2000; Silva et al. 2000; Smith et al. 1998a; Smith et al. 1998b). The nested RT-PCR and nested PCR assays were shown to be more sensitive than ELISA detection of cell culture isolated ALV (García et al. 2003). ALV subgroup A contamination of commercial poultry vaccines is reported to have been detected by nested RT-PCR assay in vaccine samples that were negative by culture isolation and COFAL assay, and were positive by ELISA assay only following thermal inactivation and dilution of samples to neutralise ELISA assay inhibitors (Zavala and Cheng 2006).

An assay for detection of surface ATPase activity has been developed for avian myeloblastosis virus (AMV); however, the assay is not useful for other types of avian retroviruses. All types and subgroups of ALV can be detected by the RT activity assay. The assay format has been adapted to a highly sensitive, PCR-based RT (PERT) assay. The PERT assay has been used to screen human vaccines that are produced in CEF or embryonated eggs in order to demonstrate freedom from avian retroviruses (Hussain et al. 2001; Tsang et al. 1999). As for human vaccines, the PERT assay could similarly be applied to the generic screening of veterinary vaccines for retrovirus contamination.

The 9 CFR 113.31 methods describe the detection of ALV by culture isolation in CEF followed by the COFAL testing for ALV subgroups A and B. The 9 CFR 113.31 protocol for isolation and specific testing for ALV will not be sufficient for detection of ALV. The *USDA Center for Veterinary Biologics Notice No. 03-13* was issued in 2003 to notify interested parties of contamination of a number of vaccines produced in North America and Europe with both exogenous and endogenous ALV. The vaccines were found to be contaminated with ALV by ELISA testing and were negative by the COFAL test, which is the current method outlined in 9 CFR 113.31. The USDA instigated proposed amendments to the testing requirements for ALV on January 31st 2007 (Docket No. APHIS-2007-0001). The proposed amendments to the 9 CFR 113.31 protocol are for ALV testing 'using a method that will detect extraneous replicating ALV and that is acceptable to APHIS'. The amendments do not specify testing requirements, simply that approval of the test is required by APHIS.

The European Pharmacopoeia 2.6.24 recommends culture isolation of ALV in CEF of a susceptible genotype for at least 9 days, followed by specific testing by either COFAL or ELISA assays. The

protocol does not provide optimal culture conditions and the sensitivity of the COFAL test is unacceptably low.

Conclusion

The generalised culture method of 9 CFR 113.31 for isolation and specific testing for ALV is not acceptable.

Primary isolation of ALV in CEF harvested from 9–11 day old embryos that are genetically susceptible to ALV subgroups A, B and J is recommended. CEF from C/E strain chickens are resistant to infection with endogenous ALV and support the growth of exogenous ALV. Test samples are required to be stored at –70 °C prior to inoculation onto confluent monolayers of CEF for 1 hour adsorption incubation, followed by incubation for at least 21 days at 35–37 °C.

Specific testing is required for ALV by nested PCR assay targeting the sequence within the *env* locus and the LTR. Generic screening of veterinary vaccines using the PERT assay for retrovirus contamination is recommended as a method for detection of ALV contaminants.

The method described by European Pharmacopoeia 2.6.24 for culture isolation of ALV does not provide optimal culture conditions and the sensitivity of the COFAL test is unacceptably low.

Avian nephritis virus 1 and 2

Family *Astroviridae*, genus *Avastrovirus*, species *Chicken astrovirus*

Primary isolation of avian nephritis virus (ANV) is possible in chicken hepatocellular carcinoma (LMH) and CKC cells or by inoculation of 1 day old chickens. Cell culture should be performed in serum-free medium. ANV is detected by plaque titration using agar overlays. The threshold dose detectable by experimental infection of chickens is 5 logs of virus, and therefore this method is not sufficiently sensitive for detection of the low levels of viral contaminants expected to be present in biologicals (Imada and Kawamura 1997; Mandoki 2006).

Irregular plaques are visible in CKC monolayers from 4 days after infection. CPE is evident as granular eosinophilic inclusions in the cytoplasm and virus can be detected by EM (Baxendale and Mebatsion 2004; Frazier 1990; Mandoki 2006; Takase et al. 1994).

The route of inoculation of ECE is a significant variable in the detection sensitivity; the order of sensitivity being inoculation by yolk sac, CAM, and lastly the allantoic route. The breed of chicken from which the CKC or ECE culture systems are derived also significantly affects the detection sensitivity (Baxendale and Mebatsion 2004; Frazier 1990).

ANV isolates vary in the CPE observed in culture systems. Non-cytopathic isolates of ANV have been described, and persistent culture infections are reported, characterised by low viral titres and absence of CPE. Specific testing is required to detect persistent or non-cytopathic ANV infections. Specific testing for ANV is possible by VN, AC-ELISA, FA, AGID and RT-PCR.

An FA has been developed for detection of ANV infected CKC monolayers on coverslips using virus specific mAbs (Baxendale and Mebatsion 2004; Imada et al. 1981; Imada et al. 1982; Mockett et al. 1993; Shirai et al. 1990; Takase 1989). A study systematically comparing serological detection of ANV reports the order of sensitivity of assays to be VN>ELISA>FA>AGID (Baxendale and Mebatsion 2004; Mockett et al. 1993).

A RT-PCR assay targeting the *gp1* gene has been developed for detection of ANV. The RT-PCR assay has a detection threshold of 10 copies of genome. Validation of the RT-PCR assay is reported for surveillance of chicken carcass samples, but the assay has not been sufficiently validated for diagnostic purposes (Baxendale and Mebatsion 2004; Mándoki et al. 2006; Pantin-Jackwood et al. 2008). The sequence hypervariability in the *gp1* region is problematic for the design of molecular diagnostic assays that can detect all isolates (Mándoki et al. 2006). A multiplex RT-PCR assay has been developed for detection of ANV that can differentiate ANV from other astroviruses of chicken and turkey origin (Day et al. 2007).

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 for extraneous agent testing will not be sufficient for primary isolation of ANV. The 9 CFR guidelines do not include specific testing for ANV.

The European Pharmacopoeia 2.6.24 refers to general methods for detection of extraneous agents and specific methods for detection of ANV. The general methods are gross pathology and histopathology following inoculation of test material into the allantoic cavity, CAM and yolk sac of embryonated eggs, and by CPE detection in CKC monolayers. Specific isolation of ANV is by inoculation of chickens 2 week old and testing of serum for ANV by FA. Chickens are significantly more susceptible at 1 day old than 2 weeks old, and the sensitivity of this assay is inadequate for innocuity testing (Imada and Kawamura 1997; Mandoki 2006). The sensitivity of both the general and specific methods is very low and they are not acceptable.

Conclusion

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 are not acceptable.

Primary isolation of ANV in CKC and detection by plaque assay is recommended.

Specific testing is required for ANV by VN assay.

The sensitivity of the method described by European Pharmacopoeia 2.6.24 for the detection of ANV is very low and this method is not adequate for extraneous agent testing.

Avian orthoreovirus

Family *Reoviridae*, genus *Orthoreovirus*

Avian orthoreovirus is more commonly known as avian reovirus. Primary isolation of avian reovirus is possible in CELi and CEF. Avian reovirus grows readily in CEF and forms identifiable plaques. The size and extent of plaques correlates with the virulence of the virus, with attenuated and low-pathogenic strains having reduced plaque formation in CEF. For these strains detection by inoculation of CEF is not sufficiently sensitive and specific testing is required.

Specific testing is possible by EM, IEM, FA, ELISA, RT-PCR and real-time RT-PCR. EM, FA and ELISA assays have low sensitivity and specificity.

A number of PCR assays have been developed for reovirus targeting different segments. A PCR assay has been developed for avian reoviruses targeting the S2 and S4 segments (Bruhn et al. 2005). The detection limit of the assay was 0.8 TCID₅₀, the sensitivity for field isolates was 80% and 55%

for the S4 and S2 targets respectively, and specificity was reported as 100%. The assay was validated for use in extraneous agent testing of veterinary therapeutics.

A multiplex real-time SYBR green RT-PCR assay has been developed for avian reoviruses to simultaneously detect sequence from the S1 segment of chicken–origin reovirus and from the S3 segment of turkey–origin reovirus (Ke et al. 2006; Spackman et al. 2005). The detection sensitivity for the real-time RT-PCR is greater than that recorded for virus isolation and for ELISA or FA, and 3 logs greater than for the classical RT-PCR assay. The real-time RT-PCR assay has a detection threshold of 39 copies/μl of genome and is highly sensitive and 100% specific. The assay is highly reproducible and had minimal inter- and intra-assay variation. Variant and vaccine strains of reovirus can be differentiated using the melt curve analysis.

A nested PCR assay has also been described for avian reoviruses based on the σ C gene on the S1 segment (Liu et al. 1997; Liu et al. 1999). The detection sensitivity of the classical PCR assay was 10 TCID₅₀ and increased by 3 logs in the nested format.

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 for extraneous agent testing will not be sufficient for primary isolation of avian reovirus. The 9 CFR guidelines do not include specific testing for avian reovirus.

The European Pharmacopoeia 2.6.24 refers to general methods for detection of extraneous agents by gross pathology and histopathology following inoculation of test material into the allantoic cavity, CAM and yolk sac of embryonated eggs, and by CPE detection in CKC monolayers. The European Pharmacopoeia 2.6.24 does not refer to specific testing for avian reovirus. The sensitivity of the general methods is very low and they are not acceptable.

Conclusion

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 are not acceptable.

Primary isolation of avian reovirus in CEF is recommended.

Specific testing is required for avian reovirus by real-time SYBR green RT-PCR assay targeting the S1 segment.

The sensitivity of the general methods described by European Pharmacopoeia 2.6.24 for the detection of avian reovirus is very low and they are not acceptable.

Chicken anaemia virus

Family *Circoviridae*, genus *Gyrovirus*

Primary isolation of chicken anaemia virus (CAV) is possible in Marek's disease virus (MDV) transformed chicken T lymphoblastoid (MDCC-MSB1 and MDCC-CU147) cell lines. MDCC-CU147 are reported to have 10 times greater detection sensitivity when compared to 26 MDV-transformed cell lines and are the most sensitive cell lines for primary isolation (Calnek et al. 2000).

FA is the diagnostic standard for flock surveillance; however, molecular assays have greater sensitivity and specificity for low level detection (McNeilly et al. 1991; Toro et al. 1997).

PCR and nested PCR assays have been developed targeting the *VP1* gene of CAV (Imai et al. 1998; Miller et al. 2003; Tham and Stanislawek 1992a; Tham and Stanislawek 1992b). The detection limit of the assay is reported to be $10^{-1.5}$ TCID₅₀ (Imai et al. 1998). The assay sensitivity was increased by 100 times for the nested PCR assay (Imai et al. 1998; Soiné et al. 1993). PCR combined with dot blot hybridization using a radioactive probe has a detection sensitivity of 1 fg DNA (Todd et al. 1992). PCR assay is highly sensitive and specific and has been validated in a number of studies.

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 for extraneous agent testing will not be sufficient for primary isolation of CAV. The 9 CFR guidelines do not include specific testing for CAV.

The European Pharmacopoeia 2.6.24 refers to primary isolation of chicken anaemia virus in MDCC-MSB1 cells for at least 24 days and 8 passages followed by FA. PCR assay has been reported to be considerably more sensitive and specific than FA for culture-isolated CAV (Todd et al. 1992).

Conclusion

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 are not acceptable.

Primary isolation of CAV in MDCC-CU147 cells is recommended.

Specific testing is required for CAV by PCR assay targeting the *VP1* gene of CAV.

The sensitivity of the culture isolation and FA method described by European Pharmacopoeia 2.6.24 for the detection of chicken anaemia virus is considerably lower than PCR and is not acceptable.

Duck viral hepatitis virus 1

Family *Picornaviridae* (Unassigned to a genus)

There are at least 3 aetiological agents responsible for duck hepatitis. Duck viral hepatitis virus 1 (DVHV-1), is the most significant aetiological agent and the only agent considered here.

Primary isolation of DVHV-1 is possible in duck embryo liver (DEL), duck embryo kidney (DEK), DEF, EDE, and ducklings. The virus has poor growth in cell culture and only grows to low titres. The latent period for virus growth is 24 hours and several passages are required to ensure detection. CPE is not obvious and specific detection is required. An adsorption incubation of 30 minutes is required for virus inoculation onto DEL monolayers. DEL is more susceptible to DVHV-1 growth than DEK or DEF cell cultures (Hwang 1965; Hwang 1966). Plaque formation is more readily identified for cell culture-adapted virus isolates, whereas more virulent field isolates may grow in DEL without evidence of CPE or plaque formation (Woolcock 1986). Virus growth can be identified by FA or RT-PCR methods in these non-cytopathic cultures. Culture-adapted isolates are also less sensitive to growth inhibition by serum components.

A multi-stage isolation process is most successful for DVHV-1 to ensure culture adaptation occurs. Isolation of DVHV-1 is by initial subcutaneous or intramuscular inoculation of 1–7 day old SPF ducklings. Characteristic clinical signs and death occur within 18–48 hours and virus is then re-isolated from liver homogenised in 20% buffered saline with 5% chloroform added. The second stage of the isolation process is allantoic inoculation of the harvested liver homogenate into 10–14

day old EDE and virus is re-isolated from the liver of embryos at 72 hours. EDE are more susceptible to DVHV-1 than ECE, and ECE are not recommended for primary isolation of DVHV-1. Alternatively, the second stage isolation can also be performed in DEL cells which are highly susceptible to DVHV-1 (Woolcock et al. 1982; Woolcock 1986). DVHV-1 infection of DEL monolayers with agarose overlays produces 1mm diameter plaques at 24 hours. Serum or foetal calf serum at concentrations $\leq 0.1\%$ in the growth medium reduces DVHV-1 plaque formation and at concentrations $\geq 0.2\%$ inhibits virus growth completely. The non-specific growth inhibition by mammalian serum components on DVHV-1 is attributed to an effect of the albumen fraction on the virus itself, rather than on virus attachment to cell culture receptors and virus entry (Chalmers and Woolcock 1984; OIE 2010; Woolcock et al. 1982; Woolcock 1986). Specific testing for DVHV-1 is required due to the difficulty of primary isolation and the high frequency of non-cytopathic isolates in DEL culture.

Specific testing for DVHV-1 is possible by FA, SN, AGID, immunohistochemical assay (IHA), ELISA, RT-PCR, and real-time RT-PCR.

The SN test is performed either *in ovo* or as a plaque reduction assay *in vitro* using hyperimmune duck serum (Woolcock 1986). Caution should be used in the interpretation of the SN assay to control for the common occurrence of non-specific growth inhibition of DVHV-1 by serum components (Chalmers and Woolcock 1984). The SN test suffers from non-specific reactions and false positives and variability for repetitions of the assay.

A study comparing the performance of the ELISA, SN and AGID tests for detection of DVHV-1 found equivalent specificity for all 3 tests; however, the sensitivities of the assays were 68.8%, 68.8% and 18.8% respectively (Zhao et al. 1991).

A one-step RT-PCR assay has been developed that detects the *pol* (3D) gene of DVHV-1 with 100% specificity and a detection threshold of either 10 median embryo lethal doses (ELD₅₀/ml, 100 pg RNA or 10³ viral genomic copies (Kim et al. 2008a; Kim et al. 2007a)). A SYBR green real-time RT-PCR assay has been developed for DVHV-1 combined with a melting curve analysis to demonstrate specificity, with a reported 100% sensitivity and 100% specificity (Wang et al. 2002). The RT-PCR and real-time SYBR green RT-PCR assays have not been adequately validated for diagnostic purposes.

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 for extraneous agent testing will not be sufficient for primary isolation of DVHV-1. The 9 CFR guidelines do not include specific testing for DVHV-1.

The European Pharmacopoeia 2.6.24 requires inoculation of 1 day old ducklings followed by SN assay for DVHV-1. The SN test has low specificity, poor repeatability and interpretation of output from the SN assay is problematic.

Conclusion

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 are not acceptable.

A multi-stage isolation process for DVHV-1 by initial subcutaneous or intramuscular inoculation of 1–7 day old SPF ducklings, followed by re-isolation of virus from liver and either allantoic inoculation of EDE or isolation in DEL is recommended.

Specific testing is required for DVHV-1 by either ELISA or RT-PCR.

The method described by European Pharmacopoeia 2.6.24 for the detection of DVHV-1 is not acceptable as the SN test has low specificity, poor repeatability and interpretation of output from the SN assay is problematic.

Fowlpox virus

Family *Poxviridae*, genus *Avipoxvirus*

Primary isolation of fowlpox virus is possible in CEF, CEK, chicken embryo dermis and quail fibrosarcoma (QT-35) cells, or ECE. CPE is readily visible as large intracytoplasmic inclusion bodies (Bollinger bodies) that contain smaller elementary bodies (Borrel bodies) in haematoxylin and eosin (H&E) stained preparations. EM can be used to confirm the presence of pox viruses with characteristic morphology. Some attenuated strains of fowlpox virus do not form obvious plaques in cell culture. Fowlpox virus requires cell culture adaptation to grow in primary chicken cell lines such as CEF, CEK, QT-35 or dermal cells. Fowlpox virus isolates can form latent infections and atypical fowlpox virus is difficult to diagnose. Fowlpox virus can be isolated by inoculation onto the CAM of 9–12 day old ECE, incubation for 5–7 days, and then examination for small focal white pock lesions (OIE 2010).

Specific testing is required to confirm virus detection. Specific testing is by FA, EM, ELISA, AGID, HI, immunoblot, DNA hybridization, or PCR.

FA and EM assays are used to confirm CPE or pock formation in infected cultures. The AC-ELISA is 400–800 times more sensitive than the AGID test for serological detection in inoculated embryos (Zhang et al. 2005).

A PCR assay has been developed for fowlpox virus targeting the 4b core protein (Lee and Lee 1997). The detection threshold for the PCR assay was 10^{-1} TCID₅₀ or 150 genomic copies. A nested PCR has been developed that uses as external primers those used by Lee & Lee (1997) and an internal pair of primers (Fallavena et al. 2002). The nested PCR format increased the detection sensitivity relative to the classical PCR assay and the detection threshold was 0.28 EID₅₀. Specificity of the nested PCR was established using a range of other pox viruses. The nested PCR assay was sufficiently sensitive to detect low titre infections such as chronic or latent fowlpox virus infections that were not detectable by the classical PCR assay.

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 for extraneous agent testing will not be sufficient for primary isolation of fowlpox virus. The 9 CFR guidelines do not include specific testing for fowlpox virus.

The European Pharmacopoeia does not have specific testing requirements for fowlpox virus beyond generalized extraneous agent testing. The European Pharmacopoeia 2.6.24 refers to general methods for detection of extraneous agents by gross pathology and histopathology following inoculation of test material into the allantoic cavity, CAM and yolk sac of embryonated eggs, and by CPE detection in CKC monolayers. The sensitivity of the general methods is very low and they are not acceptable.

Conclusion

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 are not acceptable.

Primary isolation of fowlpox virus in CEF, CEK, chicken embryo dermis cells, QT-35, or ECE is recommended. Fowlpox virus is detected by pock formation on the CAM of ECE, or CPE in culture is confirmed by FA or EM assay.

Specific testing is required for fowlpox virus by nested PCR assay targeting the 4b core protein.

The sensitivity of the general methods described by European Pharmacopoeia 2.6.24 for the detection of fowlpox virus is very low and they are not acceptable.

Infectious bronchitis virus

Family *Coronaviridae*, genus *Coronavirus*

Primary isolation of infectious bronchitis virus (IBV) is possible in 9–11 day old ECE by allantoic inoculation. The allantoic fluid is collected after 3–7 days and used to re-inoculate ECE. Three serial passages in embryos are required. Growth of IBV in embryos is evident by teratogenic pathology and specific testing is required to confirm IBV infection. FA using a group-specific mAb is used to identify virus infection of the CAM. IBV can grow in TOC but this method is not recommended for primary isolation. IBV requires culture adaptation by passage in eggs before isolates will grow in tissue culture with identifiable CPE. TOC are prepared as suspensions of tissue from 20 day old ECE and can be used for infection of culture-adapted IBV(OIE 2010).

Specific assays for IBV are real-time RT-PCR, RT-PCR combined with RFLP, immunoblot, EIA, AGID, FA, HI, VN, or ELISA.

The AGID test lacks sensitivity and HI and ELISA have poor reproducibility and low sensitivity. Specific testing of the allantoic fluid from IBV infected ECE is possible by RT-PCR, detection by a DNA probe in a dot blot hybridization assay, FA, ELISA or ISH. MAbs used for detection of IBV in FA, ELISA, VN or ISH assays are limited in application by the continued emergence of new antigenic types of IBV that are not reactive (Karaca et al. 1992). ELISA is the most sensitive serological assay available for IBV (Hawkes et al. 1983; Karaca et al. 1992; Mockett and Darbyshire 1981).

A RT-PCR assay has been developed targeting the S1 subunit of the spike gene of IBV (Jackwood et al. 1997). The spike gene encodes a hypervariable region of the genome that correlates with the intra-species variation in neutralising antigenic epitopes. The RT-PCR assay is combined with RFLP for typing of virus isolates (Abreu et al. 2006; Jackwood et al. 2005; Jackwood et al. 2007). The assay specificity is enhanced by the inclusion of an internal control RNA, and the assay is sufficiently sensitive to detect IBV from field samples without the necessity for primary isolation in ECE. The assay has been adapted to a real-time SYBR green RT-PCR assay; however, this assay has not been sufficiently validated and the sensitivity or specificity of the assay has not been established for diagnostic purposes (Pantin-Jackwood et al. 2005). A 1 step real-time fluorogenic RT-PCR assay has been developed targeting ORF 1b, for generic detection of coronaviruses, including IBV (Escutenaire et al. 2007). The generic coronavirus real-time RT-PCR has a detection threshold of 10 genomic copies, is reported to detect 32 different species of coronavirus, and is highly specific to coronaviruses.

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 for extraneous agent testing will not be sufficient for primary isolation of IBV. The 9 CFR guidelines do not include specific testing for IBV.

The European Pharmacopoeia 2.6.24 refers to inoculation of 2 week old chickens followed by EIA or HI assay for IBV. This method is not recommended due to the high frequency of failures for isolation of IBV in 2 week old chickens and the low sensitivity of detection of seroconversion by EIA and HI (de Wit 2000). Newly emergent antigenic variants of IBV occur at high frequency and will not be detected by the EIA method. The European Pharmacopoeia 2.6.24 also refers to general methods for detection of extraneous agents by gross pathology and histopathology following inoculation of test material into the allantoic cavity, CAM and yolk sac of embryonated eggs, and by CPE detection in CKC monolayers. The sensitivity of both the general and specific methods is very low and they are not acceptable.

Conclusion

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 are not acceptable.

Primary isolation of IBV in ECE and 3 serial passages in embryos is recommended. FA is used to confirm virus infection of the CAM or cells within the allantoic fluid.

Specific testing is required by ELISA or RT-PCR targeting the S1 subunit of the spike gene of IBV.

The methods described by European Pharmacopoeia 2.6.24 for the detection of IBV are inadequate for extraneous agent testing.

Infectious bursal disease virus

Family *Birnaviridae*, genus *Avibirnavirus*

There are 2 serotypes of infectious bursal disease virus (IBDV): serotype 1 is pathogenic for poultry and is the serotype targeted by diagnostic assays, whereas serotype 2 is not pathogenic for poultry.

Primary isolation of IBDV is possible in QT-35, Vero, BGM-70, chicken B-lymphoblastoid cell lines transformed by Rous-associated virus 2 (LSCC-RP9 and LSCC-RP12), chicken macrophage (MQ-NCSU), B-lymphoblastoid cells, ECE, or CEF cultures. ECE, CEF, BGM-70, Vero and QT-35 cell lines are most frequently used for IBDV isolation. IBDV infected cultures should be incubated for 3 days and passaged at least 3 times to ensure detection. CPE is apparent at 48–72 hours as cellular aggregation, rounding, granulation and necrosis with plaque formation in monolayers of cells (OIE 2010).

Attempts at primary isolation of IBDV in chickens at 2 and 3 weeks of age showed variable success and resulted in a high frequency of false negatives (Abdel-Alim and Saif 2001b). IBDV inoculation of 1 day old chickens has lower detection sensitivity than inoculation of ECE (Abdel-Alim and Saif 2001b).

IBDV serotype 1 strains are classified as classical (cIBDV), variant (vIBDV) or very virulent (vvIBDV). Some isolates of vvIBDV require passaging in ECE and cannot be adapted to growth in QT-35, Vero, BGM-70, LSCC-RP9, MQ-NCSU, LSCC-RP12, or B-lymphoblastoid cells (Abdel-Alim and Saif 2001a; Hussain and Rasool 2005).

IBDV undergoes culture-adaptation and attenuation with serial passages in cell culture. Culture-adaptation has been associated with changes in 3 residues of the VP2 structural protein. Culture-adapted isolates are difficult to detect as they have reduced pathogenicity indices in ECE or in chickens, and reduced CPE in cell culture. Several studies report that IBDV infection could not be

detected in chickens by IEM, IF, AC-ELISA or RT-PCR following inoculation with a culture adapted virus (Abdel-Alim and Saif 2001b). Specific testing for IBDV is therefore required because of the difficulties of primary isolation.

Specific testing for IBDV is possible by AC-ELISA, IEM, AGP, VN, IP, FA, RT-PCR or real-time RT-PCR assays.

The AGP and FA have low sensitivity, and the VN test lacks reactivity with the full range of IBDV strains. The ELISA is the most sensitive of the immunological techniques but has lower sensitivity than molecular assays. Cross reaction of serotype 2, non-pathogenic IBDV has been reported for the 5 commercially available ELISA kits, limiting their usefulness for IBDV serotype 1 detection (Hussain and Rasool 2005).

A number of RT-PCR and real-time RT-PCR assays have been developed with very high sensitivity and specificity. A RT-PCR assay targeting the *VP4* gene of IBDV failed to differentiate between serotype 1 and 2 (Wu et al. 1992). A RT-PCR assay has been developed for IBDV detection targeting the *VP2* structural gene sequence that is specific for serotype 1 (Lin et al. 1994). This assay was adapted to a slot-blot format and also adapted to a QC-PCR assay (Akin et al. 1993; Wu et al. 1997). The *VP2* region amplified in the RT-PCR assay incorporates a hypervariable region used for identification of strain markers by RT-PCR-RE and RT-PCR-RFLP assays (Akin et al. 1993; Jackwood et al. 2003; Jackwood and Sommer 2002; Jackwood and Sommer 2005; Lin et al. 1994; Peters et al. 2005; Sapats and Ignjatovic 2002; Wu et al. 1997; Wu et al. 2007). It has not been possible to identify markers that consistently identify IBDV strains according to pathotypes. The RT-PCR assay has been further adapted to a SYBR green real-time RT-PCR assay targeting the *VP2* gene (Li et al. 2007). Another real-time Taqman RT-PCR assay has been developed utilising probes specific to the classical, variant and virulent groups of virus and targeting the *VP4* gene sequence (Peters et al. 2005). The real-time Taqman RT-PCR assay targeting the *VP4* gene had 100% specificity for IBDV serotype 1 and a detection threshold of 300 genomic copies. A fluorescence resonance energy transfer (FRET) real-time RT-PCR assay targeting the *VP2* gene has been developed that is used to differentiate between the subgroups of IBDV by melt curve analysis and this assay has been used for diagnostic surveillance (Jackwood et al. 2003).

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 for extraneous agent testing will not be sufficient for primary isolation of IBDV. The 9 CFR guidelines do not include specific testing for IBDV.

The European Pharmacopoeia 2.6.24 refers to general methods for detection of extraneous agents and specific methods for detection of IBDV. The general methods are gross pathology and histopathology following inoculation of test material into the allantoic cavity, CAM and yolk sac of embryonated eggs, and by CPE detection in CKC monolayers. Specific isolation of IBDV is by inoculation of chickens 2 weeks old followed by AGP, EIA or VN assay for IBDV. Inoculation of 2 week old chickens has an unacceptably high failure rate and will not be sufficiently sensitive for isolation of culture-adapted strains (Abdel-Alim and Saif 2001b). The AGP, EIA and VN assays have lower sensitivity than the molecular assays described above. The sensitivity of both the general and specific methods is very low and they are not acceptable.

Conclusion

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 are not acceptable.

Primary isolation of IBDV by both inoculation of ECE, and isolation in CEF, BGM-70, Vero or QT-35 cell lines is recommended. Cultures should be passaged 3 times.

Specific testing is required for IBDV by real-time Taqman RT-PCR targeting the *VP4* gene or real-time SYBR green RT-PCR targeting the *VP2* gene.

The methods described by European Pharmacopoeia 2.6.24 for the detection of IBDV are inadequate for extraneous agent testing.

Infectious laryngotracheitis virus

Family *Herpesviridae*, genus *Iltovirus*

The taxonomically correct name for Infectious laryngotracheitis virus is gallid herpesvirus 1. Primary isolation of infectious laryngotracheitis virus (ILTV) is possible in ECE, CELi, CEK or CKC cells. CELi cells are the most sensitive culture system for ILTV isolation. Infected cultures require 2 hours incubation to allow virus adsorption to cells, and cultures are then incubated for 7 days and examined daily for evidence of CPE. Three blind passages in culture are required for isolation. CPE is evident as the formation of syncytia and intranuclear inclusions. CPE can be confirmed by FA using hyperimmune, polyclonal chicken antiserum or mAbs specific to ILTV, to stain the intranuclear inclusions (Ide 1978). Isolation in ECE is by inoculation onto the dropped CAM of 10–12 day old ECE. The CAM is examined for pock formation at 7 days after inoculation (OIE 2010).

ILTV can establish persistent, latent, low-level, inapparent infections of infected cultures that are difficult to detect. Primary isolation of ILTV can be inhibited by overgrowth with bacteria or adenovirus (Williams et al. 1994). Specific testing is required because of the high frequency of false negative results for culture isolation of ILTV.

Specific testing is possible by FA, EM, AGID, ELISA, SN and nested PCR. FA using a polyclonal hyperimmune antiserum from convalescent chickens has good sensitivity for virulent strains but a high rate of false negatives for avirulent or attenuated strains of ILTV (Ide 1978). The FA has poor repeatability between laboratories and strains.

A PCR assay was developed for ILTV targeting the *tk* gene (Abbas et al. 1996). The specificity of the PCR assay was increased by combining it with a hybridization assay using a biotinylated DNA probe. The PCR assay demonstrated 100% specificity when tested against the CELi culture and a range of avian pathogens. The specificity of the DNA probe hybridization assay was highly dependent on the stringency of the assay conditions. The detection limit for the PCR assay was 1 µg DNA.

A nested PCR assay has been developed for detection of ILTV targeting the unique short region of the genome (Humberd et al. 2002). The nested PCR assay is reported to have 100% specificity when tested against a panel of avian pathogens, and a detection threshold of 50–500 fg/ml of DNA. The sensitivity of virus isolation was 33 % greater when compared to molecular detection by nested PCR, and the molecular assay could detect ILTV in samples without evidence of CPE (Humberd et al. 2002; Williams et al. 1994).

A number of PCR-RFLP assays have been developed for ILTV targeting the *tk*, *gC*, glycoprotein X (*gX*), and *ICP4* genes, with the objective of differentiating between field and vaccine strains of

ILTV (Chang et al. 1997; Graham et al. 2000; Han and Kim 2001a; Han and Kim 2001b). The *ICP4* gene has the greatest polymorphism of the tested target genes and is therefore the most useful target for strain discrimination (Chang et al. 1997). The detection limit for the PCR assay targeting *ICP4* was 30 pg DNA or 10 PFU, and the assay was found to be 100% specific. The PCR-RFLP assay targeting the *ICP4* gene has been successfully applied to diagnostic surveys of ILTV strains across wide geographic areas.

The PCR-RFLP assay has been adapted to a real-time SYBR green PCR-RFLP assay targeting the *ICP4* gene and combined with a melt curve analysis (Creelan et al. 2006; Johnson et al. 1995). The assay is reported to have 100% specificity for ILTV strains, and is combined with RFLP analysis to include strain discrimination. The detection threshold of the real-time SYBR green PCR assay is 140 genomic copies/μl and the assay had 100% sensitivity for detection of field cases of ILTV.

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 for extraneous agent testing will not be sufficient for primary isolation of ILTV. The 9 CFR guidelines do not include specific testing for ILTV.

The European Pharmacopoeia 2.6.24 refers to general methods for detection of extraneous agents and specific methods for detection of ILTV. The general methods are gross pathology and histopathology following inoculation of test material into the allantoic cavity, CAM and yolk sac of embryonated eggs, and by CPE detection in CKC monolayers. Specific isolation of ILTV is by inoculation of 2 week old chickens followed by SN, EIA or FA for ILTV. Inoculation of 2 week old chickens is less sensitive than isolation in ECE or CELi cells. Specific testing by SN, EIA or FA is not suitable due to the high rate of false negative results with these tests. The sensitivity of both the general and specific methods is very low and they are not acceptable.

Conclusion

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 are not acceptable.

Primary isolation of ILTV in CELi cells is recommended. Adsorption incubation for 2 hours is required, cultures should be maintained for 7 days and 3 passages are required.

Specific testing is required for ILTV by real-time SYBR green PCR targeting the *ICP4* gene of ILTV.

The methods described by European Pharmacopoeia 2.6.24 for the detection of ILTV are inadequate for extraneous agent testing.

Marek's disease virus 1 and 2

Family *Herpesviridae*, genus *Mardivirus*

The taxonomically correct name for Marek's disease virus 1 and 2 is gallid herpesvirus 2 and 3 respectively. Primary isolation of MDV is possible on CKC, DEF and CEF. DEF cells are the most sensitive culture system for detection of MDV-1. The inoculum is adsorbed for 40 minutes prior to the addition of maintenance medium. Plaque formation occurs within 3 days of inoculation and can be confirmed by FA using mAbs specific to MDV-1 (Lee et al. 1983). Cultures are maintained for 7 days with medium changed every 2 days and 3 passages are required. Virus isolation is unreliable and has poor repeatability due to latency of virus (OIE 2010). MDV is highly cell-associated and cannot be purified in a cell-free medium. Specific testing is required due to the difficulty of primary isolation.

Specific testing is possible by PCR, AGID, FA, VN and ELISA. Serological diagnosis of MDV is problematic as turkey herpesvirus (HVT) shares common antigens with MDV and HVT cross reacts in serological assays (Davidson et al. 1991; Davidson et al. 1995a).

A PCR assay has been developed for MDV-1 targeting the 132 bp tandem repeat located in the *Bam*HI-H fragment of the genome (Becker et al. 1992; Becker et al. 1993; Davidson et al. 1995b; Davidson et al. 2002; Zhu et al. 1992). The size of the PCR product is dependent on the number of repeats present and correlates with the virulence of the isolate as either an oncogenic or attenuated strain. The PCR assay targeting the 132 bp tandem repeat region is reported to have 100% specificity for MDV-1 and was more sensitive than virus isolation for detection of MDV-1 in commercial flocks (Davidson et al. 1995b).

A second target for PCR assays for MDV detection has been the *ICP4* gene, and this assay has been adapted to a quantitative competitive PCR (QC-PCR) using fluorescent primers (Anderson et al. 1992; Bumstead et al. 1997; Burgess and Davison 1999). The detection limit for the QC-PCR is reported to be 40 copies of DNA but the assay range was too narrow to be useful. The QC-PCR assay had reasonable repeatability with 15% variance between replicate results, and was 100% specific to MDV-1 (Bumstead et al. 1997).

A QC-PCR assay was developed targeting the *gB* gene of MDV and using a competitor DNA fragment (Reddy et al. 2000). The assay detection limit was 5 fg DNA and it was 100% specific to MDV. Because MDV is highly cell-associated it is difficult to establish the detection limit of the assay relative to a bioassay for MDV.

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 for extraneous agent testing will not be sufficient for primary isolation of MDV. The 9 CFR guidelines do not include specific testing for MDV.

The European Pharmacopoeia 2.6.24 refers to general methods for detection of extraneous agents and specific methods for detection of MDV. The general methods are gross pathology and histopathology following inoculation of test material into the allantoic cavity, CAM and yolk sac of embryonated eggs, and by CPE detection in CKC monolayers. Specific isolation of MDV is by inoculation of 2 week old chickens and testing of serum by AGP. The sensitivity of both the general and specific methods is very low and they are not acceptable. The European Pharmacopoeia 2.6.25 *Avian live virus vaccines: tests for extraneous agents in batches of finished product* refers to primary isolation of MDV in CEF cultivated for 21 days in which there are at least 3 passages. The cultures are tested by FA using a MDV-specific mAb. Isolation in CEF is less sensitive than in DEF or CKC.

Conclusion

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 are not acceptable.

Primary isolation of MDV in DEF and confirmation of plaques by FA is recommended. Cultures should be maintained for 7 days with medium changed every 2 days and 3 passages are required.

Specific testing is required for MDV by PCR assay targeting the 132 bp tandem repeat region.

The methods described by European Pharmacopoeia 2.6.24 and 2.6.25 for the detection of MDV are inadequate for extraneous agent testing.

Newcastle disease virus

Family *Paramyxoviridae*, genus *Avulavirus*

Primary isolation of Newcastle disease virus (NDV) is by allantoic inoculation of 9–11 day old embryonated SPF fowl eggs. The recommendation is for incubation of inoculated eggs for 4–7 days between passages. Allantoic fluid is harvested and tested for NDV. Allantoic fluid testing negative is passaged into fresh eggs at least 3 more times.

Specific testing is required to detect NDV in the allantoic fluid. Specific testing for NDV is recommended by HA and RT-PCR assays on samples from the final passage together with an earlier passage.

The generalised culture method described in 9 CFR 113.37: *Detection of pathogens by the chicken embryo inoculation test* will not provide optimal growth conditions for NDV and will not be sufficient for isolation of NDV. The protocol 9 CFR 113.37 has an incubation period of 7 days for the inoculated eggs, which is insufficient for isolation of low titre viruses. The 9 CFR guidelines do not include specific testing for NDV detection.

The European Pharmacopoeia 2.6.24 refers to general methods for detection of extraneous agents and specific methods for detection of NDV. The general methods are gross pathology and histopathology following inoculation of test material into the allantoic cavity, CAM and yolk sac of embryonated eggs, and by CPE detection in CKC monolayers. Specific isolation is inoculation of chickens 2 weeks old and testing of serum by HI and EIA. The sensitivity of both the general and specific methods is very low and they are not acceptable.

Conclusion

The generalised culture method of 9 CFR 113.37: *Detection of pathogens by the chicken embryo inoculation test* is not acceptable.

Primary isolation of NDV by allantoic inoculation of embryonated SPF fowl eggs 9–11 days old is recommended. Inoculated eggs should be incubated for 4–7 days between passages, and 4 passages in eggs are required.

Specific testing for NDV is required for the harvested allantoic fluid by HA and RT-PCR assays.

The methods described by European Pharmacopoeia 2.6.24 for the detection of NDV are inadequate for extraneous agent testing.

Reticuloendotheliosis virus

Family *Retroviridae*, genus *Gammaretrovirus*

Primary isolation of reticuloendotheliosis virus (REV) is possible in DEF, CEF and 1 day old SPF chickens. CPE can be detected in infected CEF 5 days after inoculation and is confirmed by FA using mAbs (Bagust and Dennet 1977). REV isolates may establish non-cytolytic infections that are difficult to detect, and therefore specific testing is required.

Specific testing is possible by FA, ELISA, complement fixation procedure for assay of avian REV (COFAR), PCR and real-time PCR.

Therefore PCR detection in conjunction with the FA and primary isolation is recommended.

A PCR assay has been developed targeting the LTR region of REV (Aly et al. 1993). The PCR has been shown to be at least 10 times more sensitive than FA. FA and PCR were reported to be more sensitive than ELISA for detection of REV isolated in CEF, whereas ELISA was most sensitive for detection of REV isolated in chickens (Fadly and Witter 1997). A second PCR assay has been developed for REV targeting the *env* gene and is reported to have equivalent sensitivity to the assay targeting the LTR in direct comparisons (Davidson and Malkinson 1996). The detection limit for the PCR assay targeting the ENV gene was found to be 23.5 pg and when combined with a hybridization probe the limit of detection was 2.35 pg.

A real-time SYBR green PCR assay has also been developed for detection of REV targeting the regions encoding the ENV and the LTR (Tadese et al. 2008). Confirmation of assay specificity is achieved by melt curve analysis or sequencing. The real-time assay was reported to be 100% specific for REV and the detection limit was 3–5 target copies. Adaption of this assay to either MGB or FRET probes did not increase the assay sensitivity but did allow strain discrimination for REV. The real-time SYBR green RT-PCR assay has greater sensitivity than FA assay.

Contamination of veterinary vaccines with REV has been a significant source of virus infection in vaccinated chickens and extraneous agent testing for REV is therefore an important consideration (Fadly and Witter 1997). REV contamination of fowl pox virus vaccines is due to integrated REV provirus in the fowl pox virus genome (Bagust and Dennet 1977; Bendheim 1973; Tadese et al. 2008). A real-time SYBR green PCR method has been developed for differentiation of the REV integrated into the fowl pox virus genome from non-fowl pox virus associated provirus targeting the integration site of fowl pox virus-REV (Tadese et al. 2008).

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 for extraneous agent testing will not be sufficient for primary isolation of REV. The 9 CFR guidelines do not include specific testing for REV.

The European Pharmacopoeia 2.6.24 specifies primary isolation of REV in CEF cultivated for at least 10 days in which there were at least 3 passages. Virus is detected by FA staining of monolayers at the third passage. The method described by European Pharmacopoeia 2.6.24 will not detect non-cytolytic infections.

Conclusion

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 are not acceptable.

Primary isolation of REV in CEF and detection by FA is recommended.

Specific testing is required for REV by real-time SYBR green RT-PCR assay targeting the LTR and ENV coding regions of REV.

The method described in European Pharmacopoeia 2.6.24 is acceptable for culture of REV; however, specific testing is required as it will not detect non-cytolytic infections.

Turkey rhinotracheitis virus

Family *Paramyxoviridae*, genus *Metapneumovirus*

The taxonomically correct name for turkey rhinotracheitis virus is avian metapneumovirus; however, it is also known as avian pneumovirus. The most sensitive method for the primary isolation of turkey rhinotracheitis virus (TRV) is a 2-stage isolation process in ECE and Vero cells. In the first stage, sample is inoculated into the yolk sac of 6 day old ECE. Allantoic fluid and yolk sac are harvested 8 days later, homogenised and used for 3 serial passages in ECE (Cook and Cavanagh 2002). In the second stage, the egg fluid is harvested and homogenised and is then inoculated onto Vero, CELi or CEF. Vero cells are more sensitive than CEF or CELi for the second stage of isolation. CPE in Vero cells is readily evident as pinpoint foci of cytolysis at 5 days after inoculation, cytoplasmic eosinophilic inclusions and polykaryocytes. Some isolates fail to grow in monolayer cultures or establish inapparent non-cytolytic infections. Specific testing is therefore required.

Alternatively primary isolation is possible in TOC. Four passages at 4 day intervals are required in TOC, because initially there is ciliostasis and inhibition of culture propagation. The inhibitory effect on TOC can give unreliable results and therefore the 2-stage isolation method in ECE and Vero cells is recommended as the method of choice (Cook and Cavanagh 2002).

Specific testing for TRV is possible by FA, EM, EIA, ELISA and RT-PCR. FA, EM, ELISA and EIA are reported to have low detection sensitivities and are not suitable for extraneous agent testing.

FA can be used to confirm TRV-induced CPE in infected monolayers. This technique has been applied widely to studies of TRV; however, there have been a number of formats for the assays used and there has not been a consistent study to establish the validity of the assay and its sensitivity and specificity for TRV diagnosis (Baxter-Jones et al. 1986; Cook 2000; Jones et al. 1986; Jones et al. 1987). The FAs developed have used turkey convalescent sera (Jones et al. 1988), hyperimmune serum raised in rabbits (Majó et al. 1995; O'Loan and Allan 1990) or mouse-anti-TRV mAbs (Catelli et al. 1998; Cook 2000).

There are a number of subtypes of TRV: A, B, C, D, E and F. A RT-PCR assay has been developed that is capable of detecting all subtypes of TRV, targeting the *N* gene, and combined with a *G* gene-based sub-typing RT-PCR assay (Bäyon-Auboyer et al. 1999). Other RT-PCRs have been developed that are subtype specific. In a comparison of RT-PCR targets, it is reported that only the assay targeting the *N* gene had 100% sensitivity for isolates obtained from 4 different countries (Bäyon-Auboyer et al. 1999). The RT-PCR targeting the *N* gene detected 100% of samples tested, whereas in direct comparison Vero cell culture isolation detected only 27% and ELISA detection from experimental infections detected only 63% of positives (Bäyon-Auboyer et al. 1999).

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 for extraneous agent testing will not be sufficient for primary isolation of TRV. The 9 CFR guidelines do not include specific testing for TRV.

The European Pharmacopoeia 2.6.24 refers to primary isolation of TRV in chickens 2 weeks old followed by EIA on serum. The sensitivity of primary isolation in chickens 2 weeks old is low and this method is therefore not suitable for extraneous agent testing (Cook and Cavanagh 2002). The European Pharmacopoeia 2.6.25 part 5 refers to testing for TRV in CEF monolayers from 9 day old embryos followed by FA. CEF cells have lower sensitivity than Vero cells for TRV isolation.

Conclusion

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 are not acceptable.

Primary isolation of TRV by a 2-stage process consisting of primary isolation by yolk sac inoculation of ECE and 3 serial passages in ECE, followed by growth in Vero cell cultures is recommended.

Specific testing is required for TRV by RT-PCR targeting the *N* gene of TRV.

The methods described by European Pharmacopoeia 2.6.24 and 2.6.25 for the detection of TRV are inadequate for extraneous agent testing.

Bacteria — *Brucella* spp.

Brucella abortus

Detection of *Brucella* spp. is based on the growth of characteristic 1–2 mm pearly white, round colonies on tellurite glycine agar (TGA) by 4 days. Uniform coccobacilli are found on bacterial smears, which are not acid-fast but resistant to decolourisation using Stamp's stain.

Low levels of *Brucella* spp. may not be detected on TGA due to overgrowth by other agents. Inhibition by components of the test material such as lipid droplets is a significant consideration for the testing of vaccine materials as adjuvants are mostly lipid-based formulations. It is recommended that isolation is performed in selective medium to reduce overgrowth and inoculation of enrichment medium to increase sensitivity of detection. The most widely used selective medium for isolation of *Brucella abortus* is Farrell's medium which is prepared by the addition of 6 antimicrobials (polymyxin B sulphate, bacitracin, natamycin, nalidixic acid, nystatin, and vancomycin) to the basal medium. The addition of 2–5% equine or bovine serum is necessary for the growth of strains such as *B. abortus* biovar 2. Incubation of cultures should be at 37 °C and in 5–10% CO₂ for up to 6 weeks. Colony identification is by the following biochemical tests; urease, oxidase and catalase tests. A slide agglutination test is then conducted using anti-*Brucella* polyclonal serum. However, the slide agglutination test has poor specificity (OIE 2010; Roop, II et al. 1987).

Specific testing for *B. abortus* is recommended by either immune staining with a fluorochrome-conjugated mAb or by PCR. A colony blot ELISA that uses the mAb BRU 38 directed against the O side chain of lipopolysaccharide (LPS) is rapid, highly sensitive and has greatly improved specificity for detection of *B. abortus*.

Brucella genus-specific PCR assays directed against either the BCSP31 or 16S ribosomal RNA (rRNA) genes have been developed and standardised and are widely used. These PCR assays will not distinguish between the 6 species of the *Brucella* genus. Species specific and biovar specific *B. abortus* assays are mostly based on single nucleotide polymorphisms. The multi-locus AMOS PCR targeting the *ery* locus of *Brucella* spp. (AMOS-ERY PCR (AMOS is an acronym for *B. abortus*, *B. melitensis*, *B. ovis* and *B. suis*)), based on the multi-copy insertion element IS711 (also known as IS6501), is a multiplex assay that can differentiate *B. abortus* biovars 1, 2, 3b, 4, 5, 6 and 9 as well as the vaccine strains S19 and RB15. A PCR-RFLP assay based on the *omp2* locus has been developed that differentiates *B. abortus* from other members of the genus, and when combined with RFLP can distinguish between biovars of *B. abortus*. Both the AMOS-ERY PCR and the PCR-RFLP methods have been widely adopted by the veterinary laboratories. Recently a Taqman real-time PCR assay using fluorogenic probes has been developed based on the sequence spanning the

alkB gene and IS711. The real-time PCR assay has a detection threshold of 7.5 fg DNA (Bricker 2002; Michaux-Charachon et al. 1997; Moreno et al. 2002; Newby et al. 2003; Whatmore et al. 2005).

The 9 CFR 113.32: *Detection of Brucella contamination* describes the isolation of *Brucella* spp. in selective tryptose growth medium and detection of characteristic colonies. The 9 CFR 113.32 guidelines are not sufficiently specific or detailed, and the general method of culture isolation is not sufficiently sensitive for isolation of *B. abortus*.

The European Pharmacopoeia monographs *Vaccines for veterinary use*, 2.6.12 *Microbiological examination of non-sterile products: microbial enumeration tests* and 2.6.13 *Microbiological examination of non-sterile products: test for specified microorganisms* describe generalised methods for microbiological testing and selective methods of testing for specified microorganisms. The generalised methods described in the monograph 2.6.12 are not suitable for *B. abortus* detection. The monograph 2.6.13 does not include guidelines for *B. abortus*.

Conclusion

The culture method in 9 CFR 113.32: *Detection of Brucella contamination* is not acceptable for isolation of *B. abortus*.

Primary isolation of *B. abortus* on selective Farrell's medium supplemented with 2–5% equine or bovine serum is recommended. Test material should also be inoculated into fluid enrichment medium in parallel. Colony identification is by specific biochemical tests and a slide agglutination test.

Specific testing by PCR is required using either the AMOS-ERY PCR based on the multi-copy element IS711 or the PCR-RFLP assay based on the *omp2* locus.

The generalised methods described in European Pharmacopoeia monograph 2.6.12 are not suitable for *B. abortus* detection.

Brucella canis

Culture isolation of *Brucella canis* can be performed on blood or on tryptose agar plates under aerobic conditions at 37 °C for 7 days (Keid et al. 2007). Initial inoculation of enrichment broth increases the success rate for culture isolation of *B. canis*.

Specific testing is recommended as bacteriological isolation is problematic and has a high rate of false negatives for *B. canis* (Kim et al. 2006).

A PCR assay has been developed for the 16S/23S rRNA interspace region that can detect as little as 3.8 fg of DNA. Systematic studies comparing PCR to culture isolation have shown that PCR has better sensitivity than culture isolation alone (Keid et al. 2007).

The 9 CFR 113.32: *Detection of Brucella contamination* describes the isolation of *Brucella* spp. in standard tryptose growth medium and detection of characteristic colonies. The 9 CFR 113.32 culture methods are problematic due to unacceptably high rates of false negatives and specific testing is required. The 9 CFR guidelines do not include specific testing for *B. canis*.

The European Pharmacopoeia monographs *Vaccines for veterinary use*, 2.6.12 *Microbiological examination of non-sterile products: microbial enumeration tests* and 2.6.13 *Microbiological examination of non-sterile products: test for specified microorganisms* describe generalised methods for microbiological testing and selective methods of testing for specified microorganisms. The generalised methods described in the monograph 2.6.12 are not suitable for *B. canis* detection. The monograph 2.6.13 does not include guidelines for *B. canis*.

Conclusion

The culture method of 9 CFR 113.32: *Detection of Brucella contamination* is acceptable for culture isolation of *B. canis*.

Culture isolation of *B. canis* by initial inoculation of enrichment broth prior to plating on tryptose agar plates is recommended. Cultures should be maintained for 7 days and incubated at 35 °C. Colony identification is by Gram stained bacterial stains.

Specific testing for *B. canis* is required by PCR assay targeting the 16S/23S rRNA gene.

The generalised methods described in European Pharmacopoeia monograph 2.6.12 are not suitable for *B. canis* detection.

Brucella melitensis

Culture isolation of *Brucella melitensis* is possible on standard solid media such as tryptose agar under aerobic conditions at 37 °C. Additional isolation on the modified Thayer-Martin medium ensures detection of some strains of *B. melitensis* that are inhibited by nalidixic acid and bacitracin present in the Farrell's medium (Marin et al. 1996b; Marin et al. 1996a; Marín et al. 1999). Enrichment in broth culture prior to plating onto agar is recommended to enhance detection. Cultures should be maintained for 21 days and passaged at 7 day intervals to ensure detection. Cultures are incubated at 35 °C in 10% CO₂. Colony identification is by Gram-stained bacterial smears in which *B. melitensis* appears as small gram-negative coccobacilli. *B. melitensis* is positive for urea, catalase and oxidase in biochemical tests.

Specific testing is required for *B. melitensis* as low level detection is problematic even with the use of selective medium. Specific testing is possible by FA or PCR.

Confirmation of *B. melitensis* in bacterial smears is possible using specific reference antiserum in a FA.

PCR assays for *B. melitensis* have been developed targeting the 16S rRNA, bscp31 and IS 6501/711 molecular markers (Garin-Bastuji et al. 2006). Specific testing by real-time PCR targeting the IS711 gene can differentiate the 7 main clades or species of *Brucella* based on SNPs. Foster (2008) reports a detection threshold of 10 fg of DNA, detection sensitivity of 100% and specificity of 100% (Foster et al. 2008).

The 9 CFR 113.32: *Detection of Brucella contamination* describes the isolation of *Brucella* spp. in standard tryptose growth medium and detection of characteristic colonies. The 9 CFR 113.32 guidelines are not sufficiently specific or detailed, and the general method of culture isolation is not sufficiently sensitive for *B. melitensis*.

The European Pharmacopoeia monographs *Vaccines for veterinary use*, 2.6.12 *Microbiological examination of non-sterile products: microbial enumeration tests* and 2.6.13 *Microbiological examination of non-sterile products: test for specified microorganisms* describe generalised methods for microbiological testing and selective methods of testing for specified microorganisms. The generalised methods described in the monograph 2.6.12 are not suitable for *B. melitensis* detection. The monograph 2.6.13 does not include guidelines for *B. melitensis*.

Conclusion

The culture method in 9 CFR 113.32: *Detection of Brucella contamination* is not acceptable for isolation of *B. melitensis*.

Culture isolation of *B. melitensis* by initial inoculation of enrichment broth prior to plating on tryptose agar, Farrell's medium and Thayer-Martin medium is recommended. Cultures should be maintained for 21 days and passaged every 7 days. Cultures should be incubated at 35 °C in 10% CO₂. Colony identification is by Gram-stained bacterial smears.

Specific testing for *B. melitensis* is required by either FA or real-time PCR targeting the IS711 gene.

The generalised methods described in European Pharmacopoeia monograph 2.6.12 are not suitable for *B. melitensis* detection.

Brucella suis

Brucella suis is cultured on enriched tryptic soy agar under aerobic conditions with 5% CO₂. *B. suis* grows very slowly and cultures should be maintained for 4 weeks. On gram smears *B. suis* are gram-negative coccobacilli. Confirmation of *B. suis* is by slide-agglutination using anti-*Brucella* serum.

Genus specific PCR assays for *Brucella* have been developed targeting the OMP and 16S rRNA genes (Fekete et al. 1990; Romero et al. 1995) and genus specific probes have been used to differentiate *Brucella* spp. from *Agrobacterium* spp. (Fayazi et al. 2002; Herman and de Ridder 1992). A species specific PCR has been developed to differentiate *Brucella abortus* and *Brucella suis* based on the IS711 insertion sequence (Herman and de Ridder 1992). The PCR assay targeting the IS711 insertion sequence has been adapted to a real-time Taqman PCR assay using dual-labelled probes specific to *B. melitensis*, *B. abortus* and *B. suis*. The real-time Taqman PCR assay is highly sensitive, specific and accurate (Redkar et al. 2001).

The 9 CFR 113.32: *Detection of Brucella contamination* protocol describes the isolation of *Brucella* spp. in selective tryptose growth medium and detection of characteristic colonies. The 9 CFR culture method describes optimal growth conditions for the culture isolation of *B. suis*. Confirmation of culture positives as *B. suis* would require specific testing.

The European Pharmacopoeia monographs *Vaccines for veterinary use*, 2.6.12 *Microbiological examination of non-sterile products: microbial enumeration tests* and 2.6.13 *Microbiological examination of non-sterile products: test for specified microorganisms* describe generalised methods for microbiological testing and selective methods of testing for specified microorganisms. The generalised methods described in the monograph 2.6.12 are not suitable for *B. suis* detection. The monograph 2.6.13 does not include guidelines for *B. suis*.

Conclusion

The culture method of 9 CFR 113.32: *Detection of Brucella contamination* is acceptable for culture isolation of *B. suis*.

Culture isolation of *B. suis* on enriched tryptic soy agar is recommended. Cultures should be maintained for 4 weeks and the identity of cultures confirmed by bacterial smears and slide agglutination assay.

Specific testing is required for confirmation of *B. suis* by real-time Taqman PCR targeting the IS711 insertion sequence.

The generalised methods described in European Pharmacopoeia monograph 2.6.12 are not suitable for *B. suis* detection.

Bacteria — *Salmonella* spp.

Salmonella Enteritidis, *Salmonella* Gallinarum and *Salmonella* Pullorum

Culture isolation of *Salmonella* spp. is possible in Rappaport-Vassiliadis and tetrathionate (TET) selective enrichment broths. Selective culture methods have been adapted to ensure sensitive culture isolation without inhibition of subsequent PCR reactions for *Salmonella* spp. (Oliveira et al. 2003; Stone et al. 1994). The Rappaport-Vassiliadis PCR method was found to result in the highest detection sensitivity and least inhibitory action for field samples when compared to non-selective, selenite cystine (SC) broth, TET and buffered peptone water (BPW) broth culture methods (Oliveira et al. 2003; Stone et al. 1994). Cultures are characterised by serological and biochemical typing methods.

A systematic study across 5 laboratories for contamination of meat samples compared the detection sensitivity of culture isolation alone (sensitivity 56.67 %) and culture isolation followed by specific detection by ELISA or PCR (sensitivity 71%) (Dickel et al. 2005). Specific testing significantly enhanced the sensitivity of *Salmonella* Pullorum detection. There was no difference found between the sensitivities for ELISA and PCR.

An AGID test is widely used for flock detection of *Salmonella* spp. and discrimination between standard and variant strains. The AGID test has poor repeatability, low specificity and low sensitivity.

PCR assays for *Salmonella* spp. detection have been developed targeting the *invA* and *rfbS* genes (Oliveira et al. 2003). A PCR-RFLP assay targeting the *rfbS* gene has been developed to detect and differentiate *Salmonella* Gallinarum and *Salmonella* Pullorum (Luk et al. 1993; Luk et al. 1997). The PCR assay has also been adapted to a PCR-ELISA assay using a digoxigenin-labelled probe (Luk et al. 1997). The detection limit of the PCR-ELISA was 10 bacteria and the assay has been validated for field isolates. An allele-specific PCR based on the *rfbS* gene has been developed for the detection and differentiation of both *Salmonella* Gallinarum and *Salmonella* Pullorum (Desai et al. 2005; Kim et al. 2008b). The *rfbS* gene encodes paratose synthetase present only in *Salmonella* spp. of serogroup D and containing species specific polymorphisms (Liu et al. 1991; Verma et al. 1988). The assay is highly serotype-specific for *Salmonella* type D and the detection sensitivity is 100 pg DNA. This assay has been adapted to a capillary gel electrophoresis and microchip format (Jeon et al. 2007).

A Taqman PCR developed for detection of *Salmonella* spp. targeting the flagellin gene (fliC) is reported to be 4 logs more sensitive than conventional PCR detection (Lee et al. 2002). The Taqman assay has not been validated for extraneous agent testing.

The 9 CFR 113.30²² specifies detection of *Salmonella* spp. by inoculation of liquid broth medium (tryptose and either selenite F or TET) and incubation for 18–24 hours at 35–37 °C. The inoculum is transferred to either MacConkey agar or *Salmonella-Shigella* agar and incubated for 18–24 hours. The 9 CFR culture method provides the optimal growth conditions for isolation of *Salmonella* spp.; however, does not meet the requirements for specific testing.

The European Pharmacopoeia monograph 2.6.24 specifies isolation of *Salmonella* Pullorum by inoculation of chickens 2 weeks old followed by agglutination assay. The AGID test has poor repeatability, low specificity and low sensitivity and is not sufficiently sensitive for the purposes of extraneous agent detection. The European Pharmacopoeia monograph 2.6.24 does not specify testing for *Salmonella* Enteritidis or *Salmonella* Gallinarum.

Conclusion

The culture method of 9 CFR 113.30 is acceptable for culture isolation of *Salmonella* spp..

Culture isolation of *Salmonella* spp. in selective enrichment Rappaport-Vassiliadis broth is recommended.

Specific testing is required by allele-specific PCR targeting the *rfbS* gene using one of the classical, microchip, capillary electrophoresis or PCR-ELISA platforms.

The method in European Pharmacopoeia 2.6.24 for the isolation of *Salmonella* Pullorum is not sufficiently sensitive for the purposes of extraneous agent detection.

Bacteria — other

Burkholderia mallei

Isolation of *Burkholderia mallei* is by culture on glycerol agar under aerobic conditions for 48 hours. Colonies are confluent, cream coloured, smooth, moist and viscid. Gram-stained smears are characterised by gram-negative, non-sporulating, non-encapsulated rods. The commercial analytical profile index (API) system test will confirm *B. mallei* as a member of the *Pseudomonas* group, but unlike members of the *Pseudomonas* genus, *B. mallei* are non-motile (OIE 2010). Therefore specific testing is not required.

The generic protocols of 9 CFR 113.26: *Detection of viable bacteria and fungi except in live vaccines* and 9 CFR 113.27: *Detection of extraneous viable bacteria and fungi in live vaccines* do not provide the growth conditions required for *B. mallei* and are not suitable for primary isolation of *B. mallei*. The 9 CFR guidelines do not require specific testing for *B. mallei*.

The European Pharmacopoeia monographs *Vaccines for veterinary use*, 2.6.12 *Microbiological examination of non-sterile products: microbial enumeration tests* and 2.6.13 *Microbiological examination of non-sterile products: test for specified microorganisms* describe generalised methods for extraneous agent testing using methods that are sensitive to the agent being tested, and

²² Detection of *Salmonella* contamination.

detection using specific testing methods that are sensitive. The monographs do not specify details of a culture system or assay for *B. mallei* detection, simply that the methods should be sensitive.

Conclusion

The generic protocols of 9 CFR 113.26: *Detection of viable bacteria and fungi except in live vaccines* and 9 CFR 113.27: *Detection of extraneous viable bacteria and fungi in live vaccines* are not acceptable for primary isolation of *B. mallei*.

Isolation of *B. mallei* by culture on glycerol agar under aerobic conditions for 48 hours is recommended.

Specific testing is not required.

Coxiella burnetii

Coxiella burnetii is a fastidious, facultative intracellular organism that multiplies within vacuoles of phagolysosomal origin. Susceptible cell culture systems are human embryonic lung (HEL), BHK-21, monkey kidney, Vero, baby grivet monkey kidney (BGM), L929 and J774 cells. *C. burnetii* can also be isolated by yolk sac inoculation of 5 day old ECE and harvested at 10-15 days, or by intraperitoneal inoculation of mice or guinea pigs (Fournier et al. 1998; Marrie 1990; OIE 2010).

Primary isolation of *C. burnetii* by the centrifugation shell–vial technique is the most sensitive and reliable technique available. Samples are inoculated into HEL monolayers growing in shell vials. *C. burnetii* is detected 6–15 days later either by FA or by PCR of cultures. It is critical to wash away serum components from inoculated cells to prevent fibrin interference with diagnosis. HEL cells have contact inhibition and so can be maintained as confluent monolayers for extended periods. The centrifugation shell-vial technique using HEL monolayers is the recommended method as it has been shown to increase the detection sensitivity by 137% when compared to conventional culture (Gil-Grande et al. 1995; Raoult et al. 1990).

It is important to exclude antimicrobials from culture medium prior to and during primary isolation as this has been shown to markedly reduce the rates of primary isolation.

Specific testing is required as primary culture isolation is difficult, unreliable and is not sufficiently sensitive for detection of *C. burnetii*. Persistent infection of cell lines by *C. burnetii* can occur without any obvious cytopathology.

There are several PCR assays for *C. burnetii* available and in common use. The sensitivity of PCR amplification of the plasmid-encoded *htpAB*-associated repetitive element is extremely high as there are 19 copies present. PCR against genomic superoxide dismutase has been shown to have very high sensitivity and can detect as little as 10 copies. These assays are highly recommended for specific testing of culture-isolated *C. burnetii* (Stein and Raoult 1992; Willems et al. 1993).

The AC-ELISA for *C. burnetii* has good sensitivity but lacks specificity (Henning and Sting 2002).

The FA for *C. burnetii* uses anti-*C. burnetii* mAbs to detect rod-shaped bacteria within the cells and has been shown to have comparable sensitivity to the PCR but relies upon culture isolation which is problematic.

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 do not include selective culture methods for the primary isolation of *C. burnetii*. The 9 CFR guidelines do not include specific testing for *C. burnetii*.

The European Pharmacopoeia general monograph *Vaccines for veterinary use* and 5.2.4 does not include the selective methods required for *C. burnetii*.

Conclusion

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 are not acceptable.

Primary isolation of *C. burnetii* by the centrifugation shell–vial technique using HEL monolayers is recommended.

Specific testing is required by PCR detection of the *htpAB* element or the superoxide dismutase gene.

Francisella tularensis

Isolation of *Francisella tularensis* requires special culture media: Francis medium, McCoy and Chapin media, cysteine heart agar, glucose cysteine chocolate agar, buffered charcoal yeast extract agar (BCYE), or modified Thayer–Martin agar. *F. tularensis* does not grow in standard culture as it is an obligate intracellular pathogen and requires cysteine supplementation (Splettstoesser et al. 2005). Characteristic colonies appear by 48 hours after inoculation. *F. tularensis* cannot be grown in enrichment broth, and growth is slow even in specialised media. Broth cultures should be maintained for 10 days. Overgrowth is a problem with culture isolation of *F. tularensis*.

In stained smears *F. tularensis* bacteria are non-motile, non-sporulating, bipolar staining, and of uniform appearance in 24 hour cultures, but become pleomorphic in older cultures (OIE 2010).

Specific testing is required due to the low sensitivity of culture isolation for *F. tularensis*. Specific testing is possible by slide-agglutination, FA, ELISA, ISH, PCR or real-time PCR.

FAs have been developed for tularaemia using immunoblots developed with fluorophore-conjugated polyclonal or monoclonal *F. tularensis*-specific antibodies (Dennis et al. 2001; Zeidner et al. 2004). The FA is highly specific and sensitive but the detection limit of 10^6 bacterial cells is too high to be suitable for extraneous agent testing.

PCR assays have been developed for *F. tularensis* targeting the *tul4* gene and the 16S rRNA gene (Forsman et al. 1994; Junhui et al. 1996; Long et al. 1993). Comparative studies have reported that in general the classical PCR assays have higher sensitivity than immunological assays such as FA and ELISA, but the sensitivity is lower than required for an acceptable diagnostic assay and the detection threshold was reported to be 1000 colony forming units (CFU)/ml (Splettstoesser et al. 2005). A nested PCR assay targeting the *fopA* gene had better sensitivity than the classical PCR assay and a detection threshold of 100 CFU/ml of spiked blood (Fulop et al. 1996). A multiplex PCR assay targeting both the *tul4* gene and the 16S rRNA genes includes primer sets to identify *F. tularensis* to genus and species level, and to identify the 4 subspecies of *F. tularensis*; *F. tularensis* subsp. *tularensis* (Jellison type A); *F. tularensis* subsp. *holarctica* (Jellison type B); *F. tularensis* subsp. *mediasiatica* and *F. tularensis* subsp. *novicida* (Forsman et al. 1994). Field surveys of diagnostic human samples report a 75% detection sensitivity using the multiplex PCR targeting the 16S rRNA gene and a 62% culture detection sensitivity (Johansson et al. 2000; Sjöstedt et al. 1997).

Similar studies have not been carried out to validate the application of conventional PCR for *F. tularensis* in rabbits.

The PCR assay for *F. tularensis* targeting the *fopA* gene has been adapted to a PCR-EIA assay with an associated increase in sensitivity (82%) and specificity (79%), a detection threshold of 1 pg of DNA or 22 bacterial cells, but the assay has poor repeatability (Higgins et al. 2000). The efficacy of the assay was established for a range of environmental samples and host tissues. A real-time Taqman PCR targeting the *fopA* gene had comparable sensitivity and specificity to the PCR-EIA assay and a detection threshold of 1 pg DNA or 100 bacterial cells (Higgins et al. 2000). The sensitivity of the real-time Taqman PCR has been further improved in multi-target assays. Emanuel *et al* (2003) developed a multi-target Taqman assay targeting *fopA* and *tul4* genes, and Versage *et al* (2003) developed a multi-target Taqman assay targeting *ISFtu2*, 23 kDa, *tul4* and *fopA* genes. The sensitivity of the multi-target real-time Taqman PCR is 7 logs greater than for conventional PCR and for infected carcasses and tissues the sensitivity is reported to be 87% (Versage et al. 2003).

A number of molecular typing methods have been developed for *F. tularensis* such as repetitive extragenic palindromic element PCR (REP-PCR), enterobacterial repetitive intergenic consensus sequence PCR (ERIC-PCR), random amplified polymorphic DNA (RAPD) PCR and long primers RAPD-PCR. All these molecular assays lack reproducibility and demonstrate inter-laboratory variability (Speltstoeser et al. 2005).

The 9 CFR 113.52, 113.53 and 113.55 protocols do not specify culture and assay conditions for *F. tularensis*.

The European Pharmacopoeia monographs *Vaccines for veterinary use*, 2.6.12 *Microbiological examination of non-sterile products: microbial enumeration tests* and 2.6.13 *Microbiological examination of non-sterile products: test for specified microorganisms* describe generalised methods for microbiological testing and selective methods of testing for specified microorganisms. The monograph 2.6.13 does not include guidelines for *F. tularensis*.

Conclusion

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 are not acceptable.

Primary isolation of *F. tularensis* in a specialised medium supplemented with cysteine, such as Francis medium, McCoy and Chapin media, cysteine heart agar, glucose cysteine chocolate agar, BCYE, or modified Thayer-Martin agar is recommended. Broth cultures should be maintained for 10 days.

Specific testing is required for *F. tularensis* by the multi-target, real-time Taqman PCR targeting the *ISFtu2*, 23 kDa, *tul4* and *fopA* genes.

The generalised methods described in the monograph 2.6.12 are not suitable for detection of *F. tularensis*. The monograph 2.6.13 does not include guidelines for *F. tularensis*.

Leptospira interrogans* var. *canicola

Leptospira interrogans var. *canicola* can be grown in Fletcher's medium supplemented with 10% rabbit serum in an aerobic atmosphere at 28 °C. Cultures should be grown for 6 weeks and checked weekly by dark-field microscopy.

A genus-specific PCR assay detects the 16S rRNA gene (Heinemann et al. 1999; Heinemann et al. 2000; Merien et al. 1992; Richtzenhain et al. 2002; Savio et al. 1994; Woodward et al. 1991; Woodward and Redstone 1993). The PCR detection threshold for clinical samples was shown to be 20 bacteria/ml and the detection sensitivity is significantly greater than for culture isolation alone. Other tests include commonly used microscopic agglutination test (MAT), ELISA and FA detection. The MAT uses a panel of rabbit anti-*Leptospira* sera and allows typing of *Leptospira* strains (Rossetti et al. 2005).

The generic protocols of 9 CFR 113.26: *Detection of viable bacteria and fungi except in live vaccines* and 9 CFR 113.27: *Detection of extraneous viable bacteria and fungi in live vaccines* will not be sufficient for primary isolation of *L. interrogans* var. *canicola*. The 9 CFR guidelines do not include specific testing for *L. interrogans* var. *canicola*.

The European Pharmacopoeia monographs *Vaccines for veterinary use*, 2.6.12 *Microbiological examination of non-sterile products: microbial enumeration tests* and 2.6.13 *Microbiological examination of non-sterile products: test for specified microorganisms* describe generalised methods for microbiological testing and selective methods of testing for specified microorganisms. The monograph 2.6.13 does not include guidelines for *L. interrogans* var. *canicola*.

Conclusion

The generic protocols of 9 CFR 113.26: *Detection of viable bacteria and fungi except in live vaccines* and 9 CFR 113.27: *Detection of extraneous viable bacteria and fungi in live vaccines* are not acceptable.

Primary isolation of *L. interrogans* var. *canicola* in Fletcher's medium supplemented with 10% rabbit serum is recommended. Cultures should be maintained for 6 weeks in an aerobic atmosphere at 28 °C. Confirmation of growth is required by dark-field microscopy.

Specific testing is required for *L. interrogans* var. *canicola*.

The generalised methods described in the monograph 2.6.12 are not suitable for detection of *L. interrogans* var. *canicola*. The monograph 2.6.13 does not include guidelines for *L. interrogans* var. *canicola*.

Ornithobacterium rhinotracheale

Culture isolation of *Ornithobacterium rhinotracheale* requires enriched media such as blood agar supplemented with gentamycin and polymyxin. Cultures require microaerophilic conditions (5–10% carbon dioxide). For the identification of *O. rhinotracheale* a combination of AGP and biochemical testing by the API-ZONE identification strip (Bio Merieux, France) or RapID NF Plus system (Innovative Diagnosis, USA) can be used (Post et al. 1999).

Bacteriological isolation of *O. rhinotracheale* is problematic. Identification can be confounded by *Haemophilus* spp. or *Pasteurella* spp. as all 3 genera are gram-negative, non-sporulating, non-motile, pleomorphic rods. A suitable selective medium is not available for *O. rhinotracheale* and overgrowth with *E. coli* is common. Identification of *O. rhinotracheale* by conventional biochemical or morphological means is difficult as isolates have variable colony morphology and variable biochemical reactivity and HA activity (Hafez 2000; Hafez 2002; Hafez and Sting 1999).

PCR amplification of the 16S rRNA gene is highly sensitive and specific and the only definitive diagnostic assay available (van Empel and Hafez 1999). RFLP patterns generated from the PCR products of the 16S rRNA assay do not give consistent diagnostic fingerprints (Hafez 2002).

The generic protocols of 9 CFR 113.26: *Detection of viable bacteria and fungi except in live vaccines* and 9 CFR 113.27: *Detection of extraneous viable bacteria and fungi in live vaccines* will not be sufficient for the primary isolation of *O. rhinotracheale*. The 9 CFR guidelines do not include specific testing for *O. rhinotracheale*.

The European Pharmacopoeia monographs *Vaccines for veterinary use*, 2.6.12 *Microbiological examination of non-sterile products: microbial enumeration tests* and 2.6.13 *Microbiological examination of non-sterile products: test for specified microorganisms* describe generalised methods for microbiological testing and selective methods of testing for specified microorganisms. The monograph 2.6.13 does not include guidelines for *O. rhinotracheale*. The monograph 2.6.24 does not require specific testing for *O. rhinotracheale*.

Conclusion

The generic protocols of 9 CFR 113.26: *Detection of viable bacteria and fungi except in live vaccines* and 9 CFR 113.27: *Detection of extraneous viable bacteria and fungi in live vaccines* are not acceptable.

Culture isolation of *O. rhinotracheale* is not recommended.

Specific testing is required for *O. rhinotracheale* by PCR assay targeting the 16S rRNA gene.

The generalised methods described in the monograph 2.6.12 are not suitable for detection of *O. rhinotracheale*. The monograph 2.6.13 does not include guidelines for *O. rhinotracheale*.

Taylorella equigenitalis

Primary isolation of *Taylorella equigenitalis* is difficult due to the fastidious nature of the organism and the potential for bacterial or fungal overgrowth. The preferred isolation method for both biotypes *T. equigenitalis* and *T. asinigenitalis* is on 5% (v/v) heated blood or 'chocolate' agar plates under microaerophilic conditions, and at 37 °C. When cooled to 45–50 °C, trimethoprim (1 µg/ml), clindamycin (5 µg/ml), and amphotericin B (5 µg/ml) are added to the medium. Lysed horse blood is also added to 5% to counteract the inactivation of trimethoprim by thymidine in the peptone medium. Lysed horse blood contains thymidine phosphorylase, which will inactivate thymidine. An initial enrichment culture step will improve detection sensitivity. The reference strain of *T. equigenitalis* must be cultured in parallel with the test samples to ensure that the culture conditions are optimal for isolation of this organism.

At least 3 days is required before colonies of *T. equigenitalis* become visible, after which time daily inspection for 14 days is needed. A standard incubation time of at least 7 days is advisable before certifying cultures negative for *T. equigenitalis*. Plates should be examined for contaminants after the first 24 hours incubation.

Colonies of *T. equigenitalis* are up to 2–3 mm in diameter, smooth with an entire edge, glossy and yellowish grey. *T. equigenitalis* is a gram negative coccoid, pleomorphic rod that exhibits bipolar staining and is characterised biochemically by catalase, phosphatase and oxidase production (OIE 2010).

Specific testing is recommended because of the difficulties of culture isolation. Specific testing is possible by FA, PCR or real-time PCR assays.

FAs have been described using either polyclonal antibody to whole killed *T. equigenitalis* or mAbs.

PCR assays have been developed to specifically detect the 16S rRNA gene from *T. equigenitalis* which is present in multiple copies in the genome. The 16S rRNA gene PCR assay can detect 10–15 CFU. Semi-nested and nested PCR assays have also been developed targeting the same region. PCR assays are species-specific and do not cross react with other contaminating members of the genus *Taylorella* that may be present in test samples. Systematic studies comparing the sensitivity of primary isolation with PCR detection have found approximately 10 times greater sensitivity for PCR detection (Anzai et al. 1999; Anzai et al. 2001; Anzai et al. 2002; Bleumink-Pluym et al. 1993; Duquesne et al. 2007; Niwa et al. 2007; Wakeley et al. 2006). A real-time PCR assay using a fluorogenic probe against the same region of the 16S rRNA gene had similar sensitivity to the classical PCR assay (Wakeley et al. 2006).

The generic protocols of 9 CFR 113.26: *Detection of viable bacteria and fungi except in live vaccines* and 9 CFR 113.27: *Detection of extraneous viable bacteria and fungi in live vaccines* do not provide the growth conditions required for *T. equigenitalis* and are not suitable for primary isolation of *T. equigenitalis*. The 9 CFR guidelines do not require specific testing for *T. equigenitalis*.

The European Pharmacopoeia monographs *Vaccines for veterinary use*, 2.6.12 *Microbiological examination of non-sterile products: microbial enumeration tests* and 2.6.13 *Microbiological examination of non-sterile products: test for specified microorganisms* describe generalised methods for extraneous agent testing using methods that are sensitive to the agent being tested, and detection using specific testing methods that are sensitive. The monographs do not specify details of a culture system or assay for *T. equigenitalis* detection, simply that the methods should be sensitive.

Conclusion

The generic protocols of 9 CFR 113.26: *Detection of viable bacteria and fungi except in live vaccines* and 9 CFR 113.27: *Detection of extraneous viable bacteria and fungi in live vaccines* are not acceptable.

Isolation of *T. equigenitalis* is by inoculation of test material into enrichment broth and then isolation on chocolate agar supplemented with antimicrobials and lysed horse blood is recommended.

Specific testing by PCR targeting the 16S rRNA gene is required.

Treponema paraluisccuniculi

Treponema paraluisccuniculi is non-cultivable and is an obligate intracellular pathogen. Inoculation of rabbit testicles is used for detection and propagation of *T. paraluisccuniculi*. Rabbits are inoculated with a 1 ml sample into the testicle and a firm orchitis develops in 7–11 days. The treponemes are harvested by longitudinal sectioning of the testicle and gentle rotation in 10% Venereal Disease Research Laboratory (VDRL) non-reactive rabbit serum and 0.14% saline, followed by washing then centrifugation to pellet the treponemes. Dark-field microscopy or silver staining can be used to visualise *T. paraluisccuniculi*, which appears as a flagellated, helical

bacterium with rotational motility (Hougen et al. 1973; Jenkins 2008; Lukehart et al. 1980). A second passage is required by testicular inoculation of rabbits.

Serological assays, ELISA, the fluorescent treponemal antigen (FTA) test and rapid plasma regain (RPR) card tests, are widely used for clinical diagnosis but are not suitable for extraneous agent testing. Confirmation of *T. paraluiscuniculi* is possible by FA using hyperimmune pooled rabbit syphilitic serum or by EM (Miller et al. 1966).

T. paraluiscuniculi is closely related to the human agent of syphilis *Treponema pallidum* subsp. *pallidum*. *T. paraluiscuniculi* cross-reacts with antiserum raised against *T. pallidum* subsp. *pallidum* and microarray analysis of the subspecies genomic differences has identified that heterogeneity is localised in the *tp* loci (Strouhal et al. 2007). Several PCR, RT-PCR and real-time PCR assays have been developed for the diagnosis of *T. pallidum* subsp. *pallidum*; however, no such assay has been developed for *T. paraluiscuniculi*. Genus-specific PCR assays targeting the 16S rRNA gene of *Treponema* have been developed to identify spirochaetes in a range of mammalian hosts and in environmental samples (Weisburg et al. 1991; Wilson 1994). PCR targeting the 16S rRNA gene have low sensitivity in diagnostic studies and the assay is inhibited by the presence of contaminating extraneous bacterial DNA (Fox et al. 1992; Stackebrandt and Goebel 1994). A PCR assay targeting the highly conserved *rpoB* gene has been developed with the capacity to detect spirochaetes from the genera *Borrelia*, *Treponema*, and *Leptospira*. The PCR assay targeting the *rpoB* gene has the advantage of improved sensitivity and is not inhibited by bacterial contaminants (Renesto et al. 2000; Strouhal et al. 2007). This assay has the capacity to detect *T. paraluiscuniculi* to the genus level.

The CFR 113.52, 113.53 and 113.55 protocols do not specify culture and assay conditions for *T. paraluiscuniculi*.

The European Pharmacopoeia monographs *Vaccines for veterinary use*, 2.6.12 *Microbiological examination of non-sterile products: microbial enumeration tests* and 2.6.13 *Microbiological examination of non-sterile products: test for specified microorganisms* describe generalised methods for microbiological testing and selective methods of testing for specified microorganisms. The monograph 2.6.13 does not include guidelines for *T. paraluiscuniculi*.

Conclusion

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 are not acceptable.

Primary isolation of *T. paraluiscuniculi* by inoculation of rabbit testicles is recommended. Treponemes harvested from the testicle after 10 days are confirmed by specific testing using dark field microscopy and FA. The harvested treponemes are passaged a second time in rabbit testicles.

Specific testing for *T. paraluiscuniculi* is required by the genus-specific PCR assay targeting the *rpoB* gene.

Fungi

Histoplasma capsulatum* var. *farcinosum

Culture isolation of *Histoplasma capsulatum* var. *farcinosum* is possible on a number of selective media: mycobiotic agar, Sabouraud's dextrose agar medium enriched with 2.5% glycerol, brain-heart infusion agar supplemented with 10% horse blood, and pleuropneumonia-like organism

(PPLO) nutrient agar enriched with 2% dextrose and 2.5% glycerol, pH 7.8. The mycelial form grows slowly (2–8 weeks at 26 °C) and therefore it is recommended to add cycloheximide (0.5 g/litre) and chloramphenicol (0.5 g/litre) to prevent overgrowth.

A generic approach to enrichment and identification of fungal pathogens has been described using BacT/ALERT, BACTEC or BBL MGIT media, followed by specific testing (Pryce et al. 2006). The generic approach would be suitable for isolation of *H. capsulatum* var. *farciminosum*.

Colonies are dry, yellow to dark brown, granular, wrinkled mycelia. Aerial forms occur, but are rare. Microscopically, hyphae from cultured colonies are septate, branched, pleomorphic and stain variable with Gram stain.

As a confirmatory test the yeast form of *H. capsulatum* var. *farciminosum* can be induced by subculturing some of the mycelium into brain-heart infusion agar containing 5% horse blood or by using Pine's medium alone at 35–37 °C. Yeast colonies are flat, raised, wrinkled, white to greyish brown, and pasty in consistency (OIE 2010).

Specific testing is required for detection of *H. capsulatum* var. *farciminosum* as culture for 8 weeks to detect a fungal colony is not a highly reliable technique for detection of contaminants in biological products given the propensity for opportunistic fungal growth on laboratory medium over extended periods. Opportunistic fungal overgrowth is an issue here because the 8 week incubation period required for growth means there is a high propensity for environmental fungal contamination. This is not primarily a risk introduced by having heavily contaminated samples but a risk arising from the long incubation period giving opportunity for other contaminants to grow faster than the slow growing *H. capsulatum* var. *farciminosum*. If a vaccine material is required to be tested for the fungal pathogen *H. capsulatum* var. *farciminosum* then this implies the processing of the material is insufficient to mitigate the risk of contamination with the pathogen. By extension the vaccine material is therefore insufficiently processed to be certain of elimination of other fungi that are even more likely to be present and not sterilised by processing as they are less fastidious in growth requirements and more rapid in growth than *H. capsulatum* var. *farciminosum*.

Detection is possible by experimental inoculation of laboratory animals followed by serological diagnosis using FA, IFA or ELISA.

Specific testing by a nested PCR assay has been developed targeting the fungal rRNA gene (Ueda et al. 2003). PCR and sequencing when combined with generic fungal enrichment and isolation using BacT/ALERT, BACTEC and BBL MGIT media has established this method as a sensitive and specific method for detection of *H. capsulatum* var. *farciminosum* (Pryce et al. 2006).

The generic protocols of 9 CFR 113.26: *Detection of viable bacteria and fungi except in live vaccines* and 9 CFR 113.27: *Detection of extraneous viable bacteria and fungi in live vaccines* do not provide the growth conditions required for *H. capsulatum* var. *farciminosum* and are not suitable for primary isolation of *H. capsulatum* var. *farciminosum*. The 9 CFR guidelines do not require specific testing for *H. capsulatum* var. *farciminosum*.

The European Pharmacopoeia monographs *Vaccines for veterinary use*, 2.6.12 *Microbiological examination of non-sterile products: microbial enumeration tests* and 2.6.13 *Microbiological examination of non-sterile products: test for specified microorganisms* describe generalised methods for extraneous agent testing using methods that are sensitive to the agent being tested, and detection using specific testing methods that are sensitive. The monographs do not specify details of

a culture system or assay for *H. capsulatum* var. *farciminosum* detection, simply that the methods should be sensitive.

Conclusion

The generic protocols of 9 CFR 113.26: *Detection of viable bacteria and fungi except in live vaccines* and 9 CFR 113.27: *Detection of extraneous viable bacteria and fungi in live vaccines* are not acceptable for primary isolation of *H. capsulatum* var. *farciminosum*.

Primary isolation of *H. capsulatum* var. *farciminosum* using BacT/ALERT, BACTEC and BBL MGIT media is recommended. Broths should be incubated for 14 days prior to subculturing onto selective media. Selective media include: mycobiotic agar, Sabouraud's dextrose agar medium enriched with 2.5% glycerol, brain-heart infusion agar supplemented with 10% horse blood, and PPLO nutrient agar enriched with 2% dextrose and 2.5% glycerol, pH 7.8.

Specific testing is required by nested PCR targeting the fungal rRNA gene.

Protozoa

Theileria equi* and *Babesia caballi

Babesia caballi and *Theileria equi* can be cultured *in vitro* in 10% equine RBC in supportive medium supplemented with 40% horse serum and in a microaerophilic environment. Giemsa-stained blood smears are prepared from cultures daily for 7 days (Avarzed et al. 1997; Ikadai et al. 2001). Culture isolation of *T. equi* is more sensitive than for *B. caballi*. *B. caballi* is characterised by paired merozoites connected at one end. *T. equi* is characterised by a tetrad formation of merozoites or 'Maltese cross'. Confirmation of the diagnosis is by FA (OIE 2010).

Molecular diagnosis is recommended for the testing of biological products that do not contain whole blood or organs. Molecular diagnosis by PCR or LAMP assay are the most sensitive and specific testing methods for detection of the agents of equine piroplasmosis (Alhassan et al. 2007). PCR assays have been developed targeting the EMA-2, 16S rRNA and Bc48 genes (Alhassan et al. 2005; Alhassan et al. 2007; Bashiruddin et al. 1999; Nicolaiewsky et al. 2001; Rampersad et al. 2003) and a nested PCR targeting the EMA-1 gene. A systematic study comparing the LAMP assay targeting the 16S rRNA gene with PCR targeting the EMA-2 and 16S rRNA gene had comparable sensitivity and specificity (Alhassan et al. 2007).

The 9 CFR 113.52, 113.53 and 113.55 protocols do not specify culture and assay conditions for *B. caballi* and *T. equi*.

The European Pharmacopoeia general monograph *Vaccines for veterinary use* and 5.2.4 *Cell cultures for the production of veterinary vaccines* describe generalised methods for extraneous agent testing using methods that are sensitive to the agent being tested, and detection using specific testing methods that are sensitive. The monographs do not specify details of a culture system or assay for *B. caballi* and *T. equi* detection, simply that the methods should be sensitive.

Conclusion

Culture isolation of *B. caballi* and *T. equi* in 10% RBC for 7 days followed by detection from Giemsa-stained blood smears is recommended.

Specific testing is required by PCR or LAMP assay.

Trypanosoma evansi

There are no cell culture methods suitable for the primary isolation of *Trypanosoma evansi*; however, *T. evansi* can be isolated in laboratory animals such as mice or rats.

Following isolation in laboratory animals *T. evansi* can be identified as motile parasites in Giemsa-stained or phase-contrast views of wet blood films (OIE 2010).

As there are no suitable culture methods for isolation of *T. evansi* specific testing is required. A nested PCR has been developed for detection of *T. evansi* targeting the nuclear repetitive gene (Aradaib and Majid 2006). This assay is highly sensitive and the detection threshold is reported to be 10 fg of DNA. The sensitivity of an alternative PCR assay targeting a repetitive element is reported to be much lower and comparable to that of cell culture detection (Panyim et al. 1993; Wuyts et al. 1994; Wuyts et al. 1995).

The 9 CFR 113.52, 113.53 and 113.55 protocols do not specify culture and assay conditions for *T. evansi*.

The European Pharmacopoeia general monograph *Vaccines for veterinary use* and 5.2.4 *Cell cultures for the production of veterinary vaccines* describe generalised methods for extraneous agent testing using methods that are sensitive to the agent being tested, and detection using specific testing methods that are sensitive. The monographs do not specify details of a culture system or assay for *T. evansi* detection, simply that the methods should be sensitive.

Conclusion

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 are not acceptable.

Culture isolation of *T. evansi* is not recommended.

Specific testing is required for *T. evansi* by nested PCR targeting the nuclear repetitive gene element.

Rickettsia

Ehrlichia canis

Ehrlichia canis is typically isolated from clinical cases by blood culture; however, this approach is not suitable for extraneous agent testing. *E. canis* can be propagated in transformed canine macrophage/monocyte (DH82) cells once isolated by blood culture; however, primary isolation in DH82 cells has a low success rate (Cheng et al. 2008). Culture isolation is therefore not recommended for extraneous agent testing and specific testing is required.

FA is used on Giemsa-stained infected DH82 monolayers for detection of morula; however, this method is dependent on successful culture isolation.

Specific testing can be performed by nested PCR targeting the 16S rRNA sequence in a 2-stage process. Genus-specific primers are used for the first step and species-specific primers for the second step. The nested PCR assay can detect as little as 20 pg of DNA and specificity of the assay

is achieved in the second step. The PCR assay targeting the 16S rRNA sequence has been adapted to a PCR and chemiluminescent hybridization (CH) assay with a complementary internal 287-bp oligonucleotide probe. The PCR/CH assay improved detection sensitivity by 1000 times relative to PCR detection alone and the detection limit is reported to be 30 pg of DNA (McBride et al. 1996). A Taqman real-time PCR assay has been developed targeting the 16S rRNA sequence and using magnetic capture for enrichment of pathogen rRNA and RT-PCR conversion. The assay is reported to be 100 times more sensitive than PCR for diagnosis of *E. canis* in canine blood samples; however, its sensitivity for the detection of *E. canis* in biologicals has not been assessed (Sirigireddy and Ganta 2005).

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 for extraneous agent testing will not be sufficient for primary isolation of *E. canis*. The 9 CFR guidelines do not include specific testing for *E. canis*.

The European Pharmacopoeia general monograph *Vaccines for veterinary use* and 5.2.4 *Cell cultures for the production of veterinary vaccines* describe generalised methods for extraneous agent testing using cell culture monolayers that are sensitive to the agent being tested, and detection using specific testing methods that are sensitive to the agent. The monographs do not specify details of a culture system or assay for *E. canis* detection, simply that the methods should be sensitive.

Conclusion

Culture isolation of *E. canis* is not recommended.

Specific testing is required for *E. canis* by nested PCR assay targeting the 16S rRNA sequence.

Neorickettsia risticii

Primary isolation of *Neorickettsia risticii* can be performed by co-cultivation of infected blood monocytes. This technique is of limited value to the extraneous agent testing of biological products and is of low sensitivity.

Specific testing for *N. risticii* is required and is possible by FA or nested PCR.

A nested PCR assay for detection of *N. risticii* has been developed targeting the 16S rRNA gene (Barlough et al. 1997). A systematic comparison of the performance of the nested PCR and FA for detection of *N. risticii* indicated the nested PCR was more sensitive (Mott et al. 1997; Mott et al. 2002).

The 9 CFR 113.52, 113.53 and 113.55 protocols do not specify culture and assay conditions for *N. risticii*.

The European Pharmacopoeia general monograph *Vaccines for veterinary use* and 5.2.4 *Cell cultures for the production of veterinary vaccines* describe generalised methods for extraneous agent testing using methods that are sensitive to the agent being tested, and detection using specific testing methods that are sensitive. The monographs do not specify details of a culture system or assay for *N. risticii* detection, simply that the methods should be sensitive.

Conclusion

Culture isolation of *N. risticii* is not recommended.

Specific testing is required for *N. risticii* by nested PCR assay targeting the 16S rRNA gene.

Mycoplasma

Mycoplasma agalactiae

Culture isolation of *Mycoplasma agalactiae* is recommended in modified Hayflick broth and cultures should be maintained for 3 weeks (Cottew and Leach 1969; Hayflick 1969; Masover et al. 1974; Stanbridge et al. 1971).

M. agalactiae is reported to grow moderately well as a pure culture on all mycoplasma media; however, with lower sensitivity due to a high frequency of overgrowth. The sensitivity of isolation in selective heart infusion broth will be too poor for detection of low level contaminants, as would occur in the case of extraneous agent testing (Washburn and Somerson 1979).

Specific testing is required for *M. agalactiae* because of the low rate of success for culture isolation and is possible by PCR or real-time PCR assays.

A real-time PCR assay has been developed for detection of *M. agalactiae* targeting the 16S rRNA gene. The assay has a sensitivity of 100% and specificity of 99% relative to culture, and can detect as little as 10 genomic copies (Cai et al. 2005; Chavez-Gonzalez et al. 1995; Lorusso et al. 2007; McAuliffe et al. 2005; McAuliffe et al. 2006; Tola et al. 1997). Sequence alignments of the 16S rRNA gene from *M. bovis* and *M. agalactiae* show there are only 8 scattered nucleotide differences across the ORF. For assays targeting this ORF, cross-reaction with *M. bovis* could be a problem, but is not an issue in the context of extraneous agent testing. Confirmation of the identity of positives from the assay should be by SNP melt analysis, RFLP, sequencing or using hybridization probes.

The protocol 9 CFR 113.28: *Detection of mycoplasma contamination* requires culture isolation in modified selective heart infusion broth. The 9 CFR 113.28 protocol does not provide the growth conditions required for *M. agalactiae* and is not suitable for primary isolation of *M. agalactiae*. The 9 CFR guidelines do not require specific testing for *M. agalactiae*.

The European Pharmacopoeia monograph 2.6.7 *Mycoplasmas* describes the culture method and indicator cell culture method for identification of mycoplasma. The monograph does not refer specifically to *M. agalactiae* but does provide a generalised protocol for isolation of *Mycoplasma* and for identification of inhibitory conditions for isolation. The generalised culture method described is not sufficient for identification of *M. agalactiae*. The monograph includes guidelines for the use of nucleic acid amplification techniques (NAT) either as complementary tests or in place of culture methods for *Mycoplasma* detection. The monograph does not refer to specific assays but to methods of establishing the suitability of a NAT assay for this purpose. Therefore to meet the requirements of the monograph the validation of the assay must be reported.

Conclusion

The culture method in 9 CFR 113.28 is not acceptable.

Culture isolation of *M. agalactiae* in modified Hayflick broth is recommended. Cultures should be maintained for 3 weeks.

Specific testing for *M. agalactiae* is required by real-time PCR targeting the 16S rRNA gene.

The generalised culture method described in European Pharmacopoeia monograph 2.6.7 *Mycoplasmas* is not acceptable.

Mycoplasma capricolum* subsp. *capripneumoniae

Culture isolation of *Mycoplasma capricolum* subsp. *capripneumoniae* is as described above for *M. agalactiae*. However, *M. capricolum* subsp. *capripneumoniae* is more fastidious and difficult to isolate than *M. agalactiae*.

Culture isolation of *M. capricolum* subsp. *capripneumoniae* is recommended in modified Hayflick broth, and cultures should be maintained for 3 weeks (Cottew and Leach 1969; Hayflick 1969; Masover et al. 1974; Stanbridge et al. 1971).

Specific testing is required for *M. capricolum* subsp. *capripneumoniae* because of the low rate of success for culture isolation. Specific testing is possible by real-time PCR assays.

A specific real-time PCR assay has been developed for the detection of *M. capricolum* subsp. *capripneumoniae* (Persson et al. 1999; Pettersson et al. 1996; Pettersson et al. 1998; Pettersson et al. 2001; Woubit et al. 2004).

The protocol 9 CFR 113.28: *Detection of mycoplasma contamination* requires culture isolation in modified selective heart infusion broth. The 9 CFR 113.28 protocol does not provide the growth conditions required for *M. capricolum* subsp. *capripneumoniae* and is not suitable for primary isolation of *M. capricolum* subsp. *capripneumoniae*. The 9 CFR guidelines do not require specific testing for *M. capricolum* subsp. *capripneumoniae*.

The European Pharmacopoeia monograph 2.6.7 *Mycoplasmas* describes the culture method and indicator cell culture method for identification of mycoplasma. The monograph does not refer specifically to *M. capricolum* subsp. *capripneumoniae* but does provide a generalised protocol for isolation of mycoplasma and for identification of inhibitory conditions for isolation. The generalised culture method described is not sufficient for identification of *M. capricolum* subsp. *capripneumoniae*. The monograph includes guidelines for the use of NAT either as complementary tests or in place of culture methods for mycoplasma detection. The monograph does not refer to specific assays but to methods of establishing the suitability of a NAT assay for this purpose. Therefore to meet the requirements of the monograph the validation of the assay must be reported.

Conclusion

The culture method in 9 CFR 113.28 is not acceptable.

Culture isolation of *M. capricolum* subsp. *capripneumoniae* in modified Hayflick broth is recommended. Cultures should be maintained for 3 weeks.

Specific testing for *M. capricolum* subsp. *capripneumoniae* is required by real-time PCR targeting the 16S rRNA gene.

The generalised culture method described in European Pharmacopoeia monograph 2.6.7 *Mycoplasmas* is not acceptable.

Mycoplasma gallisepticum* and *Mycoplasma synoviae

Avian mycoplasma species, *Mycoplasma gallisepticum* and *Mycoplasma synoviae*, can be isolated on Hayflick or Frey specialised media. Hayflick medium is recommended for *M. synoviae* growth. For isolation of *M. gallisepticum* nicotinamide adenine dinucleotide may be omitted from Frey's medium. *M. gallisepticum* can also be isolated in PPLO with pig serum supplement.

Cultures should be incubated at 35–38 °C under microaerophilic conditions. *Mycoplasma* spp. growth is highly reliant on unidentified components of serum supplements and the efficiency of isolation can vary between batches of serum and batches of yeast extract. It is therefore critical to grow positive reference *Mycoplasma* spp. in parallel. Failure of culture isolation is frequently due to bacterial overgrowth during the extended incubation period required for isolation of mycoplasma. *Mycoplasma* spp. can be detected by their characteristic colony appearance and confirmed by staining using a fluorescent dye that binds nucleic acid. Growth is evident within 3–10 days as turbidity in a broth culture or fried-egg colonies on agar. It is recommended to perform 3 blind culture passages for sensitive detection (OIE 2010).

Specific testing is required because culture isolation can be unreliable. Available assays include FA, ELISA, SAT, HI, PCR-RFLP or real-time PCR.

FA is sensitive and specific for detection of colonies of *Mycoplasma* spp.; however, cross reactivity can occur with *M. imitans* and a PCR-RFLP assay is required for speciation (Kempf 1998). The FA is dependent on successful culture isolation of *Mycoplasma* spp.. The SAT, HA and ELISA tests are useful for diagnosis of clinical disease at the flock level but lack specificity and sensitivity for extraneous agent testing (Kempf 1998).

A generic RT-PCR assay has been developed targeting the 16S rRNA gene of avian mycoplasma to detect *M. gallisepticum*, *M. synoviae* and *M. iowae* (García et al. 1996). Discrimination of *Mycoplasma* spp. is by digoxigenin-labelled probes specific to *M. gallisepticum*, *M. synoviae* and *M. iowae*. The detection threshold for the *M. gallisepticum*, *M. synoviae* and *M. iowae* probes was 70, 50 and 30 CFU respectively.

A generic PCR targeting the 16S/23S ribosomal intergenic spacer region has been adapted to speciate avian mycoplasma by an RFLP analysis (Fan et al. 1995; García et al. 1996; Lauerman et al. 1995; Marois et al. 2002). RAPD and arbitrarily primed PCR (AP-PCR) methods have been developed for detection of avian mycoplasma based on DNA fingerprint patterns (Fan et al. 1995; Sanei et al. 2007). This method was successful for colony purified cultures and did not give interpretable results from mixed preparations. The PCR-RFLP assay is therefore not useful for detection of non-cultivable isolates. A commercial IDEXX PCR-based DNA probe test for *M. gallisepticum* and *M. synoviae* is widely used for flock diagnosis. A systematic study comparing the performance of the IDEXX test kits to culture detected mycoplasma not identified by culture isolation was 100% specific for avian mycoplasma (Salisch et al. 1998).

A real-time SYBR green PCR assay has been developed for the detection of *M. gallisepticum* targeting the lipoprotein gene combined with a melt curve analysis to establish specificity (Carli and Eyigor 2003; Salisch et al. 1998). The detection limit of the assay was 3 CFU.

The protocol 9 CFR 113.28: *Detection of mycoplasma contamination* requires culture isolation in modified selective heart infusion broth. The 9 CFR 113.28 protocol does not provide the growth conditions required for *M. gallisepticum* and *M. synoviae* and is not suitable for primary isolation of

M. gallisepticum and *M. synoviae*. The 9 CFR guidelines do not require specific testing for *M. gallisepticum* and *M. synoviae*.

The recommendation in the European Pharmacopoeia 2.6.7 for isolation of *Mycoplasma* spp. in Hayflick or Frey media is acceptable.

Conclusion

The culture method in 9 CFR 113.28 is not acceptable.

Culture isolation of avian *Mycoplasma* spp. in Hayflick or Frey media is recommended. Cultures should be incubated at 35–38 °C under microaerophilic conditions and 3 blind culture passages are required.

Specific testing is required²³ by generic avian mycoplasma PCR and speciation by RFLP, DNA probe or sequence analysis.

The generalised culture method described in European Pharmacopoeia monograph 2.6.7 *Mycoplasmas* is acceptable.

Mycoplasma hyopneumoniae

Isolation of *Mycoplasma hyopneumoniae* is recommended in Friss medium. *M. hyopneumoniae* is highly fastidious and because it is slow growing, cultures should be maintained for 4–8 weeks to ensure growth is detected. Supplementation with swine serum is required; however, growth rates vary with different batches of serum and growth is sensitive to antibodies against *M. hyopneumoniae* present in the serum. Presence of coinfecting pathogens can inhibit growth of *M. hyopneumoniae* (Thacker 2004).

Specific testing for *M. hyopneumoniae* is recommended because of the high probability of false negatives by culture isolation. Because of the difficulty of primary isolation, failure to isolate *M. hyopneumoniae* cannot be considered to indicate absence of the organism.

Conventional and nested PCR assays have been developed for detection of *M. hyopneumoniae* (Verdin et al. 2000). The nested PCR was reported to have a detection threshold of 1 fg or 1 organism, and the detection threshold was reported to be 400 times lower than conventional PCR. An arbitrary primed PCR method for *M. hyopneumoniae* indicates sequence heterogeneity influences the detection rate from PCR assays (Artiushin and Minion 1996). A FA has been described for culture-isolated *M. hyopneumoniae* using polyclonal porcine antiserum.

The culture protocol described in 9 CFR 113.28: *Detection of Mycoplasma contamination* does not provide the optimal growth conditions for *M. hyopneumoniae* and is not sufficient for isolation of *M. hyopneumoniae*. The 9 CFR guidelines do not include specific testing for *M. hyopneumoniae*.

The European Pharmacopoeia monograph 2.6.7 *Mycoplasmas* describes the culture method and indicator cell culture method for identification of mycoplasma. The monograph does not refer specifically to *M. hyopneumoniae* but does provide a generalised protocol for isolation of *Mycoplasma* spp. and for identification of inhibitory conditions for isolation. The generalised culture method described is not sufficient for identification of *M. hyopneumoniae*. The monograph

²³ Except when generalised culture method described in European Pharmacopoeia monograph 2.6.7 *Mycoplasmas* is used.

includes guidelines for the use of NAT either as complementary tests or in place of culture methods for *Mycoplasma* spp. detection. The monograph does not refer to specific assays but to methods of establishing the suitability of a NAT assay for this purpose. Therefore to meet the requirements of the monograph the validation of the assay must be reported.

Conclusion

The culture method in 9 CFR 113.28 is not acceptable.

Culture isolation of *M. hyopneumoniae* in Friss medium is recommended. Cultures should be maintained for 8 weeks and supplemented with swine serum that has been tested and found to be free of *M. hyopneumoniae*.

Specific testing is required for *M. hyopneumoniae* by nested PCR.

The generalised culture method described in European Pharmacopoeia monograph 2.6.7 *Mycoplasmas* is not acceptable.

***Mycoplasma mycoides* subsp. *mycoides* small colony (SC) type**

Mycoplasma mycoides subsp. *mycoides* SC (*Mmm*SC) is fastidious and difficult to isolate. Primary isolation requires PPLO heart infusion broth/agar supplemented with 10% horse serum and with inhibitors to prevent overgrowth by contaminants. Mycoplasma growth is highly reliant on unidentified components of serum supplements and the efficiency of isolation can vary between batches of serum. It is therefore critical to grow positive reference *Mycoplasma* spp. in parallel. Growth is evident within 3–10 days either as turbidity in broth or can be seen on solid medium as characteristic ‘fried-egg’ colonies. Three blind passages are recommended to ensure sensitive detection. A biochemical assay has been developed to detect *Mmm*SC based on its inability to metabolise maltose which is unique amongst the mycoides cluster (OIE 2010; Williamson et al. 2007).

Specific testing for *Mmm*SC is required in order to address the problems associated with primary isolation. Available assays include FA, growth inhibition or PCR. Assays based on antibody detection have problems with cross-reactivity with other species of mycoplasma. PCR detection has high sensitivity and specificity and is commonly used for *Mmm*SC detection.

PCR assays targeting the 16S rRNA sequence of *Mmm*SC (MYC-PCR) are not sufficiently specific to differentiate *Mmm*SC from the mycoides cluster. Single nucleotide polymorphisms differentiate the sequences of the 16S rRNA gene within the mycoides cluster and therefore a second stage assay is required such as a second stage PCR restriction enzyme analysis (PCR-REA), or PCR denaturing gradient gel electrophoresis (PCR-DGGE). The MSC-PCR selectively targets the *Mmm*SC from the mycoides cluster. A Taqman real-time PCR assay has also been developed targeting the 16S rRNA gene that uses a fluorogenic *Mmm*SC-specific probe. Several studies of the Taqman real-time PCR indicate the assay is highly sensitive and specific for *Mmm*SC and the detection limit was 10³ cfu/ml or 100 fg DNA (Lorenzon et al. 2000; Miles et al. 2006; Miserez et al. 1997).

A 2-stage protocol has been developed for PCR detection of *Mmm*SC as follows: MYC-PCR detects an *AluI* fragment that is conserved across the mycoides cluster, and the second stage combines this PCR with the specific MSC-PCR to give very high sensitivity and specificity for *Mmm*SC detection (Dedieu et al. 1995).

PCR assays directed against the multi-copy insertion elements IS1634 and IS1296 are widely used to identify *MmmSC* with high sensitivity and specificity, and to differentiate strains.

A PCR assay targeting the *lppA* sequence, which was identified using suppression-subtractive hybridization, has poor specificity for *MmmSC* detection when applied to a range of mycoplasma isolates.

The culture protocol described in 9 CFR 113.28: *Detection of mycoplasma contamination* is not sufficient for isolation of *MmmSC*. The 9 CFR guidelines do not include specific testing for *MmmSC*.

The European Pharmacopoeia monograph 2.6.7 *Mycoplasmas* describes the culture method and indicator cell culture method for identification of mycoplasma. The monograph does not refer specifically to *MmmSC* but does provide a generalised protocol for isolation of mycoplasma and for identification of inhibitory conditions for isolation. The generalised culture method described is sufficient for identification of *MmmSC*. The monograph includes guidelines for the use of NAT either as complementary tests or in place of culture methods for mycoplasma detection. The monograph does not refer to specific assays but to methods of establishing the suitability of a NAT assay for this purpose. Therefore to meet the requirements of the monograph the validation of the assay must be reported.

Conclusion

The culture method in 9 CFR 113.28 is not acceptable.

Primary isolation of *MmmSC* in PPLO heart infusion broth/agar supplemented with 10% horse serum and with inhibitors to prevent overgrowth by contaminants is recommended. Cultures should be maintained for 10 days and 3 blind passages are required.

Specific testing for *MmmSC* is required by PCR using either the 2-stage MYC-PCR/MS-PCR or PCR targeting the IS1634 and IS1296 insertion elements. Evidence of greater validation of the Taqman real-time PCR, PCR-REA, and PCR-DGGE assays is required before they could be recommended for this purpose.

The generalised culture method described in European Pharmacopoeia monograph 2.6.7 *Mycoplasmas* is acceptable.

Conclusions

Tables 7–11 provide a summary of the conclusions from review of the published test methods for the reliable and sensitive detection of extraneous agents in vaccines and vaccine raw materials.

For details, refer to the specific chapters.

Table 7: Viruses — mammalian

Viruses - mammalian	Disease (species)	9 CFR Part 113 <i>standard requirements</i>		European Pharmacopoeia <i>Vaccines for veterinary use</i> and 5.2.4 ²⁴ acceptable (Y/N/NA ²⁵)	Specific testing required (Y/N)
		113.47 ²⁶ acceptable (Y/N/NA)	113.52 ²⁷ , 113.53 ²⁸ and 113.55 ²⁹ acceptable (Y/N)		
African horse sickness virus	African horse sickness (equine)	NA	N	NA	N ³⁰
African swine fever virus	African swine fever (porcine)	NA	N	NA	Y
Akabane virus	Akabane (bovine, ovine, caprine)	NA	N	NA	Y
Bluetongue virus	Bluetongue (bovine, ovine, caprine, canine)	N	N	NA	Y

²⁴ *Cell cultures for the production of veterinary vaccines.*

²⁵ The European Pharmacopoeia general monograph *Vaccines for veterinary use* and 5.2.4 *Cell cultures for the production of veterinary vaccines* describe generalised methods for extraneous agent testing using cell culture monolayers that are sensitive to the agent being tested, and detection using specific testing methods that are sensitive to the viral agent. Where the monographs do not specify details of a culture system or assay, case-by-case assessment is required and is therefore not applicable.

²⁶ *Detection of extraneous viruses by the fluorescent antibody technique.*

²⁷ *Requirements for cell lines used for production of biologics.*

²⁸ *Requirements for ingredients of animal origin used for production of biologics.*

²⁹ *Detection of extraneous agents in Master Seed Virus.*

³⁰ Virus is readily detected by CPE following culture isolation using alternative methods.

Viruses - mammalian	Disease (species)	9 CFR Part 113 <i>standard requirements</i>		European Pharmacopoeia <i>Vaccines for veterinary use</i> and 5.2.4 ²⁴ acceptable (Y/N/NA ²⁵)	Specific testing required (Y/N)
		113.47 ²⁶ acceptable (Y/N/NA)	113.52 ²⁷ , 113.53 ²⁸ and 113.55 ²⁹ acceptable (Y/N)		
Border disease virus	Border disease/hairy shaker disease (ovine)	N	N	NA	Y
Bovine adenovirus (subgroup 1 & 2)	Respiratory and enteric disease (bovine)	N	N	NA	Y
Bovine ephemeral fever virus	Bovine ephemeral fever (bovine)	NA	N	NA	Y
Bovine herpesvirus 1	Infectious bovine rhinotracheitis/ infectious pustular vulvovaginitis/ infectious balanoposthitis (bovine)	NA	N	NA	Y
Bovine herpesvirus 2	Pseudo-lumpy skin disease/bovine herpes mammillitis (bovine)	NA	N	NA	Y
Bovine herpesvirus 4	Metritis (bovine)	NA	N	NA	Y
Bovine immunodeficiency virus	Bovine immunodeficiency (bovine)	NA	N	NA	Y
Bovine leukaemia virus	Enzootic bovine leukosis (bovine)	NA	N	NA	Y
Bovine parainfluenza virus 3	Calf pneumonia/shipping fever (bovine)	NA	Y ³¹	NA	Y ³²
Bovine parvovirus	Parvovirus (bovine)	Y ³³	N	NA	Y
Bovine respiratory syncytial virus	Bovine respiratory disease complex (bovine)	Y ³⁴	N	NA	Y

³¹ Provided the recommended sensitive cell lines BEK or BEL are used.

³² There are both cytopathic and non-cytopathic isolates and CPE is unreliable for detection of non-cytopathic isolates.

³³ If FA test performed between 8–16 hours.

³⁴ Provided that FA is performed with a polyclonal antiserum directed against BRSV whole virus antigen.

Viruses - mammalian	Disease (species)	9 CFR Part 113 <i>standard requirements</i>		European Pharmacopoeia <i>Vaccines for veterinary use</i> and 5.2.4 ²⁴ acceptable (Y/N/NA ²⁵)	Specific testing required (Y/N)
		113.47 ²⁶ acceptable (Y/N/NA)	113.52 ²⁷ , 113.53 ²⁸ and 113.55 ²⁹ acceptable (Y/N)		
Bovine viral diarrhoea virus 1 & 2 (bovine pestiviruses)	Bovine viral diarrhoea/mucosal disease (bovine, ovine, porcine)	N	N	NA	Y
Canine adenovirus 1 and 2	Infectious canine hepatitis/kennel cough (canine)	NA	Y ³⁵	NA	Y ³⁶
Canine distemper virus	Canine distemper (canine)	N	N	NA	Y
Canine parvovirus	Parvovirus (canine)	N	N	NA	Y
Caprine and ovine pox virus	Sheep pox/goat pox (caprine, ovine)	NA	N	NA	N ³⁷
Caprine arthritis encephalitis virus and visna/maedi (maedi-visna) virus	Caprine arthritis encephalitis, maedi-visna (caprine, ovine)	NA	N	NA	Y
Classical swine fever virus	Classical swine fever (porcine)	NA	N	NA	Y
Ectromelia virus	Mouse pox (mouse)	NA	N	NA	Y
Epizootic haemorrhagic disease virus	Epizootic hemorrhagic disease (bovine, ovine)	NA	N	NA	Y
Equid herpesvirus 1, 2, 3 and 4 (equine herpes virus 1, 2, 3 and 4)	Equine rhinopneumonitis/equine viral abortion/ keratoconjunctivitis/ equine coital exanthema (equine)	Y ³⁸	N	NA	Y

³⁵ Provided the MDCK or MDCK-SP cell lines are used for isolation.

³⁶ Specific testing is required because of the difficulty of culture detection for CAcV.

³⁷ Virus can be readily identified by alternative methods for culture isolation and CPE detection.

³⁸ Provided that primary isolation is in equine foetal kidney cells or equine fibroblasts.

Viruses - mammalian	Disease (species)	9 CFR Part 113 <i>standard requirements</i>		European Pharmacopoeia <i>Vaccines for veterinary use</i> and 5.2.4 ²⁴ acceptable (Y/N/NA ²⁵)	Specific testing required (Y/N)
		113.47 ²⁶ acceptable (Y/N/NA)	113.52 ²⁷ , 113.53 ²⁸ and 113.55 ²⁹ acceptable (Y/N)		
Equine adenovirus	Respiratory and enteric disease (equine)	NA	N	NA	Y
Equine arteritis virus	Equine viral arteritis (equine)	Y	N	NA	Y
Equine encephalitis viruses (eastern equine encephalitis virus, western equine encephalitis virus, Venezuelan equine encephalitis virus)	Eastern equine encephalitis, Western equine encephalitis, Venezuelan equine encephalitis (equine)	NA	N	NA	Y
Equine infectious anaemia virus	Equine infectious anaemia (equine)	NA	N	NA	Y
Equine influenza virus	Equine influenza (equine)	NA	N	NA	Y
Feline calicivirus	Feline calicivirus (feline)	NA	N	NA	Y
Felid herpesvirus 1 (feline rhinotracheitis virus)	Feline rhinotracheitis (feline)	NA	N	NA	Y
Feline immunodeficiency virus	Feline immunodeficiency (feline)	NA	N	NA	Y
Feline infectious peritonitis virus	Feline infectious peritonitis (feline)	N	N	NA	Y
Feline leukaemia virus	Feline leukaemia (feline)	NA	N	NA	Y
Feline panleukopaemia virus	Feline panleukopaemia (feline)	N	N	NA	Y
Foot-and-mouth disease virus	Foot-and-mouth disease (bovine, caprine, ovine, porcine)	NA	N	NA	Y

Viruses - mammalian	Disease (species)	9 CFR Part 113 <i>standard requirements</i>		European Pharmacopoeia <i>Vaccines for veterinary use</i> and 5.2.4 ²⁴ acceptable (Y/N/NA ²⁵)	Specific testing required (Y/N)
		113.47 ²⁶ acceptable (Y/N/NA)	113.52 ²⁷ , 113.53 ²⁸ and 113.55 ²⁹ acceptable (Y/N)		
Hantaan virus (Korean haemorrhagic fever virus)	Haemorrhagic fever with renal syndrome/Korean haemorrhagic fever (rodents)	NA	N	NA	Y
Horse pox virus	Horse pox (equine)	NA	N	NA	Y
Jaagsiekte sheep retrovirus (ovine pulmonary adenocarcinoma virus, pulmonary adenomatosis virus)	Ovine pulmonary adenomatosis/Ovine pulmonary adenocarcinoma/Jaagsiekte (caprine, ovine)	NA	N	NA	Y
Japanese encephalitis virus and West Nile virus	Japanese encephalitis, West Nile fever (equine)	NA	N	NA	Y
Louping ill virus	Louping ill (ovine)	NA	N	NA	Y
Lumpy skin disease virus	Lumpy skin disease (bovine)	NA	Y ³⁹	NA	N
Lymphocytic choriomeningitis virus (Arenavirus)	Lymphocytic choriomeningitis (rodents)	NA	N	NA	Y
Murine adenovirus	Subclinical infection (rodent)	NA	Y	NA	Y
Orf virus	Contagious pustular dermatitis (caprine, ovine)	NA	N	NA	Y
Ovine adenovirus	Respiratory and enteric disease (ovine)	Y ⁴⁰	Y ⁴¹	NA	Y

³⁹ Provided LT cells or OA3.Ts cells are used.

⁴⁰ Stipulates testing of ovine lines for bovine adenovirus and does not include testing specific to OAdV.

⁴¹ Provided SEK, LT, SK or OFT cells are used.

Viruses - mammalian	Disease (species)	9 CFR Part 113 <i>standard requirements</i>		European Pharmacopoeia <i>Vaccines for veterinary use</i> and 5.2.4 ²⁴ acceptable (Y/N/NA ²⁵)	Specific testing required (Y/N)
		113.47 ²⁶ acceptable (Y/N/NA)	113.52 ²⁷ , 113.53 ²⁸ and 113.55 ²⁹ acceptable (Y/N)		
Peste-des-petits-ruminants virus	Peste-des-petits-ruminants (caprine, ovine)	NA	Y	NA	Y ⁴²
Porcine adenovirus	Respiratory and enteric disease (porcine)	Y	Y ⁴³	NA	Y ⁴⁴
Porcine circovirus 2	Postweaning multisystemic wasting syndrome (porcine)	NA	N	NA	Y
Porcine epidemic diarrhoea virus	Porcine epidemic diarrhoea (porcine)	NA	N	NA	Y
Porcine haemagglutinating encephalomyelitis virus	Vomiting and wasting disease, Coronaviral encephalomyelitis (porcine)	Y	N	NA	Y
Porcine parvovirus	Porcine parvovirus (porcine)	N	N	NA	Y
Porcine reproductive and respiratory syndrome virus	Porcine reproductive and respiratory syndrome (porcine)	NA	N	NA	Y
Porcine respiratory coronavirus	Subclinical respiratory disease (porcine)	N	N	NA	Y
Porcine teschovirus 1 (Polioencephalomyelitis virus)	Teschen disease, Talfan disease, porcine polioencephalomyelitis (porcine)	NA	N	NA	Y
Pseudorabies virus (Aujeszky's disease virus, suid herpesvirus 1)	Aujeszky's disease/Pseudorabies (porcine, canine)	NA	N	NA	Y
Rabbit fibroma virus (Shope fibroma virus)	Shope fibromas (rabbit)	NA	N	NA	Y

⁴² It is difficult to detect the subtle CPE due to the culture isolated virus.

⁴³ Provided culture is in porcine thyroid cells.

⁴⁴ Culture isolation is not sufficiently sensitive alone to detect PAdV.

Viruses - mammalian	Disease (species)	9 CFR Part 113 <i>standard requirements</i>		European Pharmacopoeia <i>Vaccines for veterinary use</i> and 5.2.4 ²⁴ acceptable (Y/N/NA ²⁵)	Specific testing required (Y/N)
		113.47 ²⁶ acceptable (Y/N/NA)	113.52 ²⁷ , 113.53 ²⁸ and 113.55 ²⁹ acceptable (Y/N)		
Rabbit haemorrhagic disease virus (rabbit calicivirus)	Rabbit hemorrhagic disease (rabbit)	NA	N	NA	Y
Rabies virus	Rabies (bovine, canine, caprine, equine, feline, ovine, porcine, rabbit, rodent)	Y ⁴⁵	N	NA	Y
Rift Valley fever virus	Rift Valley fever (bovine, caprine, ovine)	NA	N	NA	N ⁴⁶
Rinderpest virus	Rinderpest (bovine, caprine, ovine)	NA	Y	NA	N
Rotavirus (bovine and porcine)	Diarrhoea (bovine), rotaviral enteritis (porcine)	NA	N	NA	Y
Sendai virus (murine parainfluenza virus 1)	Respiratory tract infection (rodent)	NA	N	NA	Y
Swine influenza virus	Swine influenza (porcine)	NA	N	NA	Y
Swine pox virus	Swine pox (porcine)	NA	N	NA	Y
Swine vesicular disease virus	Swine vesicular disease (porcine)	NA	N	NA	Y
Theiler's murine encephalomyelitis virus	Murine encephalomyelitis (rodent)	NA	N	NA	Y
Transmissible gastroenteritis virus	Transmissible gastroenteritis (porcine)	N	N	NA	Y
Vesicular stomatitis virus	Vesicular stomatitis (bovine, equine, caprine, ovine, porcine)	NA	Y	NA	N

⁴⁵ If combined with primary isolation in Neuro-2a cells.

⁴⁶ Due to the profound cytolytic effect of RVFV in cell culture and readily identifiable CPE.

Table 8: Viruses — avian

Viruses - avian	Disease (species)	9 CFR Part 113 <i>standard requirements</i>			European Pharmacopoeia <i>Vaccines for veterinary use</i> and 2.6.24 ⁴⁷ acceptable (Y/N/NA)	Specific testing required (Y/N)
		113.31 ⁴⁸ acceptable (Y/N/NA)	113.37 ⁴⁹ acceptable (Y/N/NA)	113.52, 113.53 and 113.55 acceptable (Y/N/NA)		
Anatid herpesvirus 1 (duck enteritis virus, duck plague herpesvirus)	Duck viral enteritis/duck plague (ducks, geese, and swans)	NA	NA	N	N	Y
Avian adenoviruses (all viruses in the genus Aviadenovirus and duck adenovirus A (egg drop syndrome virus))	Various diseases/syndromes (avian)	NA	NA	N	N	Y
Avian encephalomyelitis virus	Epidemic tremor (avian)	NA	NA	N	N	Y
Avian influenza virus	Avian influenza (avian)	NA	N	NA	N	Y
Avian leukosis virus	Avian leukosis (avian)	N	NA	NA	N	Y
Avian nephritis virus 1 & 2	Avian nephritis (avian)	NA	NA	N	N	Y
Avian orthoreovirus (avian reovirus)	Infectious viral arthritis/tenosynovitis (avian)	NA	NA	N	N	Y

⁴⁷ Avian viral vaccines: tests for extraneous agents in seed lots.

⁴⁸ Detection of avian lymphoid leukosis.

⁴⁹ Detection of pathogens by the chicken embryo inoculation test.

Viruses - avian	Disease (species)	9 CFR Part 113 <i>standard requirements</i>			European Pharmacopoeia <i>Vaccines for veterinary use</i> and 2.6.24 ⁴⁷ acceptable (Y/N/NA)	Specific testing required (Y/N)
		113.31 ⁴⁸ acceptable (Y/N/NA)	113.37 ⁴⁹ acceptable (Y/N/NA)	113.52, 113.53 and 113.55 acceptable (Y/N/NA)		
Chicken anaemia virus	Chicken infectious anaemia (avian)	NA	NA	N	N	Y
Duck viral hepatitis virus type I	Duck viral hepatitis (ducks)	NA	NA	N	N	Y
Fowl pox virus	Fowl pox (avian)	NA	NA	N	N	Y
Infectious bronchitis virus	Infectious bronchitis (avian)	NA	NA	N	N	Y
Infectious bursal disease virus	Infectious bursal disease (avian)	NA	NA	N	N	Y
Infectious laryngotracheitis virus (Gallid herpesvirus 1)	Infectious laryngotracheitis (avian)	NA	NA	N	N	Y
Marek's disease virus 1 and 2 (Gallid herpesvirus 2 and 3)	Marek's disease (avian)	NA	NA	N	N ⁵⁰	Y
Newcastle disease virus	Newcastle disease (avian)	NA	N	NA	N	Y
Reticuloendotheliosis virus	Reticuloendotheliosis (avian)	NA	NA	N	Y	Y ⁵¹
Turkey rhinotracheitis virus (avian metapneumovirus, avian pneumovirus)	Turkey rhinotracheitis (avian)	NA	NA	N	N	Y

⁵⁰ The methods described by European Pharmacopoeia 2.6.25 for the detection of MDV are also inadequate for extraneous agent testing.

⁵¹ As the method described by European Pharmacopoeia 2.6.24 will not detect non-cytolytic infections.

Table 9: Bacteria — *Brucella* spp.

Bacteria – <i>Brucella</i> spp.	Disease (species)	9 CFR 113.32 <i>Detection of Brucella contamination acceptable (Y/N)</i>	European Pharmacopoeia <i>Vaccines for veterinary use</i> , 2.6.12 ⁵² , 2.6.13 ⁵³ acceptable (Y/N/NA ⁵⁴)	Specific testing required (Y/N)
<i>Brucella abortus</i>	Brucellosis (bovine)	N	N	Y
<i>Brucella canis</i>	Brucellosis (canine)	Y	N	Y
<i>Brucella melitensis</i>	Brucellosis (caprine, ovine)	N	N	Y
<i>Brucella suis</i>	Brucellosis (porcine)	Y	Y	Y

Table 10: Bacteria — *Salmonella* spp.

Bacteria – <i>Salmonella</i> spp.	Disease (species)	9CFR 113.30 <i>Detection of Salmonella contamination. acceptable (Y/N)</i>	European Pharmacopoeia <i>Vaccines for veterinary use</i> , 2.6.24 (Y/N/NA)	Specific testing required (Y/N)
<i>Salmonella</i> Enteritidis	Salmonellosis (avian)	Y	NA	Y
<i>Salmonella</i> Gallinarum	Fowl typhoid (avian)	Y	NA	Y
<i>Salmonella</i> Pullorum	Pullorum disease (avian)	Y	N	Y

⁵² Microbiological examination of non-sterile products: microbial enumeration tests.

⁵³ Microbiological examination of non-sterile products: test for specified microorganisms.

⁵⁴ The European Pharmacopoeia monographs *Vaccines for veterinary use*, 2.6.12 *Microbiological examination of non-sterile products: total viable aerobic count* and 2.6.13 *Microbiological examination of non-sterile products: test for specified microorganisms* describe generalised methods for microbiological testing and selective methods of testing for specified microorganisms. Where the monograph 2.6.13 does not include guidelines for a specific microorganism then case-by-case assessment is required and is therefore not applicable.

Table 11: Bacteria — other, fungi, protozoa and rickettsia

Bacteria – other, fungi, protozoa and rickettsia	Disease (species)	9CFR extraneous agent testing		European Pharmacopoeia <i>Vaccines for veterinary use</i>		Specific testing required (Y/N)
		113.26 ⁵⁵ and 113.27 ⁵⁶ acceptable (Y/N/NA)	113.52, 113.53 and 113.55 acceptable (Y/N/NA)	2.6.12 and 2.6.13 acceptable (Y/N/NA ⁵⁷)	5.2.4 acceptable (Y/N/NA)	
Bacteria- other						
<i>Burkholderia mallei</i>	Glanders (equine)	N	NA	NA	NA	N
<i>Coxiella burnetii</i>	Q fever (bovine)	NA	N	NA	NA	Y
<i>Francisella tularensis</i>	Tularaemia/rabbit fever (rabbit)	NA	N	NA	NA	Y
<i>Leptospira interrogans</i> var. <i>canicola</i>	Leptospirosis (canine)	N	NA	N	NA	Y
<i>Ornithobacterium rhinotracheale</i>	Respiratory disease (avian)	N	NA	N	NA	Y
<i>Taylorella equigenitalis</i>	Contagious equine metritis (equine)	N	NA	NA	NA	Y
<i>Treponema paraluis-cuniculi</i>	Treponematosis (rabbit)	NA	N	NA	NA	Y
Fungi						
<i>Histoplasma capsulatum</i> var. <i>farciminosum</i>	Epizootic lymphangitis (equine)	N	NA	NA	NA	Y
Protozoa						

⁵⁵ Detection of viable bacteria and fungi except in live vaccine.

⁵⁶ Detection of extraneous viable bacteria and fungi in live vaccines.

⁵⁷ The European Pharmacopoeia monographs *Vaccines for veterinary use*, 2.6.12 *Microbiological examination of non-sterile products: total viable aerobic count* and 2.6.13 *Microbiological examination of non-sterile products: test for specified microorganisms* describe generalised methods for microbiological testing and selective methods of testing for specified microorganisms. Where the monograph 2.6.13 does not include guidelines for a specific microorganism then case-by-case assessment is required and is therefore not applicable.

Bacteria – other, fungi, protozoa and rickettsia	Disease (species)	9CFR extraneous agent testing		European Pharmacopoeia <i>Vaccines for veterinary use</i>		Specific testing required (Y/N)
		113.26 ⁵⁵ and 113.27 ⁵⁶ acceptable (Y/N/NA)	113.52, 113.53 and 113.55 acceptable (Y/N/NA)	2.6.12 and 2.6.13 acceptable (Y/N/NA ⁵⁷)	5.2.4 acceptable (Y/N/NA)	
<i>Babesia caballi</i>	Equine piroplasmosis/babesiosis (equine)	NA	NA	NA	NA	Y
<i>Theileria equi</i>	Equine piroplasmosis/babesiosis (equine)	NA	NA	NA	NA	Y
<i>Trypanosoma evansi</i>	Surra (equine)	NA	NA	NA	NA	Y
Rickettsia						
<i>Ehrlichia canis</i>	Tropical canine pancytopenia (canine)	NA	NA	NA	NA	Y
<i>Neorickettsia risticii</i>	Potomac fever/equine monocytic Ehrlichiosis/equine Ehrlichial colitis (equine)	NA	NA	NA	NA	Y

Table 12: Mycoplasma

Mycoplasma	Disease (species)	9CFR 113.28: <i>Detection of mycoplasma contamination</i> acceptable (Y/N)	European Pharmacopoeia monograph 2.6.7 <i>Mycoplasmas</i> acceptable (Y/N)	Specific testing required (Y/N)
<i>Mycoplasma agalactiae</i>	Contagious agalactia (caprine, ovine)	N	N	Y
<i>Mycoplasma capricolum</i> subsp. <i>capripneumoniae</i>	Contagious caprine pleuropneumonia (caprine)	N	N	Y
<i>Mycoplasma gallisepticum</i>	Chronic respiratory disease (avian)	N	Y	Y ⁵⁸
<i>Mycoplasma hyopneumoniae</i>	Enzootic pneumonia (porcine)	N	N	Y

⁵⁸ Except when generalised culture method described in European Pharmacopoeia monograph 2.6.7 *Mycoplasmas* is used.

Mycoplasma	Disease (species)	9CFR 113.28: <i>Detection of mycoplasma contamination</i> acceptable (Y/N)	European Pharmacopoeia monograph 2.6.7 <i>Mycoplasmas</i> acceptable (Y/N)	Specific testing required (Y/N)
<i>Mycoplasma mycoides</i> subsp. <i>mycoides</i> SC	Contagious bovine pleuropneumonia (bovine)	N	Y	Y ⁵⁸
<i>Mycoplasma synoviae</i>	Infectious synovitis/infectious arthritis (avian)	N	Y	Y ⁵⁸

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Appendix 1 — Updated Annexes 1–3

ANNEX 1 — Exotic animal disease pathogens of major economic and social concern

Animal species ⁵⁹	Pathogen	Disease	OIE notifiable (Y/N)	Exotic (Y/N)	More virulent exotic strains of an endemic pathogen (Y/N)	Endemic (Y/N)
Avian	Avian influenza virus ⁶⁰	Highly pathogenic notifiable avian influenza (HPNAI) and low pathogenicity notifiable avian influenza (LPNAI)	Y	N	Y	Y ⁶¹
	Newcastle disease virus ⁶²	Virulent Newcastle disease	Y	N	Y	Y ⁶³
Bovine	Foot-and-mouth disease virus	Foot-and-mouth disease	Y	Y	N	N
	Rinderpest virus	Rinderpest	Y	Y	N	N
Caprine and ovine	Caprine/ovine pox virus	Sheep pox/goat pox	Y	Y	N	N
	Foot-and-mouth disease virus	Foot-and-mouth disease	Y	Y	N	N
	Jaagsiekte sheep retrovirus (ovine pulmonary adenocarcinoma virus, pulmonary adenomatosis)	Ovine pulmonary adenomatosis/ovine pulmonary adenocarcinoma/	N	Y	N	N

⁵⁹ And other species as determined by AQIS on application.

⁶⁰ Influenza A virus of the H5 or H7 subtypes or any AI virus with an intravenous pathogenicity index (IVPI) greater than 1.2.

⁶¹ Not present in commercial poultry flocks.

⁶² That meets one of the criteria for virulence described in the OIE Terrestrial Animal Health Code.

⁶³ Less virulent strains are present in commercial poultry flocks.

Animal species⁵⁹	Pathogen	Disease	OIE notifiable (Y/N)	Exotic (Y/N)	More virulent exotic strains of an endemic pathogen (Y/N)	Endemic (Y/N)
	virus)	Jaagsiekte				
	Peste-des-petits-ruminants virus	Peste-des-petits-ruminants	Y	Y	N	N
	Rinderpest virus	Rinderpest	Y	Y	N	N
Equine	African horse sickness virus	African horse sickness	Y	Y	N	N
Porcine	African swine fever virus	African swine fever	Y	Y	N	N
	Classical swine fever virus	Classical swine fever	Y	Y	N	N
	Foot-and-mouth disease virus	Foot-and-mouth disease	Y	Y	N	N
	Swine vesicular disease virus	Swine vesicular disease	Y	Y	N	N

ANNEX 2 — Exotic animal transmissible spongiform encephalopathies (TSE) of major economic and social concern

(Relatively low infectivity but extremely high resistance to normal inactivation processes)

Animal species	Pathogen	Disease	OIE notifiable (Y/N)	Exotic (Y/N)	More virulent exotic strains of an endemic pathogen (Y/N)	Endemic (Y/N)
Bovine	Bovine spongiform encephalopathy (BSE) agent (prion)	Bovine spongiform encephalopathy (BSE)	Y	Y	N	N
Cervids (including deer, elk and moose)	Chronic wasting disease (CWD) agent (prion)	Chronic wasting disease (CWD)	N	Y	N	N
Mink	Transmissible mink encephalopathy (TME) agent (prion)	Transmissible mink encephalopathy (TME)	N	Y	N	N
Ovine	Scrapie agent (prion)	Scrapie	Y	Y	N	N
Species⁶⁴ — other	Other agents of transmissible spongiform encephalopathies as determined by AQIS based on origin and end use	Other transmissible spongiform encephalopathies	N	Y	N	N

⁶⁴ As determined by AQIS on application.

ANNEX 3 — Other animal disease pathogens of economic and social concern

These pathogens are either exotic to Australia, exotic strains of endemic pathogens or potential contaminants of concern.

Animal species	Pathogen	Disease	OIE notifiable (Y/N)	Exotic (Y/N)	More virulent exotic strains of an endemic pathogen (Y/N)	Endemic (Y/N)
Avian — chickens and turkeys	Avian adenovirus (all viruses in the genus Aviadenovirus)	Various diseases	N	N	Y	Y
	Avian encephalomyelitis virus	Epidemic tremor	N	N	N	Y
	Avian leukosis virus	Avian leukosis	N	N	N	Y
	Avian nephritis virus 1 and 2	Avian nephritis	N	N	N	Y
	Avian orthoreovirus (avian reovirus)	Infectious viral arthritis/tenosynovitis	N	N	N	Y
	Chicken anaemia virus	Chicken infectious anaemia	N	N	N	Y
	Duck adenovirus A (egg drop syndrome virus)	Egg drop syndrome (in chickens)	N	N	N	Y
	Fowlpox virus	Fowlpox	N	N	N	Y
	Infectious bronchitis virus	Infectious bronchitis	Y	N	Y	Y
	Infectious bursal disease virus	Infectious bursal disease (Gumboro disease)	Y	N	Y	Y

Animal species	Pathogen	Disease	OIE notifiable (Y/N)	Exotic (Y/N)	More virulent exotic strains of an endemic pathogen (Y/N)	Endemic (Y/N)
	Infectious laryngotracheitis virus (gallid herpesvirus 1)	Infectious laryngotracheitis	Y	N	N	Y
	Marek's disease virus 1 and 2 (gallid herpesvirus 2 and 3)	Marek's disease	Y	N	N	Y
	<i>Mycoplasma gallisepticum</i>	Chronic respiratory disease	Y	N	N	Y
	<i>Mycoplasma synoviae</i>	Infectious synovitis/infectious arthritis	Y	N	N	Y
	<i>Ornithobacterium rhinotracheale</i>	Respiratory disease	N	Y	N	N
	Reticuloendotheliosis virus	Reticuloendotheliosis	N	N	N	Y
	<i>Salmonella</i> Enteritidis	Salmonellosis	N	N	Y	Y
	<i>Salmonella</i> Gallinarum	Fowl typhoid	Y	Y	N	N
	<i>Salmonella</i> Pullorum	Pullorum disease	Y	N	N	Y ⁶⁵
	Turkey rhinotracheitis virus (avian metapneumovirus, avian pneumovirus)	Turkey rhinotracheitis	Y	Y	N	N

⁶⁵ Australian commercial poultry are considered to be free of *S. Pullorum*.

Animal species	Pathogen	Disease	OIE notifiable (Y/N)	Exotic (Y/N)	More virulent exotic strains of an endemic pathogen (Y/N)	Endemic (Y/N)
Avian — ducks and geese, and swans	Anatid herpesvirus 1 (duck enteritis virus, duck plague herpesvirus)	Duck viral enteritis/duck plague	N	Y	N	N
	Avian adenovirus (all viruses in the genus Aviadenovirus)	Various diseases	N	N	Y	Y
	Avian encephalomyelitis virus	Epidemic tremor	N	N	N	Y
	Avian leukosis virus	Avian leukosis	N	N	N	Y
	Avian nephritis virus 1 and 2	Avian nephritis	N	N	N	Y
	Avian orthoreovirus (avian reovirus)	Infectious viral arthritis/tenosynovitis	N	N	N	Y
	Chicken anaemia virus	Chicken infectious anaemia	N	N	N	Y
	Duck adenovirus A (egg drop syndrome virus)	Egg drop syndrome (in chickens)	N	N	N	Y
	Duck viral hepatitis virus	Duck viral hepatitis	Y	Y	N	N
	Fowlpox virus	Fowlpox	N	N	N	Y
	Infectious bronchitis virus	Infectious bronchitis	Y	N	Y	Y
	Infectious bursal disease virus	Infectious bursal disease (Gumboro disease)	Y	N	Y	Y

Animal species	Pathogen	Disease	OIE notifiable (Y/N)	Exotic (Y/N)	More virulent exotic strains of an endemic pathogen (Y/N)	Endemic (Y/N)
	Infectious laryngotracheitis virus (gallid herpesvirus 1)	Infectious laryngotracheitis	Y	N	N	Y
	Marek's disease virus 1 and 2 (gallid herpesvirus 2 and 3)	Marek's disease	Y	N	N	Y
	<i>Mycoplasma gallisepticum</i>	Chronic respiratory disease	Y	N	N	Y
	<i>Mycoplasma synoviae</i>	Infectious synovitis/infectious arthritis	Y	N	N	Y
	<i>Ornithobacterium rhinotracheale</i>	Respiratory disease	N	Y	N	N
	Reticuloendotheliosis virus	Reticuloendotheliosis	N	N	N	Y
	<i>Salmonella</i> Enteritidis	Salmonellosis	N	N	Y	Y
	<i>Salmonella</i> Gallinarum	Fowl typhoid	Y	Y	N	N
	<i>Salmonella</i> Pullorum	Pullorum disease	Y	N	N	Y ⁶⁶
	Turkey rhinotracheitis virus (avian metapneumovirus, avian pneumovirus)	Turkey rhinotracheitis	Y	Y	N	N
Bovine	Akabane virus	Akabane	N	N	N	Y

⁶⁶ Australian commercial poultry are considered to be free of *S. Pullorum*.

Animal species	Pathogen	Disease	OIE notifiable (Y/N)	Exotic (Y/N)	More virulent exotic strains of an endemic pathogen (Y/N)	Endemic (Y/N)
	Bluetongue virus	Bluetongue	Y	N	Y	Y
	Bovine adenovirus (subgroups 1 & 2)	Respiratory and enteric disease	N	N	N	Y
	Bovine ephemeral fever virus	Bovine ephemeral fever	N	N	N	Y
	Bovine herpesvirus 1	Infectious bovine rhinotracheitis/ infectious pustular vulvovaginitis/ infectious balanoposthitis	Y	N	Y	Y
	Bovine herpesvirus 2	Pseudo-lumpy skin disease, bovine herpes mammillitis	N	N	N	Y
	Bovine herpesvirus 4	Unclear	N	Y	N	N
	Bovine immunodeficiency virus	Bovine immunodeficiency	N	N	N	Y
	Bovine leukaemia virus	Enzootic bovine leukosis	Y	N	N	Y
	Bovine parainfluenza virus 3	Calf pneumonia/shipping fever	N	N	N	Y
	Bovine parvovirus	Bovine parvovirus	N	N	N	Y
	Bovine respiratory syncytial virus	Bovine respiratory disease complex	N	Y	N	N
	Bovine rotavirus	Diarrhoea	N	N	N	Y
	Bovine viral diarrhoea virus 1 & 2 (bovine pestiviruses)	Bovine viral diarrhoea/mucosal disease	Y	N	Y	Y

Animal species	Pathogen	Disease	OIE notifiable (Y/N)	Exotic (Y/N)	More virulent exotic strains of an endemic pathogen (Y/N)	Endemic (Y/N)
	<i>Brucella abortus</i>	Brucellosis	Y	Y	N	N
	<i>Coxiella burnetii</i>	Q fever	Y	N	N	Y
	Epizootic hemorrhagic disease virus (EHDV)	Epizootic hemorrhagic disease (EHD)	Y	N	Y	Y
	Lumpy skin disease virus	Lumpy skin disease	Y	Y	N	N
	<i>Mycoplasma mycoides</i> subsp. <i>mycoides</i> small colony (SC) type	Contagious bovine pleuropneumonia	Y	Y	N	N
	Rabies virus	Rabies	Y	Y	N	N
	Rift Valley fever virus	Rift Valley fever	Y	Y	N	N
	Vesicular stomatitis virus	Vesicular stomatitis	Y	Y	N	N
Canine	Bluetongue virus	Bluetongue	Y	N	Y	Y
	<i>Brucella canis</i>	Brucellosis	N	Y	N	N
	Canine adenovirus 1 and 2	Infectious canine hepatitis/kennel cough	N	N	N	Y
	Canine distemper virus	Canine distemper	N	N	N	Y
	Canine parvovirus	Canine parvovirus	N	N	N	Y
	<i>Ehrlichia canis</i>	Tropical canine pancytopenia	N	Y	N	N
	<i>Leptospira interrogans</i> var. <i>canicola</i>	Leptospirosis	Y	Y	N	N

Animal species	Pathogen	Disease	OIE notifiable (Y/N)	Exotic (Y/N)	More virulent exotic strains of an endemic pathogen (Y/N)	Endemic (Y/N)
	Pseudorabies virus (suid herpesvirus 1, Aujeszky's disease virus)	Aujeszky's disease (pseudorabies)	Y	Y	N	N
	Rabies virus	Rabies	Y	Y	N	N
Caprine	Akabane virus	Akabane	N	N	N	Y
	Bluetongue virus	Bluetongue	Y	N	Y	Y
	<i>Brucella melitensis</i>	Brucellosis	Y	Y	N	N
	Caprine arthritis encephalitis virus	Caprine arthritis encephalitis	Y	N	N	Y
	<i>Mycoplasma agalactiae</i>	Contagious agalactia	Y	N	Y	Y
	<i>Mycoplasma capricolum</i> subsp. <i>capripneumoniae</i>	Contagious caprine pleuropneumonia	Y	Y	N	N
	Orf virus	Contagious pustular dermatitis	N	N	N	Y
	Ovine adenovirus	Respiratory and enteric disease	N	N	N	Y
	Rabies virus	Rabies	Y	Y	N	N
	Rift Valley fever virus	Rift Valley fever	Y	Y	N	N
	Vesicular stomatitis virus	Vesicular stomatitis	Y	Y	N	N
	Visna/maedi (Maedi-visna) virus	Maedi-visna	Y	Y	N	N
Equine	<i>Babesia caballi</i>	Equine piroplasmosis /babesiosis	Y	Y	N	N

Animal species	Pathogen	Disease	OIE notifiable (Y/N)	Exotic (Y/N)	More virulent exotic strains of an endemic pathogen (Y/N)	Endemic (Y/N)
	<i>Burkholderia mallei</i>	Glanders	Y	Y	N	N
	Equid herpesvirus 1, 2, 3 & 4	Equine viral abortion (EHV-1), equine coital exanthema (EHV-3), equine rhinopneumonitis (EHV-4)	Y	N	Y	Y
	Equine adenovirus	Respiratory and enteric disease	N	N	N	Y
	Equine arteritis virus	Equine viral arteritis	Y	N	Y	Y
	Equine encephalitis viruses (eastern equine encephalitis virus, western equine encephalitis virus, Venezuelan equine encephalitis virus)	Eastern equine encephalitis (EEE), western equine encephalitis (WEE), Venezuelan equine encephalitis (VEE)	Y	Y	N	N
	Equine infectious anaemia virus	Equine infectious anaemia	Y	N	N	Y
	Equine influenza virus	Equine influenza	Y	Y	N	N
	<i>Histoplasma capsulatum</i> var. <i>farcinosum</i>	Epizootic lymphangitis	N	Y	N	N
	Horse pox virus (vaccinia virus)	Horse pox	N	Y	N	N
	Japanese encephalitis virus	Japanese encephalitis (JE)	Y	Y	N	N

Animal species	Pathogen	Disease	OIE notifiable (Y/N)	Exotic (Y/N)	More virulent exotic strains of an endemic pathogen (Y/N)	Endemic (Y/N)
	<i>Neorickettsia risticii</i>	Potomac fever/equine monocytic ehrlichiosis/equine ehrlichial Colitis	N	Y	N	N
	Rabies virus	Rabies	Y	Y	N	N
	<i>Taylorella equigenitalis</i>	Contagious equine metritis	Y	Y	N	N
	<i>Theileria equi</i>	Equine piroplasmosis /babesiosis	Y	Y	N	N
	<i>Trypanosoma evansi</i>	Surra	Y	Y	N	N
	Vesicular stomatitis virus	Vesicular stomatitis	Y	Y	N	N
	West Nile virus	West Nile fever	Y	Y	N	N
Feline	Felid herpesvirus 1 (feline rhinotracheitis virus)	Feline rhinotracheitis	N	N	N	Y
	Feline calicivirus	Feline calicivirus	N	N	N	Y
	Feline immunodeficiency virus	Feline immunodeficiency	N	N	N	Y
	Feline infectious peritonitis virus	Feline infectious peritonitis	N	N	N	Y
	Feline leukaemia virus	Feline leukaemia	N	N	N	Y
	Feline panleukopaenia virus	Feline panleukopaenia	N	N	N	Y
	Rabies virus	Rabies	Y	Y	N	N
Ovine	Akabane virus	Akabane	N	N	N	Y

Animal species	Pathogen	Disease	OIE notifiable (Y/N)	Exotic (Y/N)	More virulent exotic strains of an endemic pathogen (Y/N)	Endemic (Y/N)
	Bluetongue virus	Bluetongue	Y	N	Y	Y
	Border disease virus	Border disease (hairy shaker disease)	N	N	N	Y
	Bovine adenovirus (subgroups 1 & 2)	Respiratory and enteric disease	N	N	N	Y
	Bovine viral diarrhoea virus 1 & 2 (bovine pestiviruses)	Bovine viral diarrhoea/mucosal disease	Y	N	Y	Y
	<i>Brucella melitensis</i>	Brucellosis	Y	Y	N	N
	Caprine arthritis encephalitis virus	Caprine arthritis encephalitis	Y	N	N	Y
	Epizootic hemorrhagic disease virus (EHDV)	Epizootic hemorrhagic disease (EHD) (clinical disease rarely seen in sheep)	Y	N	Y	Y
	Louping ill virus	Louping ill	N	Y	N	N
	<i>Mycoplasma agalactiae</i>	Contagious agalactia	Y	N	Y	Y
	<i>Mycoplasma capricolum</i> subsp. <i>capripneumoniae</i>	Contagious caprine pleuropneumonia	Y	Y	N	N
	Orf virus	Contagious pustular dermatitis	N	N	N	Y
	Ovine adenovirus	Respiratory and enteric disease	N	N	N	Y
	Rabies virus	Rabies	Y	Y	N	N
	Rift Valley fever virus	Rift Valley fever	Y	Y	N	N

Animal species	Pathogen	Disease	OIE notifiable (Y/N)	Exotic (Y/N)	More virulent exotic strains of an endemic pathogen (Y/N)	Endemic (Y/N)
	Vesicular stomatitis virus	Vesicular stomatitis	Y	Y	N	N
	Visna/maedi (Maedi-visna) virus	Maedi-visna	Y	Y	N	N
Porcine	Bovine viral diarrhoea virus 1 & 2 (bovine pestiviruses)	Clinical signs and pathological lesions indistinguishable from those observed in chronic classical swine fever	Y	N	Y	Y
	<i>Brucella suis</i>	Brucellosis	Y	N	N	Y
	<i>Mycoplasma hyopneumoniae</i>	Enzootic pneumonia	N	N	N	Y
	Porcine adenovirus	Respiratory and enteric disease	N	N	N	Y
	Porcine circovirus 2	Postweaning multisystemic wasting syndrome	N	N	Y	Y
	Porcine epidemic diarrhoea virus	Porcine epidemic diarrhoea	N	Y	N	N
	Porcine haemagglutinating encephalomyelitis virus	Vomiting and wasting disease, Coronaviral encephalomyelitis	N	N	N	Y
	Porcine parvovirus	Porcine parvovirus	N	N	N	Y
	Porcine reproductive and respiratory syndrome virus	Porcine reproductive and respiratory syndrome	Y	Y	N	N

Animal species	Pathogen	Disease	OIE notifiable (Y/N)	Exotic (Y/N)	More virulent exotic strains of an endemic pathogen (Y/N)	Endemic (Y/N)
	Porcine respiratory coronavirus	Subclinical respiratory disease	N	Y	N	N
	Porcine rotavirus	Rotaviral enteritis	N	N	N	Y
	Porcine teschovirus 1 (polioencephalomyelitis virus, porcine enterovirus)	Teschen disease, Talfan disease, porcine polioencephalomyelitis	N	N	Y	Y
	Pseudorabies virus (suid herpesvirus 1, Aujeszky's disease virus)	Pseudorabies (Aujeszky's disease)	Y	Y	N	N
	Rabies virus	Rabies	Y	Y	N	N
	Swine influenza virus	Swine influenza	N	N	Y ⁶⁷	Y
	Swine pox virus	Swine pox	N	N	N	Y
	Transmissible gastroenteritis virus	Transmissible gastroenteritis	Y	Y	N	N
	Vesicular stomatitis virus	Vesicular stomatitis	Y	Y	N	N
Rabbit	<i>Francisella tularensis</i>	Tularaemia/rabbit fever	Y	Y	N	N
	Rabbit haemorrhagic disease virus (rabbit calicivirus)	Rabbit hemorrhagic disease	Y	N	N	Y ⁶⁸

⁶⁷ The most common subtypes currently found in swine are H1N1, H1N2 and H3N2.

⁶⁸ Biological control agent for rabbits. Contamination with less virulent strains has the potential to undermine the effectiveness of the introduced strain.

Animal species	Pathogen	Disease	OIE notifiable (Y/N)	Exotic (Y/N)	More virulent exotic strains of an endemic pathogen (Y/N)	Endemic (Y/N)
	Rabbit fibroma virus (Shope fibroma virus)	Shope fibromas	N	Y ⁶⁹	N	N
	Rabies virus	Rabies	Y	Y	N	N
	<i>Treponema paraluis-cuniculi</i>	Treponematosi	N	Y	N	N
Rodents — all	Hantaan virus (Korean haemorrhagic fever virus)	Haemorrhagic fever with renal syndrome/Korean haemorrhagic fever	N	Y	N	N
	Lymphocytic choriomeningitis virus (Arenavirus)	Lymphocytic choriomeningitis	N	N	Y	Y
	Murine adenovirus	Subclinical infection	N	N	Y ⁷⁰	Y
	Rabies virus	Rabies	Y	Y	N	N
	Sendai virus (murine parainfluenza virus 1)	Respiratory tract infection	N	N	N	Y
	Theiler's murine encephalomyelitis virus	Murine encephalomyelitis	N	N	N	Y
Rodents — mice	Ectromelia virus	Mouse pox	N	N	N	Y
Species⁷¹ — other						

⁶⁹ Confers immunity to myxomatosis. Escape of the virus into wild rabbits could undermine the effectiveness of myxoma virus as a biological control agent.

⁷⁰ Serological evidence of MAV2 in south-eastern Australia but no evidence of MAV1.

⁷¹ As determined by AQIS on application.