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

Second edition
March 2013
Acknowledgement
DAFF acknowledges Dr Robert Heard and Dr Michelle Peters as the primary authors of this document. It is appropriate to acknowledge and thank Professor Glenn Browning for peer reviewing the technical information in this review.
# Table of contents

<table>
<thead>
<tr>
<th>Acronyms and abbreviations</th>
<th>vi</th>
</tr>
</thead>
<tbody>
<tr>
<td>Summary</td>
<td>xiv</td>
</tr>
<tr>
<td>Introduction</td>
<td>1</td>
</tr>
<tr>
<td>Retroviruses</td>
<td>3</td>
</tr>
<tr>
<td>Scope</td>
<td>5</td>
</tr>
<tr>
<td>Current requirements</td>
<td>6</td>
</tr>
<tr>
<td>Review of Annex 1 and 3 pathogens</td>
<td>7</td>
</tr>
<tr>
<td>Viruses — mammalian</td>
<td></td>
</tr>
<tr>
<td>African horse sickness virus</td>
<td>7</td>
</tr>
<tr>
<td>African swine fever virus</td>
<td>8</td>
</tr>
<tr>
<td>Bluetongue virus</td>
<td>8</td>
</tr>
<tr>
<td>Bovine herpesvirus 1</td>
<td>10</td>
</tr>
<tr>
<td>Bovine herpesvirus 4</td>
<td>11</td>
</tr>
<tr>
<td>Bovine respiratory syncytial virus</td>
<td>13</td>
</tr>
<tr>
<td>Bovine viral diarrhoea virus 1 and 2</td>
<td>14</td>
</tr>
<tr>
<td>Caprine and ovine pox virus</td>
<td>17</td>
</tr>
<tr>
<td>Classical swine fever virus</td>
<td>19</td>
</tr>
<tr>
<td>Epizootic haemorrhagic disease virus</td>
<td>20</td>
</tr>
<tr>
<td>Equid herpesvirus 1, 2, 3 and 4</td>
<td>21</td>
</tr>
<tr>
<td>Equine arteritis virus</td>
<td>22</td>
</tr>
<tr>
<td>Equine encephalitis viruses</td>
<td>23</td>
</tr>
<tr>
<td>Equine influenza virus</td>
<td>25</td>
</tr>
<tr>
<td>Foot-and-mouth disease virus</td>
<td>26</td>
</tr>
<tr>
<td>Hantaan virus</td>
<td>29</td>
</tr>
<tr>
<td>Horse pox virus</td>
<td>31</td>
</tr>
<tr>
<td>Jaagsieke sheep retrovirus</td>
<td>31</td>
</tr>
<tr>
<td>Japanese encephalitis virus and West Nile virus</td>
<td>32</td>
</tr>
<tr>
<td>Louping ill virus</td>
<td>34</td>
</tr>
<tr>
<td>Lumpy skin disease virus</td>
<td>35</td>
</tr>
<tr>
<td>Lymphocytic choriomeningitis virus</td>
<td>36</td>
</tr>
<tr>
<td>Murine adenovirus</td>
<td>37</td>
</tr>
<tr>
<td>Peste-des-petits-ruminants virus</td>
<td>38</td>
</tr>
<tr>
<td>Porcine circovirus 2</td>
<td>39</td>
</tr>
<tr>
<td>Porcine epidemic diarrhoea virus</td>
<td>40</td>
</tr>
<tr>
<td>Porcine reproductive and respiratory syndrome virus</td>
<td>41</td>
</tr>
<tr>
<td>Porcine respiratory coronavirus</td>
<td>42</td>
</tr>
<tr>
<td>Porcine teschovirus 1</td>
<td>42</td>
</tr>
<tr>
<td>Pseudorabies virus</td>
<td>43</td>
</tr>
<tr>
<td>Rabbit fibroma virus</td>
<td>44</td>
</tr>
<tr>
<td>Rabbit haemorrhagic disease virus</td>
<td>45</td>
</tr>
<tr>
<td>Rabies virus</td>
<td>47</td>
</tr>
<tr>
<td>Rift Valley fever virus</td>
<td>48</td>
</tr>
<tr>
<td>Rinderpest virus</td>
<td>49</td>
</tr>
<tr>
<td>Swine influenza virus</td>
<td>49</td>
</tr>
<tr>
<td>Swine vesicular disease virus</td>
<td>51</td>
</tr>
<tr>
<td>Transmissible gastroenteritis virus</td>
<td>52</td>
</tr>
<tr>
<td>Vesicular stomatitis virus</td>
<td>53</td>
</tr>
<tr>
<td>Visna/maedi virus</td>
<td>54</td>
</tr>
</tbody>
</table>
Viruses — avian .......................................................................................................................... 55
  Anatid herpesvirus 1 .................................................................................................................. 55
  Aviadenoviruses (all viruses in the genus) .................................................................................. 56
  Avian influenza virus .................................................................................................................. 57
  Duck viral hepatitis virus 1 ......................................................................................................... 58
  Infectious bronchitis virus .......................................................................................................... 60
  Infectious bursal disease virus ................................................................................................... 61
  Newcastle disease virus .............................................................................................................. 63
  Turkey rhinotracheitis virus ...................................................................................................... 64
Bacteria — Brucella spp. .............................................................................................................. 65
  Brucella abortus .......................................................................................................................... 65
  Brucella canis .............................................................................................................................. 66
  Brucella melitensis ...................................................................................................................... 67
Bacteria — Salmonella spp. .......................................................................................................... 68
  Salmonella Enteritidis, Salmonella Gallinarum and Salmonella Pullorum ................................ 68
Bacteria — other .......................................................................................................................... 69
  Burkholderia mallei .................................................................................................................... 69
  Francisella tularensis .................................................................................................................. 70
  Leptospira interrogans var. canicola ........................................................................................... 72
  Ornithobacterium rhinotracheale .............................................................................................. 72
  Taylorella equigenitalis .............................................................................................................. 73
  Treponema paraluidiscuniculi .................................................................................................... 75
Fungi ........................................................................................................................................... 76
  Histoplasma capsulatum var. farciminosum .............................................................................. 76
Protozoa ...................................................................................................................................... 77
  Theileria equi and Babesia caballi ............................................................................................ 77
  Trypanosoma evansi ................................................................................................................... 78
Rickettsia ....................................................................................................................................... 78
  Ehrlichia canis ............................................................................................................................ 78
  Neorickettsia risticii ................................................................................................................... 79
Mycoplasma .................................................................................................................................. 80
  Mycoplasma agalactiae ............................................................................................................... 80
  Mycoplasma capricolum subsp. capripneumoniae ...................................................................... 81
  Mycoplasma mycoides subsp. mycoides small colony (SC) type ............................................. 82
Conclusions .................................................................................................................................... 85
Table 1: Viruses — mammalian ..................................................................................................... 85
Table 2: Viruses — avian ............................................................................................................... 90
Table 3: Bacteria — Brucella spp. ................................................................................................. 91
Table 4: Bacteria — Salmonella spp. ........................................................................................... 91
Table 5: Bacteria — other, fungi, protozoa and rickettsia ............................................................. 92
Table 6: Mycoplasma ................................................................................................................... 93
References ...................................................................................................................................... 95
Appendix 1 — Updated Annexes 1–3 ........................................................................................ 135
ANNEX 1 — Exotic animal disease pathogens of major economic and social concern ............. 135
ANNEX 2 — Exotic animal transmissible spongiform encephalopathies (TSEs) of major economic and social concern ............................................................ 137
ANNEX 3 — Other animal disease pathogens of biosecurity concern .................................... 138
<table>
<thead>
<tr>
<th>Acronyms and abbreviations</th>
</tr>
</thead>
<tbody>
<tr>
<td>µg</td>
</tr>
<tr>
<td>µl</td>
</tr>
<tr>
<td>3201</td>
</tr>
<tr>
<td>9 CFR</td>
</tr>
<tr>
<td><em>Title 9: Animals and Animal Products</em></td>
</tr>
<tr>
<td>A549tTA</td>
</tr>
<tr>
<td>A-72</td>
</tr>
<tr>
<td>AA</td>
</tr>
<tr>
<td>AA C3/36</td>
</tr>
<tr>
<td>AAdV</td>
</tr>
<tr>
<td>AAdV-I</td>
</tr>
<tr>
<td>AAdV-II</td>
</tr>
<tr>
<td>AC-EIA</td>
</tr>
<tr>
<td>AC-ELISA</td>
</tr>
<tr>
<td>AGID</td>
</tr>
<tr>
<td>AGMK</td>
</tr>
<tr>
<td>AGP</td>
</tr>
<tr>
<td>AHSV</td>
</tr>
<tr>
<td>AIV</td>
</tr>
<tr>
<td>AK-D</td>
</tr>
<tr>
<td>AMOS</td>
</tr>
<tr>
<td>AMOS-ERY PCR</td>
</tr>
<tr>
<td>AMV</td>
</tr>
<tr>
<td>API</td>
</tr>
<tr>
<td>APVMA</td>
</tr>
<tr>
<td>AP-PCR</td>
</tr>
<tr>
<td>ASFV</td>
</tr>
<tr>
<td>ATCC</td>
</tr>
<tr>
<td>ATI</td>
</tr>
<tr>
<td>B95a</td>
</tr>
<tr>
<td>BAE</td>
</tr>
<tr>
<td>BCYE</td>
</tr>
<tr>
<td>BEC</td>
</tr>
<tr>
<td>BEK</td>
</tr>
<tr>
<td>BEL</td>
</tr>
<tr>
<td>BETC</td>
</tr>
<tr>
<td>BFS</td>
</tr>
<tr>
<td>BGM/BGM-70</td>
</tr>
<tr>
<td>BHK/BHK-W12/BHK-21/BHK-89</td>
</tr>
<tr>
<td>BH-RSV</td>
</tr>
<tr>
<td>BHV-1</td>
</tr>
<tr>
<td>BHV-4</td>
</tr>
<tr>
<td>BK</td>
</tr>
<tr>
<td>BLF</td>
</tr>
<tr>
<td>BLGFP</td>
</tr>
<tr>
<td>BNM</td>
</tr>
<tr>
<td>Abbreviation</td>
</tr>
<tr>
<td>--------------</td>
</tr>
<tr>
<td>BOMAC</td>
</tr>
<tr>
<td>BPW</td>
</tr>
<tr>
<td>BRSV</td>
</tr>
<tr>
<td>BSC-1</td>
</tr>
<tr>
<td>BT</td>
</tr>
<tr>
<td>BTV</td>
</tr>
<tr>
<td>BVDV</td>
</tr>
<tr>
<td>C13</td>
</tr>
<tr>
<td>CA</td>
</tr>
<tr>
<td>CAM</td>
</tr>
<tr>
<td>CCC</td>
</tr>
<tr>
<td>CCL64-RCDV</td>
</tr>
<tr>
<td>CCT</td>
</tr>
<tr>
<td>cDNA</td>
</tr>
<tr>
<td>CEF</td>
</tr>
<tr>
<td>CEK</td>
</tr>
<tr>
<td>CELi</td>
</tr>
<tr>
<td>CER</td>
</tr>
<tr>
<td>CFA</td>
</tr>
<tr>
<td>CFU</td>
</tr>
<tr>
<td>CH</td>
</tr>
<tr>
<td>CH-SAHA</td>
</tr>
<tr>
<td>cIBDV</td>
</tr>
<tr>
<td>CK</td>
</tr>
<tr>
<td>CKC</td>
</tr>
<tr>
<td>CM</td>
</tr>
<tr>
<td>CMK</td>
</tr>
<tr>
<td>COS</td>
</tr>
<tr>
<td>CPAE</td>
</tr>
<tr>
<td>cpBVDV</td>
</tr>
<tr>
<td>CPE</td>
</tr>
<tr>
<td>CRFK</td>
</tr>
<tr>
<td>CRIB</td>
</tr>
<tr>
<td>crmB</td>
</tr>
<tr>
<td>CRT-2</td>
</tr>
<tr>
<td>CSFV</td>
</tr>
<tr>
<td>CTC</td>
</tr>
<tr>
<td>DBS-FRhL-2</td>
</tr>
<tr>
<td>DC</td>
</tr>
<tr>
<td>dCTP</td>
</tr>
<tr>
<td>DAFF</td>
</tr>
<tr>
<td>DEAE-dextran</td>
</tr>
<tr>
<td>DEF</td>
</tr>
<tr>
<td>DEK</td>
</tr>
<tr>
<td>DEL</td>
</tr>
<tr>
<td>Detroit-6</td>
</tr>
<tr>
<td>DEV</td>
</tr>
<tr>
<td>DF-K</td>
</tr>
<tr>
<td>DH-82</td>
</tr>
</tbody>
</table>
DI defective interfering
defective interfering

DJRK mutant rabbit kidney

DLS dimer linkage structure (5’ region of genome of retroviruses
where genomic RNA dimerization occurs)

DNA deoxyribonucleic acid
deoxyribonucleic acid

DOBV Dobrava virus

DVHV duck viral hepatitis virus 1
duck viral hepatitis virus 1

EAV equine arteritis virus
equine arteritis virus

ECE embryonated chicken eggs

EDC equine dermal cells

EcoRI Restriction endonuclease EcoRI

EDE embryonated duck eggs

EEEV eastern equine encephalitis virus

EFK equine foetal kidney

EGFP enhanced green fluorescent protein
enhanced green fluorescent protein

EHDV epizootic haemorrhagic disease virus
epizootic haemorrhagic disease virus

EHV equid herpesvirus
equid herpesvirus

EHV-1 equid herpesvirus 1
equid herpesvirus 1

EHV-2 equid herpesvirus 2
equid herpesvirus 2

EHV-3 equid herpesvirus 3
equid herpesvirus 3

EHV-4 equid herpesvirus 4
equid herpesvirus 4

EIA enzyme immunoassay

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

env envelope gene

ERIC-PCR enterobacterial repetitive intergenic consensus sequence PCR

ESK swine embryo kidney

ETCC equine transitional cell carcinoma

F fusion (F 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

FE feline embryo

FEF feline embryonic fibroblast

FEK foetal equine kidney

FFA focus forming assay

FFU focus forming unit

fg ficogram

FISH fluorescence in situ hybridisation

FITC fluorescein isothiocyanate

FLK foetal lamb kidney

FMDV foot-and-mouth disease virus

fnRT-PCR fluorogenic nuclease real-time RT-PCR

FRET fluorescence resonance energy transfer
FS-L3  porcine kidney epithelial
FTA    fluorescent treponema antigen
GAdV-1 goat adenovirus 1
GAG    retroviral polyprotein
gag    genetic locus encoding GAG polyprotein
gB     glycoprotein B
GBK    Georgia bovine kidney
gC     glycoprotein C
GC     goat cells
gp     glycoprotein
gG     glycoprotein G
gH     glycoprotein H
GSA    group specific antigen
gX     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
IMPACT infectious microbe PCR amplification test
IN     intranuclear
IP     immunoperoxidase
IRES   internal ribosomal entry site
ISH    in situ hybridisation
IV     intravascular
J774   murine macrophage
JEV    Japanese encephalitis virus
Jinet  cynomolgus monkey kidney
JSRV   Jaagsiekte sheep retrovirus
KB     human nasopharyngeal carcinoma
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>kDa</td>
<td>kilo Dalton</td>
</tr>
<tr>
<td>KK</td>
<td>kitten kidney</td>
</tr>
<tr>
<td>KSE6</td>
<td>swine kidney epithelial</td>
</tr>
<tr>
<td>L</td>
<td>C3H mouse fibroblasts</td>
</tr>
<tr>
<td>L929</td>
<td>mouse fibroblast</td>
</tr>
<tr>
<td>LAMP</td>
<td>loop-mediated isothermal amplification</td>
</tr>
<tr>
<td>LAT</td>
<td>latency associated transcript</td>
</tr>
<tr>
<td>LCMV</td>
<td>lymphocytic choriomeningitis virus</td>
</tr>
<tr>
<td>LIV</td>
<td>louping ill virus</td>
</tr>
<tr>
<td>LK</td>
<td>lamb kidney</td>
</tr>
<tr>
<td>LLC-MK2</td>
<td>rhesus monkey kidney</td>
</tr>
<tr>
<td>LLC-RK1</td>
<td>rabbit kidney</td>
</tr>
<tr>
<td>LMH</td>
<td>chicken hepatocellular carcinoma</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>L-RNA</td>
<td>large viral RNA segment</td>
</tr>
<tr>
<td>LSA-1</td>
<td>feline T-lymphoblastoid</td>
</tr>
<tr>
<td>LSCC-RP9/LSCC-RP12</td>
<td>chicken B-lymphoblastoid cell lines transformed by Rous-associated virus 2</td>
</tr>
<tr>
<td>LSDV</td>
<td>lumpy skin disease virus</td>
</tr>
<tr>
<td>LT</td>
<td>lamb testis</td>
</tr>
<tr>
<td>LTR</td>
<td>long terminal repeat</td>
</tr>
<tr>
<td>LUX-PCR</td>
<td>light upon extension fluorogenic real-time PCR</td>
</tr>
<tr>
<td>M</td>
<td>molar (unit of concentration)</td>
</tr>
<tr>
<td>MA-104</td>
<td>foetal rhesus monkey kidney</td>
</tr>
<tr>
<td>M</td>
<td>Genetic locus encoding matrix protein</td>
</tr>
<tr>
<td>mAb</td>
<td>monoclonal antibody</td>
</tr>
<tr>
<td>MAP</td>
<td>mouse antibody production</td>
</tr>
<tr>
<td>MARC-145</td>
<td>monkey kidney</td>
</tr>
<tr>
<td>MAT</td>
<td>microscopic agglutination test</td>
</tr>
<tr>
<td>MDBK</td>
<td>Madin–Darby bovine kidney</td>
</tr>
<tr>
<td>MDBK-SY</td>
<td>bovine kidney cell line/subclone of MDBK</td>
</tr>
<tr>
<td>MDCK</td>
<td>Madin–Darby canine kidney</td>
</tr>
<tr>
<td>MDCK-SP</td>
<td>subclone of MDCK</td>
</tr>
<tr>
<td>MDM</td>
<td>monocyte-derived macrophages</td>
</tr>
<tr>
<td>ME</td>
<td>primary Swiss mouse embryo</td>
</tr>
<tr>
<td>MGB</td>
<td>minor groove binding</td>
</tr>
<tr>
<td>MK</td>
<td>monkey kidney</td>
</tr>
<tr>
<td>ml</td>
<td>millilitre</td>
</tr>
<tr>
<td>ML</td>
<td>myeloblastic leukaemia</td>
</tr>
<tr>
<td>MM</td>
<td>multiple myeloma</td>
</tr>
<tr>
<td>MmmSC</td>
<td>Mycoplasma mycoides subsp. mycoides small colony (SC) type</td>
</tr>
<tr>
<td>M-MSV</td>
<td>Moloney-murine sarcoma virus</td>
</tr>
<tr>
<td>MOI</td>
<td>multiplicity of infection</td>
</tr>
<tr>
<td>MQ-NCSU</td>
<td>chicken macrophage</td>
</tr>
<tr>
<td>MRC-5</td>
<td>human secondary lung fibroblast (ATCC no. CCL-171)</td>
</tr>
<tr>
<td>M-RNA</td>
<td>medium viral RNA segment</td>
</tr>
<tr>
<td>MS</td>
<td>monkey spleen</td>
</tr>
<tr>
<td>MSC-PCR</td>
<td>assay for identification of the Mycoplasma mycoides small colony type from the mycoides cluster</td>
</tr>
<tr>
<td>MuAdV</td>
<td>murine adenovirus</td>
</tr>
<tr>
<td>MVV</td>
<td>visna/maedi (maedi–visna) virus</td>
</tr>
</tbody>
</table>
PS-Y15 clone of swine kidney
PUUUV 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
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
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
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
SQMC squirrel monkey kidney, intestine and lung cells
SQMK squirrel monkey kidney
s-RCV smooth RCV
S-RNA small viral RNA segment
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ST</td>
<td>primary swine testicle</td>
</tr>
<tr>
<td>SV28</td>
<td>simian virus 28</td>
</tr>
<tr>
<td>SV40</td>
<td>simian virus 40</td>
</tr>
<tr>
<td>SVDV</td>
<td>swine vesicular disease virus</td>
</tr>
<tr>
<td>Tax</td>
<td>trans-activator x (x is undefined)</td>
</tr>
<tr>
<td>Tax-RE</td>
<td>Tax response element</td>
</tr>
<tr>
<td>TCID&lt;sub&gt;50&lt;/sub&gt;</td>
<td>median tissue culture infective dose</td>
</tr>
<tr>
<td>TET</td>
<td>tetrathionate</td>
</tr>
<tr>
<td>TGA</td>
<td>tellurite glycine agar</td>
</tr>
<tr>
<td>TGEV</td>
<td>transmissible gastroenteritis virus</td>
</tr>
<tr>
<td>TK (tk)</td>
<td>thymidine kinase</td>
</tr>
<tr>
<td>TMA</td>
<td>tissue microarray technology</td>
</tr>
<tr>
<td>TOC</td>
<td>tracheal organ cultures</td>
</tr>
<tr>
<td>TRV</td>
<td>turkey rhinotracheitis virus</td>
</tr>
<tr>
<td>UL</td>
<td>unique long</td>
</tr>
<tr>
<td>UTR</td>
<td>un-translated region</td>
</tr>
<tr>
<td>VDRL</td>
<td>Venereal Disease Research Laboratory</td>
</tr>
<tr>
<td>VEEV</td>
<td>Venezuelan equine encephalitis virus</td>
</tr>
<tr>
<td>Vero</td>
<td>African green monkey kidney</td>
</tr>
<tr>
<td>Vero F6</td>
<td>Vero cells transfected with the gene encoding herpes simplex virus-1 entry protein glycoprotein-H (gH)</td>
</tr>
<tr>
<td>Vero-DST</td>
<td>transgenic Vero</td>
</tr>
<tr>
<td>vIBDV</td>
<td>variant infectious bursal disease virus</td>
</tr>
<tr>
<td>VN</td>
<td>virus neutralisation</td>
</tr>
<tr>
<td>VP</td>
<td>viral protein gene</td>
</tr>
<tr>
<td>VSV</td>
<td>vesicular stomatitis virus</td>
</tr>
<tr>
<td>vvIBDV</td>
<td>very virulent infectious bursal disease virus</td>
</tr>
<tr>
<td>WEEV</td>
<td>western equine encephalitis virus</td>
</tr>
<tr>
<td>WI-38</td>
<td>human foetal lung (ATCC no. CCL-75)</td>
</tr>
<tr>
<td>WNV</td>
<td>West Nile virus</td>
</tr>
</tbody>
</table>
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 pathogens 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 pathogens of biosecurity concern i.e. major or significant exotic animal pathogens and more virulent exotic strains of endemic animal pathogens. This review provides clarification on the acceptable tests for extraneous pathogens of biosecurity concern 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 pathogens of biosecurity concern.

The first edition of the Review of Published Tests to Detect Pathogens in Veterinary Vaccines Intended for Importation into Australia was issued on 26 July 2011. As a result of preparing the first edition, the Department of Agriculture, Fisheries and Forestry (DAFF) and the Australian Pesticides and Veterinary Medicines Authority (APVMA) identified an inconsistency in the assessment of potential contamination of veterinary vaccines with endemic pathogens. DAFF’s import policy for veterinary vaccines generally requires specific testing whereas the Australian Pesticides and Veterinary Medicines Authority (APVMA) requires general testing in accordance with the United States Code of Federal Regulations, Title 9: Animals and Animal Products, part 113 (9 CFR 113) and the European Pharmacopoeia.

DAFF and APVMA agreed that contamination of veterinary vaccines with endemic pathogens that are not of biosecurity concern represents an animal safety issue. Consultation with vaccine manufacturers in 2012 achieved agreement that animal safety should be managed by APVMA under the Agriculture and Veterinary Chemicals Code Act 1994 (the Agvet Code) for all veterinary vaccines.

The second edition of the review contains modifications to remove test methods for endemic pathogens not of biosecurity concern, shown in the table below.
| Akabane virus | Equine infectious anaemia virus |
| Avian encephalomyelitis virus | Felid herpesvirus 1 (feline rhinotracheitis virus) |
| Avian leukosis virus | Feline calicivirus |
| Avian nephritis virus 1 and 2 | Feline immunodeficiency virus |
| Avian orthoreovirus (avian reovirus) | Feline infectious peritonitis virus |
| Border disease virus | Feline leukaemia virus |
| Bovine adenovirus (subgroups 1 & 2) | Feline panleukopaenia virus |
| Bovine ephemeral fever virus | Fowlpox virus |
| Bovine herpesvirus 2 | Infectious laryngotracheitis virus (gallid herpesvirus 1) |
| Bovine immunodeficiency virus | Marek’s disease virus 1 and 2 (gallid herpesvirus 2 and 3) |
| Bovine leukaemia virus | Mycoplasma gallisepticum |
| Bovine parainfluenza virus 3 | Mycoplasma hyopneumoniae |
| Bovine parvovirus | Mycoplasma synoviae |
| Bovine rotavirus | Orf virus |
| Brucella suis | Ovine adenovirus |
| Canine adenovirus 1 and 2 | Porcine adenovirus |
| Canine distemper virus | Porcine haemagglutinating encephalomyelitis virus |
| Canine parvovirus | Porcine parvovirus |
| Caprine arthritis encephalitis virus | Porcine rotavirus |
| Chicken anaemia virus | Reticuloendotheliosis virus |
| Coxiella burnetii | Sendai virus (murine parainfluenza virus 1) |
| Duck adenovirus A (egg drop syndrome virus) | Swine pox virus |
| Ectromelia virus | Theiler’s murine encephalomyelitis virus |
| Equine adenovirus | |

Contamination with exotic pathogens, and with more virulent exotic strains of endemic pathogens, remains a biosecurity concern and continues to be managed by DAFF under the Quarantine Act 1908, meaning test methods for these pathogens have been retained in the second edition.

This change does not compromise the rigour of the assessment for imported vaccines. Equivalence to specific testing is achieved through a combination of measures - general testing combined with Australia’s high level veterinary services and a comprehensive adverse experience reporting system. Australia’s import requirements have not materially 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 and more virulent exotic strains of 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 pathogens of biosecurity concern 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 general\(^1\) 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 pathogens 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 pathogens 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 pathogens of biosecurity concern 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 Program\(^2\), 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 pathogens can be reliably cultured using general test methods in accordance with 9 CFR 113 but detection or identification is unreliable, therefore specific testing\(^3\) 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 respiratory syncytial virus**\(^4\)
- **Brucella canis**\(^5\)
- Equid herpesvirus 1, 2, 3 and 4\(^6\)
- Equine arteritis virus
- Murine adenovirus
- Peste-des-petits-ruminants virus
- Rabies virus\(^7\)
- *Salmonella* Enteritidis
- *Salmonella* Gallinarum
- *Salmonella* Pullorum\(^8\)

---

\(^1\) 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.

\(^2\) 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 that FA is performed with a polyclonal antiserum directed against BRSV whole virus antigen.

\(^5\) The generalised methods described in European Pharmacopoeia monograph 2.6.12 are not suitable.

\(^6\) Provided that primary isolation is in equine foetal kidney cells or equine fibroblasts.

\(^7\) If combined with primary isolation in Neuro-2a cells.

\(^8\) The method in European Pharmacopoeia monograph 2.6.24 is not suitable.
The review concluded that the following Annex 1 and 3 extraneous pathogens 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.

- Lumpy skin disease virus
- Rinderpest virus
- Vesicular stomatitis virus

The review concluded that the following Annex 1 and 3 extraneous pathogens 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 pathogens 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
- Anatid herpesvirus 1 (duck enteritis virus, duck plague herpesvirus)
- Aviadenoviruses (all viruses in the genus Aviadenovirus)
- Avian influenza virus
- Babesia caballi
- Bluetongue virus
- Bovine herpesvirus 1
- Bovine herpesvirus 4
- Bovine viral diarrhoea virus 1 and 2
- Brucella abortus
- Brucella melitensis
- Classical swine fever virus
- Duck viral hepatitis virus 1
- Epizootic haemorrhagic disease virus
- Equine encephalitis viruses (eastern equine encephalitis virus (EEEV), western equine encephalitis virus (WEEV) and Venezuelan equine encephalitis virus (VEEV))
- Equine influenza virus
- Foot-and-mouth disease virus
- Francisella tularensis
- Hantaan virus
- Histoplasma capsulatum var. farciminusum
- Horse pox virus
- Infectious bronchitis virus
- Infectious bursal disease virus
- Japanese encephalitis virus
- Leptospira interrogans var. canicola
- Louping ill virus
- Lymphocytic choriomeningitis virus
- Mycoplasma agalactiae

9 Provided LT cells or OA3.Ts cells are used.
10 The generalised culture method described in European Pharmacopoeia monograph 2.6.7 Mycoplasmas is not acceptable.
Mycoplasma capricolum subsp. Capripneumoniae
Mycoplasma mycoides subsp. mycoides SC (MmmSC)
Newcastle disease virus
Porcine circovirus 2
Porcine epidemic diarrhoea virus
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)
Swine influenza virus
Swine vesicular disease virus
Taylorlera equigenitalis
Theileria equi
Treponema paraluiscuniculi
Turkey rhinotracheitis virus (avian metapneumovirus, avian pneumovirus)
West Nile virus

The review concluded that the following Annex 1 and 3 extraneous pathogens 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.

Ehrlichia canis
Jaagsiekte sheep retrovirus (ovine pulmonary adenocarcinoma virus, pulmonary adenomatosis virus)
Neorickettsia risticii
Ornithobacterium rhinotracheale
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 pathogens. 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 pathogens 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.

11 The generalised culture method described in European Pharmacopoeia monograph 2.6.7 Mycoplasmas is acceptable.
**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 pathogens 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. Adventitious 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 pathogens of biosecurity concern 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 pathogens, 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 and more virulent exotic strains) 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 and more virulent exotic strains of endemic animal pathogens that are potential or 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 pathogens. However, Australia’s requirements include additional risk management measures due to Australia’s different and favourable animal health status. This means that additional testing for extraneous pathogens of biosecurity concern 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 pathogens 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 pathogens 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 pathogen. 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 pathogens 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 pathogens of biosecurity concern.

This review provides clarification on the acceptable tests for extraneous pathogens of biosecurity concern 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 pathogens. Australia’s import requirements have not materially 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 pathogens 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 adventitious 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 exogenous\(^\text{12}\) retroviruses that require specific testing under Australia’s veterinary vaccines requirements. Live and inactivated veterinary vaccines may also be contaminated with endogenous\(^\text{13}\) retroviruses and these may not readily be detected by routine extraneous pathogen 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 considered unacceptable 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. The European regulatory body, Agence Françoise 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

\(^{12}\) Exogenous retroviruses are transmitted horizontally by infection and they infect somatic cells but not germ line cells.

\(^{13}\) 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.
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 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 pathogens of biosecurity concern 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 pathogens described in 9 CFR 113 and the European Pharmacopoeia.

Extraneous pathogens of biosecurity concern are major or significant exotic animal pathogens and more virulent exotic strains of endemic animal pathogens. Other extraneous pathogens that are endemic animal pathogens represent an animal safety issue, rather than a biosecurity issue, and therefore are managed by the APVMA under the Agvet Code for all veterinary vaccines, including those that are imported.

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 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 pathogens. AAHL’s validated test methods are assessed by the Biological Imports Program as suitable for the reliable detection of extraneous pathogens 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 pathogens of biosecurity concern. The Biological Imports Program bases its assessment on factors such as the country of origin, processing, treatment and testing of the vaccine for extraneous pathogens 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 more virulent exotic strains of an endemic pathogen. 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 pathogens of biosecurity concern. 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 113.52, 113.53 and 113.55 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 pathogen testing using cell culture monolayers that are sensitive to the pathogen being tested, and detection using specific testing methods that are sensitive to the viral pathogen. 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.

---

14 Requirements for cell lines used for production of biologics.
15 Requirements for ingredients of animal origin used for production of biologics.
16 Detection of extraneous agents in Master Seed Virus.
African swine fever virus

Family Asfarviridae, genus Asfivirus

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 pathogen testing using cell culture monolayers that are sensitive to the pathogen being tested, and detection using specific testing methods that are sensitive to the viral pathogen. 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.

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 pathogen 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 pathogen 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 pathogen testing using cell culture monolayers that are sensitive to the pathogen being tested, and detection using specific testing methods that are sensitive to the viral pathogen. 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.

**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 MOI $\geq 1.0$, and latent, non-cytopathic infection occurred for 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 pathogen testing will be resistant to cytolytic 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/50µl (van Engelenburg et al. 1993). Another PCR has been developed targeting the thymidine kinase (tk) gene of BHV-1 that can detect 1 TCID$_{50}$/50 µl. 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 TCID$_{50}$ 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 pathogen 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 pathogen testing using cell culture monolayers that are sensitive to the pathogen being tested, and detection using specific testing methods that are sensitive to the viral pathogen. 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 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 polyrepetitive 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 polyrepetitive 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 pathogen 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 pathogen testing using cell culture monolayers that are sensitive to the pathogen being tested, and detection using specific testing methods that are sensitive to the viral pathogen. 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 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 pathogen 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 pathogen testing using cell culture monolayers that are sensitive to the pathogen being tested, and
detection using specific testing methods that are sensitive to the viral pathogen. 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 pathogen 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 \(10^3\ TCID_{50}/\text{ml}\) 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. MAb 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 pathogen 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 pathogen 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 pathogen testing using cell culture monolayers that are sensitive to the pathogen being tested, and detection using specific testing methods that are sensitive to the viral pathogen. 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 pathogen 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 Brecia 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 pathogen 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 pathogen testing using cell culture monolayers that are sensitive to the pathogen being tested, and detection using specific testing methods that are sensitive to the viral pathogen. 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 pathogen testing.

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

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).

The Canadian National Centre for Foreign Animal Disease and the Australian Animal Health Laboratory have 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 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 pathogen testing using cell culture monolayers that are sensitive to the pathogen being tested, and detection using specific testing methods that are sensitive to the viral pathogen. 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.

**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 pathogen testing using cell culture monolayers that are sensitive to the pathogen being tested, and detection using specific testing methods that are sensitive to the viral pathogen. 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.
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-electropherotyping, 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 pathogen 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 pathogen 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 pathogen testing using cell culture monolayers that are sensitive to the pathogen being tested, and detection using specific testing methods that are sensitive to the viral pathogen. 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 (Dyonon 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 (Dyonon 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 (Dyonon et al. 2001).

The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 for extraneous pathogen 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 pathogen testing using cell culture monolayers that are sensitive to the pathogen being tested, and detection using specific testing methods that are sensitive to the viral pathogen. 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 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 TCID_{50} 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 (Dynon 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 pathogen 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 pathogen testing using cell culture monolayers that are sensitive to the pathogen being tested, and detection using specific testing methods that are sensitive to the viral pathogen. 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 pathogen 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 pathogen testing using cell culture monolayers that are sensitive to the pathogen being tested, and detection using specific testing methods that are sensitive to the viral pathogen. 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 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 (EID$_{50}$) 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 pathogen 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 pathogen testing using cell culture monolayers that are sensitive to the pathogen being tested, and detection using specific testing methods that are sensitive to the viral pathogen. 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.

**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 serotypes 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 unsusceptible 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 (ATTC 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 1960s, 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. Merial Animal Health Ltd., Ash Road, Pirbright, 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 $10^{2.3}$ TCID$_{50}$/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 cytolytic 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 $10^{-2.3}$ TCID$_{50}$/ml as compared to a culture detection limit of $10^{-2.3}$ TCID$_{50}$/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 pathogen testing using cell culture monolayers that are sensitive to the pathogen being tested, and detection using specific testing methods that are sensitive to the viral pathogen. 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 TCID$_{50}$/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 pathogens 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 pathogen 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 pathogen testing using cell culture monolayers that are sensitive to the pathogen being tested, and detection using specific testing methods that are sensitive to the viral pathogen. 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 pathogen 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 pathogen testing using cell culture monolayers that are sensitive to the pathogen being tested, and detection using specific testing methods that are sensitive to the viral pathogen. 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 pathogen 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 JRSV. 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 pathogen testing using cell culture monolayers that are sensitive to the pathogen being tested, and detection using specific testing methods that are sensitive to the viral pathogen. 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 pathogen 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 pathogen testing using cell culture monolayers that are sensitive to the pathogen being tested, and detection using specific testing methods that are sensitive to the viral pathogen. 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 pathogen 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 pathogen testing using cell culture monolayers that are sensitive to the pathogen being tested, and detection using specific testing methods that are sensitive to the viral pathogen. 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).

The Canadian National Centre for Foreign Animal Disease and the Australian Animal Health Laboratory have 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 pathogen testing using cell culture monolayers that are sensitive to the pathogen being tested, and detection using specific testing methods that are sensitive to the viral pathogen. 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 pathogen 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 pathogen testing using cell culture monolayers that are sensitive to the pathogen being tested, and detection using specific testing methods that are sensitive to the viral pathogen. 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 pathogens, 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 pathogen 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 pathogen testing using cell culture monolayers that are sensitive to the pathogen being tested, and detection using specific testing methods that are sensitive to the viral pathogen. 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.

**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
pathogen testing using cell culture monolayers that are sensitive to the pathogen being tested, and
detection using specific testing methods that are sensitive to the viral pathogen. 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 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 $10^{1.4}$ TCID$_{50}$/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 pathogen
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
pathogen testing using cell culture monolayers that are sensitive to the pathogen being tested, and
detection using specific testing methods that are sensitive to the viral pathogen. 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).


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 pathogen 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 pathogen 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 pathogen testing using cell culture monolayers that are sensitive to the pathogen being tested, and detection using specific testing methods that are sensitive to the viral pathogen. 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 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 (Larochelle 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 $10^{2.5}$ TCID$_{50}$/ml (Van Woensel et al. 1994). The nested RT-PCR can detect 10 TCID$_{50}$/ml and is therefore 3 times more sensitive than the RT-PCR (Christopher-
The generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 for extraneous pathogen 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 pathogen testing using cell culture monolayers that are sensitive to the pathogen being tested, and detection using specific testing methods that are sensitive to the viral pathogen. 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. 2005).
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 pathogen 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 pathogen testing using cell culture monolayers that are sensitive to the pathogen being tested, and detection using specific testing methods that are sensitive to the viral pathogen. 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 marginated chromatin. The success of isolation is variable, as low levels of virus can be inhibited by the presence of other pathogens, 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 pathogen 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 pathogen testing using cell culture monolayers that are sensitive to the pathogen being tested, and detection using specific testing methods that are sensitive to the viral pathogen. 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 pathogen 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 pathogen testing using cell culture monolayers that are sensitive to the pathogen being tested, and detection using specific testing methods that are sensitive to the viral pathogen. 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 pathogen 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 pathogen 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 pathogen testing using cell culture monolayers that are sensitive to the pathogen being tested, and detection using specific testing methods that are sensitive to the viral pathogen. 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 1 hour 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 pathogen 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 pathogen testing using cell culture monolayers that are sensitive to the pathogen being tested, and detection using specific testing methods that are sensitive to the viral pathogen. 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-FRhL-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-FRhL-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-FRhl-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 pathogen 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 pathogen testing using cell culture monolayers that are sensitive to the pathogen being tested, and detection using specific testing methods that are sensitive to the viral pathogen. 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 pathogen testing using cell culture monolayers that are sensitive to the pathogen being tested, and detection using specific testing methods that are sensitive to the viral pathogen. 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.

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 TCID_{50}/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 pathogen 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 pathogen testing using cell culture monolayers that are sensitive to the pathogen being tested, and detection using specific testing methods that are sensitive to the viral pathogen. 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 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 NPr, 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 pathogens 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 NPr 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 pathogen testing using cell culture monolayers that are sensitive to the pathogen being tested, and detection using specific testing methods that are sensitive to the viral pathogen. 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.

**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 pathogen 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 pathogen testing using cell culture monolayers that are sensitive to the pathogen being tested, and detection using specific testing methods that are sensitive to the viral pathogen. 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 cytolytic 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 pathogen 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 pathogen testing using cell culture monolayers that are sensitive to the pathogen being tested, and detection using specific testing methods that are sensitive to the viral pathogen. 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.

Visna/maedi virus

Family *Retroviridae*, genus *Lentivirus*

Primary isolation of 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 pathogen 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 proviral DNA and are most commonly used for MVV detection. The real-time PCR of the *env* gene has 96% positive and 97% negative concordance with ELISA serology for detection of 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).

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 pathogen testing will not be sufficient for primary isolation of MVV. The 9 CFR guidelines do not include specific testing for 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 pathogen testing using cell culture monolayers that are sensitive to the pathogen being tested, and detection using specific testing methods that are sensitive to the viral pathogen. The monographs do not specify details of a culture system or assay for 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 MVV is not recommended.
Specific testing is required. A PERT assay should be used as the initial diagnostic assay for 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.

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 pathogen 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.

**Aviadenoviruses (all viruses in the genus)**

Family *Adenoviridae*, genus *Aviadenovirus*

The taxonomy of species in the genus *Aviadenovirus* has evolved in recent years and may continue to do so. The genus includes five fowl adenovirus species, further divided into serotypes 1, 2, 3, 4, 5, 6, 7, 8a, 8b, 9, 10 and 11. Two parallel and different systems of nomenclature are in use by Europe and the United States to classify the serotypes, 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 (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-SAHA) cells. CH-SAHA 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-SAHA 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 $10^2$ TCID$_{50}$ 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 generalised culture methods of 9 CFR 113.52, 113.53 and 113.55 for extraneous pathogen 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.

The methods described by European Pharmacopoeia 2.6.24 are 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 pathogens 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.

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 ≤ 0.1% in the growth medium reduces DVHV-1 plaque formation and at concentrations ≥ 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 10^5 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 pathogen 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.

**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 pathogen 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 pathogens 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 pathogen 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 pathogen 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 pathogens 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 pathogen 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 pathogens 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 pathogen testing.
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 pathogen 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-TRT 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 pathogen 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 pathogen 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 pathogen 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 pathogens. 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; Marin 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*. 

Page 67
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.

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 pathogen testing.

The 9 CFR 113.30\(^{17}\) 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 pathogen 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 pathogen 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

\(^{17}\) Detection of *Salmonella* contamination.
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 pathogen testing using methods that are sensitive to the pathogen 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. 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.

**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 tularemia 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 pathogen 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 *fop4* 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.
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 (Splettstoesser 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 Meriex, 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 pathogen testing using methods that are sensitive to the pathogen 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 paraluiscuniculi**

*Treponema paraluiscuniculi* is non-cultivable and is an obligate intracellular pathogen. Inoculation of rabbit testicles is used for detection and propagation of *T. paraluiscuniculi*. 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. paraluiscuniculi*, 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 pathogen testing. Confirmation of *T. paraluiscuniculi* is possible by FA using hyperimmune pooled rabbit syphilic 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 *tpr* 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. paraluisiscuniculi* is required by the genus-specific PCR assay targeting the \textit{rpoB} gene.

**Fungi**

\textit{Histoplasma capsulatum} var. \textit{farcininosum}

Culture isolation of *Histoplasma capsulatum* var. \textit{farcininosum} 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. \textit{farcininosum}.

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. \textit{farcininosum} 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. \textit{farcininosum} 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. \textit{farcininosum}. If a vaccine material is required to be tested for the fungal pathogen *H. capsulatum* var. \textit{farcininosum} 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. \textit{farcininosum}.

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. \textit{farcininosum} (Pryce et al. 2006).

The generic protocols of 9 CFR 113.26: \textit{Detection of viable bacteria and fungi except in live vaccines} and 9 CFR 113.27: \textit{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 pathogen testing using methods that are sensitive to the pathogen 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 pathogens of equine piroplasmosis (Alhassan et al. 2007). PCR assays have been developed targeting the EMA-2, 16S rRNA and Be48 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
pathogen testing using methods that are sensitive to the pathogen 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 pathogen testing using methods that are sensitive to the pathogen 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 pathogen 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 pathogen 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 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 pathogen 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 pathogen testing using cell culture monolayers that are sensitive to the pathogen being tested, and detection using specific testing methods that are sensitive to the pathogen. 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 pathogen 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*. 

Page 79
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 pathogen testing using methods that are sensitive to the pathogen 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 pathogen 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 pathogen 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 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 **Mmm**SC 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 **Mmm**SC 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/MSC-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 1–6 provide a summary of the conclusions from review of the published test methods for the reliable and sensitive detection of extraneous pathogens in vaccines and vaccine raw materials. For details, refer to the specific chapters.

Table 1: Viruses — mammalian

<table>
<thead>
<tr>
<th>Viruses - mammalian</th>
<th>Disease (species)</th>
<th>9 CFR Part 113 standard requirements</th>
<th>European Pharmacopoeia Vaccines for veterinary use and 5.2.4¹⁸ acceptable (Y/N/NA)</th>
<th>Specific testing required (Y/N)</th>
</tr>
</thead>
<tbody>
<tr>
<td>African horse sickness virus</td>
<td>African horse sickness (equine)</td>
<td>NA</td>
<td>NA</td>
<td>N²⁴</td>
</tr>
<tr>
<td>African swine fever virus</td>
<td>African swine fever (porcine)</td>
<td>NA</td>
<td>N</td>
<td></td>
</tr>
<tr>
<td>Bluetongue virus</td>
<td>Bluetongue (bovine, ovine, caprine, canine)</td>
<td>N</td>
<td>N</td>
<td></td>
</tr>
<tr>
<td>Bovine herpesvirus 1</td>
<td>Infectious bovine rhinotracheitis/ infectious pustular vulvovaginitis/ infectious balanoposthitis (bovine)</td>
<td>NA</td>
<td>NA</td>
<td></td>
</tr>
</tbody>
</table>

¹⁸ Cell cultures for the production of veterinary vaccines.
¹⁹ 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 pathogen 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 pathogen. Where the monographs do not specify details of a culture system or assay, case-by-case assessment is required and is therefore not applicable.
²⁰ Detection of extraneous viruses by the fluorescent antibody technique.
²¹ Requirements for cell lines used for production of biologics.
²² Requirements for ingredients of animal origin used for production of biologics.
²³ Detection of extraneous agents in Master Seed Virus.
²⁴ Virus is readily detected by CPE following culture isolation using alternative methods.
<table>
<thead>
<tr>
<th>Viruses - mammalian</th>
<th>Disease (species)</th>
<th>9 CFR Part 113 standard requirements</th>
<th>European Pharmacopoeia Vaccines for veterinary use and 5.2.4&lt;sup&gt;18&lt;/sup&gt; acceptable (Y/N/NA&lt;sup&gt;19&lt;/sup&gt;)</th>
<th>Specific testing required (Y/N)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine herpesvirus 4</td>
<td>Metritis (bovine)</td>
<td>NA</td>
<td>NA</td>
<td>Y</td>
</tr>
<tr>
<td>Bovine respiratory</td>
<td>Bovine respiratory disease complex (bovine)</td>
<td>Y&lt;sup&gt;25&lt;/sup&gt;</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>syncytial virus</td>
<td></td>
<td></td>
<td>NA</td>
<td>Y</td>
</tr>
<tr>
<td>Bovine viral diarrhoea</td>
<td>Bovine viral diarrhoea/mucosal disease (bovine, ovine, porcine)</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>virus 1 &amp; 2 (bovine</td>
<td></td>
<td></td>
<td>NA</td>
<td>Y</td>
</tr>
<tr>
<td>pestiviruses)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Caprine and ovine pox</td>
<td>Sheep pox/goat pox (caprine, ovine)</td>
<td>NA</td>
<td>N</td>
<td>N&lt;sup&gt;26&lt;/sup&gt;</td>
</tr>
<tr>
<td>virus</td>
<td></td>
<td></td>
<td>NA</td>
<td>Y</td>
</tr>
<tr>
<td>Visna/maedi (maedi-visna)</td>
<td>Maedi-visna (ovine)</td>
<td>NA</td>
<td></td>
<td>Y</td>
</tr>
<tr>
<td>virus</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Classical swine fever</td>
<td>Classical swine fever (porcine)</td>
<td>NA</td>
<td></td>
<td>Y</td>
</tr>
<tr>
<td>virus</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Epizootic haemorrhagic</td>
<td>Epizootic hemorrhagic disease (bovine, ovine)</td>
<td>NA</td>
<td></td>
<td>Y</td>
</tr>
<tr>
<td>disease virus</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Equine herpesvirus 1, 2,</td>
<td>Equine rhinopneumonitis/equine viral abortion/keratoconjunctivitis/equine</td>
<td>Y&lt;sup&gt;27&lt;/sup&gt;</td>
<td></td>
<td>Y</td>
</tr>
<tr>
<td>and 4 (equineherpes</td>
<td>coital exanthema (equine)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>virus 1, 2, 3 and 4)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Equine arteritis virus</td>
<td>Equine viral arteritis (equine)</td>
<td>Y</td>
<td></td>
<td>Y</td>
</tr>
</tbody>
</table>

<sup>25</sup> Provided that FA is performed with a polyclonal antiserum directed against BRSV whole virus antigen.

<sup>26</sup> Virus can be readily identified by alternative methods for culture isolation and CPE detection.

<sup>27</sup> Provided that primary isolation is in equine foetal kidney cells or equine fibroblasts.
<table>
<thead>
<tr>
<th>Viruses - mammalian disease (species)</th>
<th>9 CFR Part 113 standard requirements</th>
<th>European Pharmacopoeia Vaccines for veterinary use and 5.2.4 acceptable (Y/N)</th>
<th>Specific testing required (Y/N)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Equine encephalitis viruses (eastern equine encephalitis virus, western equine encephalitis virus, Venezuelan equine encephalitis virus)</td>
<td>113.47&lt;sup&gt;20&lt;/sup&gt; acceptable (Y/N/NA) 113.52&lt;sup&gt;21&lt;/sup&gt;, 113.53&lt;sup&gt;22&lt;/sup&gt; and 113.55&lt;sup&gt;23&lt;/sup&gt; acceptable (Y/N)</td>
<td>NA</td>
<td>N</td>
</tr>
<tr>
<td>Equine influenza virus</td>
<td>Equine influenza (equine)</td>
<td>NA</td>
<td>N</td>
</tr>
<tr>
<td>Foot-and-mouth disease virus</td>
<td>Foot-and-mouth disease (bovine, caprine, ovine, porcine)</td>
<td>NA</td>
<td>N</td>
</tr>
<tr>
<td>Hantaan virus (Korean haemorrhagic fever virus)</td>
<td>Haemorrhagic fever with renal syndrome/Korean haemorrhagic fever (rodents)</td>
<td>NA</td>
<td>N</td>
</tr>
<tr>
<td>Horse pox virus</td>
<td>Horse pox (equine)</td>
<td>NA</td>
<td>N</td>
</tr>
<tr>
<td>Jaagsiekte sheep retrovirus (ovine pulmonary adenocarcinoma virus, pulmonary adenomatosis virus)</td>
<td>Ovine pulmonary adenomatosis/Ovine pulmonary adenocarcinoma/Jaagsiekte (caprine, ovine)</td>
<td>NA</td>
<td>N</td>
</tr>
<tr>
<td>Japanese encephalitis virus and West Nile virus</td>
<td>Japanese encephalitis, West Nile fever (equine)</td>
<td>NA</td>
<td>N</td>
</tr>
<tr>
<td>Louping ill virus</td>
<td>Louping ill (ovine)</td>
<td>NA</td>
<td>N</td>
</tr>
<tr>
<td>Lumpy skin disease virus</td>
<td>Lumpy skin disease (bovine)</td>
<td>NA</td>
<td>Y&lt;sup&gt;28&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>28</sup> Provided LT cells or OA3.Ts cells are used.
<table>
<thead>
<tr>
<th>Viruses - mammalian</th>
<th>Disease (species)</th>
<th>9 CFR Part 113 standard requirements</th>
<th>European Pharmacopoeia Vaccines for veterinary use and 5.2.4(^{18}) acceptable (Y/N/NA(^{19}))</th>
<th>Specific testing required (Y/N)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymphocytic choriomeningitis virus (Arenavirus)</td>
<td>Lymphocytic choriomeningitis (rodents)</td>
<td>NA</td>
<td>N</td>
<td>NA</td>
</tr>
<tr>
<td>Murine adenovirus</td>
<td>Subclinical infection (rodent)</td>
<td>NA</td>
<td>Y</td>
<td>NA</td>
</tr>
<tr>
<td>Peste-des-petits-ruminants virus</td>
<td>Peste-des-petits-ruminants (caprine, ovine)</td>
<td>NA</td>
<td>Y</td>
<td>NA(^{29})</td>
</tr>
<tr>
<td>Porcine circovirus 2</td>
<td>Postweaning multisystemic wasting syndrome (porcine)</td>
<td>NA</td>
<td>N</td>
<td>NA</td>
</tr>
<tr>
<td>Porcine epidemic diarrhoea virus</td>
<td>Porcine epidemic diarrhoea (porcine)</td>
<td>NA</td>
<td>N</td>
<td>NA</td>
</tr>
<tr>
<td>Porcine reproductive and respiratory syndrome virus</td>
<td>Porcine reproductive and respiratory syndrome (porcine)</td>
<td>NA</td>
<td>N</td>
<td>NA</td>
</tr>
<tr>
<td>Porcine respiratory coronavirus</td>
<td>Subclinical respiratory disease (porcine)</td>
<td>N</td>
<td>N</td>
<td>NA</td>
</tr>
<tr>
<td>Porcine teschovirus 1 (Polioencephalomyelitis virus)</td>
<td>Teschen disease, Talfan disease, porcine polioencephalomyelitis (porcine)</td>
<td>NA</td>
<td>N</td>
<td>NA</td>
</tr>
<tr>
<td>Pseudorabies virus (Aujeszky’s disease virus, suid herpesvirus 1)</td>
<td>Aujeszky's disease/Pseudorabies (porcine, canine)</td>
<td>NA</td>
<td>N</td>
<td>NA</td>
</tr>
<tr>
<td>Rabbit fibroma virus (Shope fibroma virus)</td>
<td>Shope fibromas (rabbit)</td>
<td>NA</td>
<td>N</td>
<td>NA</td>
</tr>
<tr>
<td>Rabbit haemorrhagic disease virus (rabbit calicivirus)</td>
<td>Rabbit hemorrhagic disease (rabbit)</td>
<td>NA</td>
<td>N</td>
<td>NA</td>
</tr>
</tbody>
</table>

\(^{29}\) It is difficult to detect the subtle CPE due to the culture isolated virus.
<table>
<thead>
<tr>
<th>Viruses - mammalian</th>
<th>Disease (species)</th>
<th>9 CFR Part 113 standard requirements</th>
<th>European Pharmacopoeia Vaccines for veterinary use and 5.2.4 acceptable (Y/N)</th>
<th>Specific testing required (Y/N)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabies virus</td>
<td>Rabies (bovine, canine, caprine, equine, feline, ovine, porcine, rabbit, rodent)</td>
<td>Y³⁰</td>
<td>N</td>
<td>NA</td>
</tr>
<tr>
<td>Rift Valley fever virus</td>
<td>Rift Valley fever (bovine, caprine, ovine)</td>
<td>NA</td>
<td>N</td>
<td>NA</td>
</tr>
<tr>
<td>Rinderpest virus</td>
<td>Rinderpest (bovine, caprine, ovine)</td>
<td>NA</td>
<td>Y</td>
<td>NA</td>
</tr>
<tr>
<td>Swine influenza virus</td>
<td>Swine influenza (porcine)</td>
<td>NA</td>
<td>N</td>
<td>NA</td>
</tr>
<tr>
<td>Swine vesicular disease virus</td>
<td>Swine vesicular disease (porcine)</td>
<td>NA</td>
<td>N</td>
<td>NA</td>
</tr>
<tr>
<td>Transmissible gastroenteritis virus</td>
<td>Transmissible gastroenteritis (porcine)</td>
<td>N</td>
<td>N</td>
<td>NA</td>
</tr>
<tr>
<td>Vesicular stomatitis virus</td>
<td>Vesicular stomatitis (bovine, equine, caprine, ovine, porcine)</td>
<td>NA</td>
<td>Y</td>
<td>NA</td>
</tr>
</tbody>
</table>

³⁰ If combined with primary isolation in Neuro-2a cells.
³¹ Due to the profound cytolytic effect of RVFV in cell culture and readily identifiable CPE.
<table>
<thead>
<tr>
<th>Viruses - avian</th>
<th>Disease (species)</th>
<th>9 CFR Part 113 standard requirements</th>
<th>European Pharmacopoeia Vaccines for veterinary use and 2.6.24\textsuperscript{32} acceptable (Y/N/NA)</th>
<th>Specific testing required (Y/N)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anatid herpesvirus 1 (duck enteritis virus, duck plague herpesvirus)</td>
<td>Duck viral enteritis/duck plague (ducks, geese, and swans)</td>
<td>NA</td>
<td>NA</td>
<td>N</td>
</tr>
<tr>
<td>Aviadenoviruses (all viruses in the genus Aviadenovirus)</td>
<td>Various diseases/syndromes (avian)</td>
<td>NA</td>
<td>NA</td>
<td>N</td>
</tr>
<tr>
<td>Avian influenza virus</td>
<td>Avian influenza (avian)</td>
<td>NA</td>
<td>N</td>
<td>NA</td>
</tr>
<tr>
<td>Duck viral hepatitis virus type I</td>
<td>Duck viral hepatitis (ducks)</td>
<td>NA</td>
<td>NA</td>
<td>N</td>
</tr>
<tr>
<td>Fowl pox virus</td>
<td>Fowl pox (avian)</td>
<td>NA</td>
<td>NA</td>
<td>N</td>
</tr>
<tr>
<td>Infectious bronchitis virus</td>
<td>Infectious bronchitis (avian)</td>
<td>NA</td>
<td>NA</td>
<td>N</td>
</tr>
<tr>
<td>Infectious bursal disease virus</td>
<td>Infectious bursal disease (avian)</td>
<td>NA</td>
<td>NA</td>
<td>N</td>
</tr>
<tr>
<td>Newcastle disease virus</td>
<td>Newcastle disease (avian)</td>
<td>NA</td>
<td>N</td>
<td>NA</td>
</tr>
<tr>
<td>Turkey rhinotracheitis virus (avian metapneumovirus, avian pneumovirus)</td>
<td>Turkey rhinotracheitis (avian)</td>
<td>NA</td>
<td>NA</td>
<td>N</td>
</tr>
</tbody>
</table>

\textsuperscript{32} Avian viral vaccines: tests for extraneous agents in seed lots.
\textsuperscript{33} Detection of avian lymphoid leukosis.
\textsuperscript{34} Detection of pathogens by the chicken embryo inoculation test.
### Table 3: Bacteria — *Brucella* spp.

<table>
<thead>
<tr>
<th>Bacteria — <em>Brucella</em> spp.</th>
<th>Disease (species)</th>
<th>9 CFR 113.32 Detection of <em>Brucella</em> contamination acceptable (Y/N)</th>
<th>European Pharmacopoeia Vaccines for veterinary use, 2.6.12(^{35}), 2.6.13(^{36}) acceptable (Y/N/NA(^{37}))</th>
<th>Specific testing required (Y/N)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Brucella abortus</em></td>
<td>Brucellosis (bovine)</td>
<td>N</td>
<td>N</td>
<td>Y</td>
</tr>
<tr>
<td><em>Brucella canis</em></td>
<td>Brucellosis (canine)</td>
<td>Y</td>
<td>N</td>
<td>Y</td>
</tr>
<tr>
<td><em>Brucella melitensis</em></td>
<td>Brucellosis (caprine, ovine)</td>
<td>N</td>
<td>N</td>
<td>Y</td>
</tr>
</tbody>
</table>

### Table 4: Bacteria — *Salmonella* spp.

<table>
<thead>
<tr>
<th>Bacteria — <em>Salmonella</em> spp.</th>
<th>Disease (species)</th>
<th>9CFR 113.30 Detection of <em>Salmonella</em> contamination. acceptable (Y/N)</th>
<th>European Pharmacopoeia Vaccines for veterinary use, 2.6.24 (Y/N/NA)</th>
<th>Specific testing required (Y/N)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Salmonella Enteritidis</em></td>
<td>Salmonellosis (avian)</td>
<td>Y</td>
<td>NA</td>
<td>Y</td>
</tr>
<tr>
<td><em>Salmonella Gallinarum</em></td>
<td>Fowl typhoid (avian)</td>
<td>Y</td>
<td>NA</td>
<td>Y</td>
</tr>
<tr>
<td><em>Salmonella Pullorum</em></td>
<td>Pullorum disease (avian)</td>
<td>Y</td>
<td>N</td>
<td>Y</td>
</tr>
</tbody>
</table>

\(^{35}\) Microbiological examination of non-sterile products: microbial enumeration tests.

\(^{36}\) Microbiological examination of non-sterile products: test for specified microorganisms.

\(^{37}\) 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 5: Bacteria — other, fungi, protozoa and rickettsia

<table>
<thead>
<tr>
<th>Bacteria – other, fungi, protozoa and rickettsia</th>
<th>Disease (species)</th>
<th>9CFR extraneous pathogen testing</th>
<th>European Pharmacopoeia Vaccines for veterinary use</th>
<th>Specific testing required (Y/N)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>113.26 and 113.27 acceptable (Y/N/NA)</td>
<td>113.52, 113.53 and 113.55 acceptable (Y/N/NA)</td>
</tr>
<tr>
<td>Bacteria- other</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Burkholderia mallei</em></td>
<td>Glanders (equine)</td>
<td>N</td>
<td>NA</td>
<td>N</td>
</tr>
<tr>
<td><em>Francisella tularensis</em></td>
<td>Tularaemia/rabbit fever (rabbit)</td>
<td>NA</td>
<td>N</td>
<td>NA</td>
</tr>
<tr>
<td><em>Leptospira interrogans var. canicola</em></td>
<td>Leptospirosis (canine)</td>
<td>N</td>
<td>NA</td>
<td>N</td>
</tr>
<tr>
<td><em>Ornithobacterium rhinotracheale</em></td>
<td>Respiratory disease (avian)</td>
<td>N</td>
<td>NA</td>
<td>N</td>
</tr>
<tr>
<td><em>Taylorella equigenitalis</em></td>
<td>Contagious equine metritis (equine)</td>
<td>N</td>
<td>NA</td>
<td>N</td>
</tr>
<tr>
<td><em>Treponema paraluiscuniculi</em></td>
<td>Treponematosis (rabbit)</td>
<td>NA</td>
<td>N</td>
<td>NA</td>
</tr>
<tr>
<td>Fungi</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Histoplasma capsulatum var. farciminorum</em></td>
<td>Epizootic lymphangitis (equine)</td>
<td>N</td>
<td>NA</td>
<td>N</td>
</tr>
<tr>
<td>Protozoa</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Babesia caballi</em></td>
<td>Equine piroplasmosis/babesiosis (equine)</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

38 Detection of viable bacteria and fungi except in live vaccine.
39 Detection of extraneous viable bacteria and fungi in live vaccines.
40 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>
<thead>
<tr>
<th>Bacteria – other, fungi, protozoa and rickettsia</th>
<th>Disease (species)</th>
<th>9CFR extraneous pathogen testing</th>
<th>European Pharmacopoeia Vaccines for veterinary use</th>
<th>Specific testing required (Y/N)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>113.26&lt;sup&gt;18&lt;/sup&gt; and 113.27&lt;sup&gt;19&lt;/sup&gt; acceptable (Y/N/NA)</td>
<td>113.52, 113.53 and 113.55 acceptable (Y/N/NA)</td>
<td>2.6.12 and 2.6.13 acceptable (Y/N/NA&lt;sup&gt;40&lt;/sup&gt;)</td>
</tr>
<tr>
<td>Theileria equi</td>
<td>Equine piroplasmosis/babesiosis (equine)</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Trypanosoma evansi</td>
<td>Surra (equine)</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Rickettsia</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ehrlichia canis</td>
<td>Tropical canine pancytopaenia (canine)</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Neorickettsia risticii</td>
<td>Potomac fever/equine monocytic Ehrlichiosis/equine Ehrlichial colitis (equine)</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

**Table 6: Mycoplasma**

<table>
<thead>
<tr>
<th>Mycoplasma</th>
<th>Disease (species)</th>
<th>9CFR 113.28: Detection of mycoplasma contamination acceptable (Y/N)</th>
<th>European Pharmacopoeia monograph 2.6.7 Mycoplasmas acceptable (Y/N)</th>
<th>Specific testing required (Y/N)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mycoplasma agalactiae</td>
<td>Contagious agalactia (caprine, ovine)</td>
<td>N</td>
<td>N</td>
<td>Y</td>
</tr>
<tr>
<td>Mycoplasma capricolum subsp. capripneumoniae</td>
<td>Contagious caprine pleuropneumonia (caprine)</td>
<td>N</td>
<td>N</td>
<td>Y</td>
</tr>
<tr>
<td>Mycoplasma mycoides subsp. mycoides SC</td>
<td>Contagious bovine pleuropneumonia (bovine)</td>
<td>N</td>
<td>Y</td>
<td>Y&lt;sup&gt;41&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>41</sup> Except when generalised culture method described in European Pharmacopoeia monograph 2.6.7 *Mycoplasmas* is used.
References


Hodes ME, Chang LMS (1968) Shope fibroma virus assay based on enumeration of cells containing inclusion bodies. *Virology* 34: 134-140.


Lawrence GL, Gilkerson J, Love DN, Sabine M, Whalley JM (1994) Rapid, single-step differentiation of equid herpesviruses 1 and 4 from clinical material using the


Marin CM, Jiménez de Baqués MP, Barberán M, Blasco JM (1996b) Comparison of two selective media for the isolation of *Brucella melitensis* from naturally infected sheep and goats. *The Veterinary Record* 138: 409-411.


Moore BD, Udeni BR, Balasuriya JF, Hedges JF, MacLachlan NJ (2002) Growth characteristics of a highly virulent, a moderately virulent, and an avirulent strain of equine arteritis virus in primary equine endothelial cells are predictive of their virulence to horses. *Virology* 298: 39-44.


Terpstra C (1979) Diagnosis of infectious bovine rhinotracheitis by direct immunofluorescence. *The Veterinary Quarterly* 1: 138-144.


causative agent of contagious caprine pleuropneumonia (CCPP). *Veterinary Microbiology* 104: 125-132.


## Appendix 1 — Updated Annexes 1–3

ANNEX 1 — Exotic animal disease pathogens of major economic and social concern

<table>
<thead>
<tr>
<th>Animal species</th>
<th>Pathogen</th>
<th>Disease</th>
<th>OIE notifiable (Y/N)</th>
<th>Exotic (Y/N)</th>
<th>More virulent exotic strains of an endemic pathogen (Y/N)</th>
<th>Endemic (Y/N)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Avian</td>
<td>Avian influenza virus&lt;sup&gt;43&lt;/sup&gt;</td>
<td>Highly pathogenic notifiable avian influenza (HPNAI) and low pathogenicity notifiable avian influenza (LPNAI)</td>
<td>Y</td>
<td>N</td>
<td>Y</td>
<td>Y&lt;sup&gt;44&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Newcastle disease virus&lt;sup&gt;45&lt;/sup&gt;</td>
<td>Virulent Newcastle disease</td>
<td>Y</td>
<td>N</td>
<td>Y</td>
<td>Y&lt;sup&gt;46&lt;/sup&gt;</td>
</tr>
<tr>
<td>Bovine</td>
<td>Foot-and-mouth disease virus</td>
<td>Foot-and-mouth disease</td>
<td>Y</td>
<td>Y</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td></td>
<td>Rinderpest virus</td>
<td>Rinderpest</td>
<td>Y</td>
<td>Y</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>Caprine and ovine</td>
<td>Caprine/ovine pox virus</td>
<td>Sheep pox/goat pox</td>
<td>Y</td>
<td>Y</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td></td>
<td>Foot-and-mouth disease virus</td>
<td>Foot-and-mouth disease</td>
<td>Y</td>
<td>Y</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td></td>
<td>Jaagsiekte sheep retrovirus (ovine pulmonary adenocarcinoma virus, pulmonary adenomatosis virus)</td>
<td>Ovine pulmonary adenomatosis/ovine pulmonary adenocarcinoma/ Jaagsiekte</td>
<td>N</td>
<td>Y</td>
<td>N</td>
<td>N</td>
</tr>
</tbody>
</table>

<sup>42</sup> And other species as determined by AQIS on application.
<sup>43</sup> Influenza A virus of the H5 or H7 subtypes or any AI virus with an intravenous pathogenicity index (IVPI) greater than 1.2.
<sup>44</sup> Not present in commercial poultry flocks.
<sup>45</sup> That meets one of the criteria for virulence described in the OIE Terrestrial Animal Health Code.
<sup>46</sup> Less virulent strains are present in commercial poultry flocks.
<table>
<thead>
<tr>
<th>Animal species&lt;sup&gt;42&lt;/sup&gt;</th>
<th>Pathogen</th>
<th>Disease</th>
<th>OIE notifiable (Y/N)</th>
<th>Exotic (Y/N)</th>
<th>More virulent exotic strains of an endemic pathogen (Y/N)</th>
<th>Endemic (Y/N)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Peste-des-petits-ruminants virus</td>
<td>Peste-des-petits-ruminants</td>
<td>Y</td>
<td>Y</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td></td>
<td>Rinderpest virus</td>
<td>Rinderpest</td>
<td>Y</td>
<td>Y</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>Equine</td>
<td>African horse sickness virus</td>
<td>African horse sickness</td>
<td>Y</td>
<td>Y</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>Porcine</td>
<td>African swine fever virus</td>
<td>African swine fever</td>
<td>Y</td>
<td>Y</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td></td>
<td>Classical swine fever virus</td>
<td>Classical swine fever</td>
<td>Y</td>
<td>Y</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td></td>
<td>Foot-and-mouth disease virus</td>
<td>Foot-and-mouth disease</td>
<td>Y</td>
<td>Y</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td></td>
<td>Swine vesicular disease virus</td>
<td>Swine vesicular disease</td>
<td>Y</td>
<td>Y</td>
<td>N</td>
<td>N</td>
</tr>
</tbody>
</table>
ANNEX 2 — Exotic animal transmissible spongiform encephalopathies (TSEs) of major economic and social concern

(Relatively low infectivity but extremely high resistance to normal inactivation processes)

<table>
<thead>
<tr>
<th>Animal species</th>
<th>Pathogen</th>
<th>Disease</th>
<th>OIE notifiable (Y/N)</th>
<th>Exotic (Y/N)</th>
<th>More virulent exotic strains of an endemic pathogen (Y/N)</th>
<th>Endemic (Y/N)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine</td>
<td>Bovine spongiform encephalopathy (BSE) agent (prion)</td>
<td>BSE</td>
<td>Y</td>
<td>Y</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>Cervids (including deer, elk and moose)</td>
<td>Chronic wasting disease (CWD) agent (prion)</td>
<td>CWD</td>
<td>N</td>
<td>Y</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>Mink</td>
<td>Transmissible mink encephalopathy (TME) agent (prion)</td>
<td>TME</td>
<td>N</td>
<td>Y</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>Ovine</td>
<td>Scrapie agent (prion)</td>
<td>Scrapie</td>
<td>Y</td>
<td>Y</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>Species — other</td>
<td>Other agents of transmissible spongiform encephalopathies as determined by AQIS based on origin and end use</td>
<td>Other transmissible spongiform encephalopathies</td>
<td>N</td>
<td>Y</td>
<td>N</td>
<td>N</td>
</tr>
</tbody>
</table>

---

47 As determined by AQIS on application.
ANNEX 3 — Other animal disease pathogens of biosecurity concern

These pathogens are either exotic to Australia or more virulent exotic strains of endemic pathogens.

<table>
<thead>
<tr>
<th>Animal species</th>
<th>Pathogen</th>
<th>Disease</th>
<th>OIE notifiable (Y/N)</th>
<th>Exotic (Y/N)</th>
<th>More virulent exotic strains of an endemic pathogen (Y/N)</th>
<th>Endemic (Y/N)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Avian — chickens and turkeys</td>
<td>Aviadenoviruses (all viruses in the genus)</td>
<td>Various diseases</td>
<td>N</td>
<td>N</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td></td>
<td>Infectious bronchitis virus</td>
<td>Infectious bronchitis</td>
<td>Y</td>
<td>N</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td></td>
<td>Infectious bursal disease virus</td>
<td>Infectious bursal disease (Gumboro disease)</td>
<td>Y</td>
<td>N</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td></td>
<td><em>Ornithobacterium rhinotracheale</em></td>
<td>Respiratory disease</td>
<td>N</td>
<td>Y</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td></td>
<td><em>Salmonella</em> Enteritidis</td>
<td>Salmonellosis</td>
<td>N</td>
<td>N</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td></td>
<td><em>Salmonella</em> Gallinarum</td>
<td>Fowl typhoid</td>
<td>Y</td>
<td>Y</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td></td>
<td><em>Salmonella</em> Pullorum</td>
<td>Pullorum disease</td>
<td>Y</td>
<td>N</td>
<td>N</td>
<td>Y&lt;sup&gt;48&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Turkey rhinotracheitis virus (avian metapneumovirus, avian pneumovirus)</td>
<td>Turkey rhinotracheitis</td>
<td>Y</td>
<td>Y</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>Avian — ducks and geese, and swans</td>
<td>Anatid herpesvirus 1 (duck enteritis virus, duck plague herpesvirus)</td>
<td>Duck viral enteritis/duck plague</td>
<td>N</td>
<td>Y</td>
<td>N</td>
<td>N</td>
</tr>
</tbody>
</table>

<sup>48</sup> Australian commercial poultry are considered to be free of *S. Pullorum.*
<table>
<thead>
<tr>
<th>Animal species</th>
<th>Pathogen</th>
<th>Disease</th>
<th>OIE notifiable (Y/N)</th>
<th>Exotic (Y/N)</th>
<th>More virulent exotic strains of an endemic pathogen (Y/N)</th>
<th>Endemic (Y/N)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Aviadenoviruses (all viruses in the genus)</td>
<td>Various diseases</td>
<td>N</td>
<td>N</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td></td>
<td>Duck viral hepatitis virus</td>
<td>Duck viral hepatitis</td>
<td>Y</td>
<td>Y</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td></td>
<td>Infectious bronchitis virus</td>
<td>Infectious bronchitis</td>
<td>Y</td>
<td>N</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td></td>
<td>Infectious bursal disease virus</td>
<td>Infectious bursal disease (Gumboro disease)</td>
<td>Y</td>
<td>N</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td></td>
<td>Ornithobacterium rhinotracheale</td>
<td>Respiratory disease</td>
<td>N</td>
<td>Y</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td></td>
<td>Salmonella Enteritidis</td>
<td>Salmonellosis</td>
<td>N</td>
<td>N</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td></td>
<td>Salmonella Gallinarum</td>
<td>Fowl typhoid</td>
<td>Y</td>
<td>Y</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td></td>
<td>Salmonella Pullorum</td>
<td>Pullorum disease</td>
<td>Y</td>
<td>N</td>
<td>N</td>
<td>Y*49</td>
</tr>
<tr>
<td></td>
<td>Turkey rhinotracheitis virus (avian metapneumovirus, avian pneumovirus)</td>
<td>Turkey rhinotracheitis</td>
<td>Y</td>
<td>Y</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td><strong>Bovine</strong></td>
<td>Bluetongue virus</td>
<td>Bluetongue</td>
<td>Y</td>
<td>N</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td></td>
<td>Bovine herpesvirus 1</td>
<td>Infectious bovine rhinotracheitis/ infectious pustular vulvovaginitis/ infectious balanoposthitis</td>
<td>Y</td>
<td>N</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td></td>
<td>Bovine herpesvirus 4</td>
<td>Unclear</td>
<td>N</td>
<td>Y</td>
<td>N</td>
<td>N</td>
</tr>
</tbody>
</table>

*49 Australian commercial poultry are considered to be free of S. Pullorum.
<table>
<thead>
<tr>
<th>Animal species</th>
<th>Pathogen</th>
<th>Disease</th>
<th>OIE notifiable (Y/N)</th>
<th>Exotic (Y/N)</th>
<th>More virulent exotic strains of an endemic pathogen (Y/N)</th>
<th>Endemic (Y/N)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine respiratory syncytial virus</td>
<td>Bovine respiratory disease complex</td>
<td>N</td>
<td>Y</td>
<td>N</td>
<td>N</td>
<td></td>
</tr>
<tr>
<td>Bovine viral diarrhoea virus 1 &amp; 2 (bovine pestiviruses)</td>
<td>Bovine viral diarrhoea/mucosal disease</td>
<td>Y</td>
<td>N</td>
<td>Y</td>
<td>Y</td>
<td></td>
</tr>
<tr>
<td><em>Brucella abortus</em></td>
<td>Brucellosis</td>
<td>Y</td>
<td>Y</td>
<td>N</td>
<td>N</td>
<td></td>
</tr>
<tr>
<td>Epizootic hemorrhagic disease virus (EHDV)</td>
<td>Epizootic hemorrhagic disease (EHD)</td>
<td>Y</td>
<td>N</td>
<td>Y</td>
<td>Y</td>
<td></td>
</tr>
<tr>
<td>Lumpy skin disease virus</td>
<td>Lumpy skin disease</td>
<td>Y</td>
<td>Y</td>
<td>N</td>
<td>N</td>
<td></td>
</tr>
<tr>
<td><em>Mycoplasma mycoides</em> subsp. <em>mycoides</em> small colony (SC) type</td>
<td>Contagious bovine pleuropneumonia</td>
<td>Y</td>
<td>Y</td>
<td>N</td>
<td>N</td>
<td></td>
</tr>
<tr>
<td>Rabies virus</td>
<td>Rabies</td>
<td>Y</td>
<td>Y</td>
<td>N</td>
<td>N</td>
<td></td>
</tr>
<tr>
<td>Rift Valley fever virus</td>
<td>Rift Valley fever</td>
<td>Y</td>
<td>Y</td>
<td>N</td>
<td>N</td>
<td></td>
</tr>
<tr>
<td>Vesicular stomatitis virus</td>
<td>Vesicular stomatitis</td>
<td>Y</td>
<td>Y</td>
<td>N</td>
<td>N</td>
<td></td>
</tr>
<tr>
<td><strong>Canine</strong></td>
<td><strong>Bluetongue virus</strong></td>
<td><strong>Bluetongue</strong></td>
<td>Y</td>
<td>N</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td><em>Brucella canis</em></td>
<td>Brucellosis</td>
<td>N</td>
<td>Y</td>
<td>N</td>
<td>N</td>
<td></td>
</tr>
<tr>
<td><em>Ehrlichia canis</em></td>
<td>Tropical canine pancytopenia</td>
<td>N</td>
<td>Y</td>
<td>N</td>
<td>N</td>
<td></td>
</tr>
<tr>
<td><em>Leptospira interrogans</em> var. <em>canicola</em></td>
<td>Leptospirosis</td>
<td>Y</td>
<td>Y</td>
<td>N</td>
<td>N</td>
<td></td>
</tr>
<tr>
<td>Animal species</td>
<td>Pathogen</td>
<td>Disease</td>
<td>OIE notifiable (Y/N)</td>
<td>Exotic (Y/N)</td>
<td>More virulent exotic strains of an endemic pathogen (Y/N)</td>
<td>Endemic (Y/N)</td>
</tr>
<tr>
<td>----------------</td>
<td>----------</td>
<td>---------</td>
<td>----------------------</td>
<td>--------------</td>
<td>----------------------------------------------------------</td>
<td>--------------</td>
</tr>
<tr>
<td></td>
<td>Pseudorabies virus (suid herpesvirus 1, Aujeszky’s disease virus)</td>
<td>Aujeszky’s disease (pseudorabies)</td>
<td>Y</td>
<td>Y</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>Caprine</td>
<td>Rabies virus</td>
<td>Rabies</td>
<td>Y</td>
<td>Y</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td></td>
<td>Bluetongue virus</td>
<td>Bluetongue</td>
<td>Y</td>
<td>N</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td></td>
<td>Brucella melitensis</td>
<td>Brucellosis</td>
<td>Y</td>
<td>Y</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td></td>
<td>Mycoplasma agalactiae</td>
<td>Contagious agalactia</td>
<td>Y</td>
<td>N</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td></td>
<td>Mycoplasma capricolum subsp. capripneumoniae</td>
<td>Contagious caprine pleuropneumonia</td>
<td>Y</td>
<td>Y</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td></td>
<td>Rabies virus</td>
<td>Rabies</td>
<td>Y</td>
<td>Y</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td></td>
<td>Rift Valley fever virus</td>
<td>Rift Valley fever</td>
<td>Y</td>
<td>Y</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td></td>
<td>Vesicular stomatitis virus</td>
<td>Vesicular stomatitis</td>
<td>Y</td>
<td>Y</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td></td>
<td>Visna/maedi (Maedi-visna) virus</td>
<td>Maedi-visna</td>
<td>Y</td>
<td>Y</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>Equine</td>
<td>Babesia caballi</td>
<td>Equine piroplasmosis /babesiosis</td>
<td>Y</td>
<td>Y</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td></td>
<td>Burkholderia mallei</td>
<td>Glanders</td>
<td>Y</td>
<td>Y</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td></td>
<td>Equid herpesvirus 1, 2, 3 &amp; 4</td>
<td>Equine viral abortion (EHV-1), equine coital exanthema (EHV-3), equine rhinopneumonitis (EHV-4)</td>
<td>Y</td>
<td>N</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td></td>
<td>Equine arteritis virus</td>
<td>Equine viral arteritis</td>
<td>Y</td>
<td>N</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>Animal species</td>
<td>Pathogen</td>
<td>Disease</td>
<td>OIE notifiable (Y/N)</td>
<td>Exotic (Y/N)</td>
<td>More virulent exotic strains of an endemic pathogen (Y/N)</td>
<td>Endemic (Y/N)</td>
</tr>
<tr>
<td>----------------</td>
<td>----------</td>
<td>---------</td>
<td>---------------------</td>
<td>--------------</td>
<td>--------------------------------------------------------</td>
<td>--------------</td>
</tr>
<tr>
<td>Equine encephalitis viruses (eastern equine encephalitis virus, western equine encephalitis virus, Venezuelan equine encephalitis virus)</td>
<td>Eastern equine encephalitis (EEE), western equine encephalitis (WEE), Venezuelan equine encephalitis (VEE)</td>
<td>Y</td>
<td>Y</td>
<td>N</td>
<td>N</td>
<td></td>
</tr>
<tr>
<td>Equine influenza virus</td>
<td>Equine influenza</td>
<td>Y</td>
<td>Y</td>
<td>N</td>
<td>N</td>
<td></td>
</tr>
<tr>
<td><em>Histoplasma capsulatum</em> var. <em>farciminosum</em></td>
<td>Epizootic lymphangitis</td>
<td>N</td>
<td>Y</td>
<td>N</td>
<td>N</td>
<td></td>
</tr>
<tr>
<td>Horse pox virus (vaccinia virus)</td>
<td>Horse pox</td>
<td>N</td>
<td>Y</td>
<td>N</td>
<td>N</td>
<td></td>
</tr>
<tr>
<td>Japanese encephalitis virus</td>
<td>Japanese encephalitis (JE)</td>
<td>Y</td>
<td>Y</td>
<td>N</td>
<td>N</td>
<td></td>
</tr>
<tr>
<td><em>Neorickettsia risticii</em></td>
<td>Potomac fever/equine monocytic ehrlichiosis/equine ehrlichial Colitis</td>
<td>N</td>
<td>Y</td>
<td>N</td>
<td>N</td>
<td></td>
</tr>
<tr>
<td>Rabies virus</td>
<td>Rabies</td>
<td>Y</td>
<td>Y</td>
<td>N</td>
<td>N</td>
<td></td>
</tr>
<tr>
<td><em>Taylorella equigenitalis</em></td>
<td>Contagious equine metritis</td>
<td>Y</td>
<td>Y</td>
<td>N</td>
<td>N</td>
<td></td>
</tr>
<tr>
<td><em>Theileria equi</em></td>
<td>Equine piroplasmosis /babesiosis</td>
<td>Y</td>
<td>Y</td>
<td>N</td>
<td>N</td>
<td></td>
</tr>
<tr>
<td><em>Trypanosoma evansi</em></td>
<td>Surra</td>
<td>Y</td>
<td>Y</td>
<td>N</td>
<td>N</td>
<td></td>
</tr>
<tr>
<td>Vesicular stomatitis virus</td>
<td>Vesicular stomatitis</td>
<td>Y</td>
<td>Y</td>
<td>N</td>
<td>N</td>
<td></td>
</tr>
<tr>
<td>Animal species</td>
<td>Pathogen</td>
<td>Disease</td>
<td>OIE notifiable (Y/N)</td>
<td>Exotic (Y/N)</td>
<td>More virulent exotic strains of an endemic pathogen (Y/N)</td>
<td>Endemic (Y/N)</td>
</tr>
<tr>
<td>----------------</td>
<td>----------</td>
<td>---------</td>
<td>----------------------</td>
<td>--------------</td>
<td>---------------------------------------------------------</td>
<td>--------------</td>
</tr>
<tr>
<td>West Nile virus</td>
<td>West Nile fever</td>
<td>Y</td>
<td>Y</td>
<td>N</td>
<td>N</td>
<td></td>
</tr>
<tr>
<td><strong>Feline</strong></td>
<td>Rabies virus</td>
<td>Rabies</td>
<td>Y</td>
<td>Y</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td><strong>Ovine</strong></td>
<td>Bluetongue virus</td>
<td>Bluetongue</td>
<td>Y</td>
<td>N</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td></td>
<td>Bovine viral diarrhoea virus 1 &amp; 2 (bovine pestiviruses)</td>
<td>Bovine viral diarrhoea/mucosal disease</td>
<td>Y</td>
<td>N</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td></td>
<td><em>Brucella melitensis</em></td>
<td>Brucellosis</td>
<td>Y</td>
<td>Y</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td></td>
<td>Epizootic hemorrhagic disease virus (EHDV)</td>
<td>Epizootic hemorrhagic disease (EHD) (clinical disease rarely seen in sheep)</td>
<td>Y</td>
<td>N</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td></td>
<td>Louping ill virus</td>
<td>Louping ill</td>
<td>N</td>
<td>Y</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td></td>
<td><em>Mycoplasma agalactiae</em></td>
<td>Contagious agalactia</td>
<td>Y</td>
<td>N</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td></td>
<td><em>Mycoplasma capricolum subsp. capripneumoniae</em></td>
<td>Contagious caprine pleuropneumonia</td>
<td>Y</td>
<td>Y</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td></td>
<td>Rabies virus</td>
<td>Rabies</td>
<td>Y</td>
<td>Y</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td></td>
<td>Rift Valley fever virus</td>
<td>Rift Valley fever</td>
<td>Y</td>
<td>Y</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td></td>
<td>Vesicular stomatitis virus</td>
<td>Vesicular stomatitis</td>
<td>Y</td>
<td>Y</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td></td>
<td>Visna/maedi (Maedi-visna) virus</td>
<td>Maedi-visna</td>
<td>Y</td>
<td>Y</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td><strong>Porcine</strong></td>
<td>Bovine viral diarrhoea virus 1 &amp; 2 (bovine pestiviruses)</td>
<td>Clinical signs and pathological lesions indistinguishable from those observed in chronic classical swine fever</td>
<td>Y</td>
<td>N</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>Animal species</td>
<td>Pathogen</td>
<td>Disease</td>
<td>OIE notifiable (Y/N)</td>
<td>Exotic (Y/N)</td>
<td>More virulent exotic strains of an endemic pathogen (Y/N)</td>
<td>Endemic (Y/N)</td>
</tr>
<tr>
<td>----------------</td>
<td>----------------------------------------------</td>
<td>----------------------------------------------</td>
<td>----------------------</td>
<td>--------------</td>
<td>----------------------------------------------------------</td>
<td>--------------</td>
</tr>
<tr>
<td></td>
<td>Porcine circovirus 2</td>
<td>Postweaning multisystemic wasting syndrome</td>
<td>N</td>
<td>N</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td></td>
<td>Porcine epidemic diarrhoea virus</td>
<td>Porcine epidemic diarrhoea</td>
<td>N</td>
<td>Y</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td></td>
<td>Porcine reproductive and respiratory syndrome virus</td>
<td>Porcine reproductive and respiratory syndrome</td>
<td>Y</td>
<td>Y</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td></td>
<td>Porcine respiratory coronavirus</td>
<td>Subclinical respiratory disease</td>
<td>N</td>
<td>Y</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td></td>
<td>Porcine teschovirus 1 (polioencephalomyelitis virus, porcine enterovirus)</td>
<td>Teschen disease, Talfan disease, porcine polioencephalomyelitis</td>
<td>N</td>
<td>N</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td></td>
<td>Pseudorabies virus (suid herpesvirus 1, Aujeszky's disease virus)</td>
<td>Pseudorabies (Aujeszky's disease)</td>
<td>Y</td>
<td>Y</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td></td>
<td>Rabies virus</td>
<td>Rabies</td>
<td>Y</td>
<td>Y</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td></td>
<td>Swine influenza virus</td>
<td>Swine influenza</td>
<td>N</td>
<td>N</td>
<td>Y&lt;sup&gt;50&lt;/sup&gt;</td>
<td>Y</td>
</tr>
<tr>
<td></td>
<td>Transmissible gastroenteritis virus</td>
<td>Transmissible gastroenteritis</td>
<td>Y</td>
<td>Y</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td></td>
<td>Vesicular stomatitis virus</td>
<td>Vesicular stomatitis</td>
<td>Y</td>
<td>Y</td>
<td>N</td>
<td>N</td>
</tr>
</tbody>
</table>

<sup>50</sup> The most common subtypes currently found in swine are H1N1, H1N2 and H3N2.
<table>
<thead>
<tr>
<th>Animal species</th>
<th>Pathogen</th>
<th>Disease</th>
<th>OIE notifiable (Y/N)</th>
<th>Exotic (Y/N)</th>
<th>More virulent exotic strains of an endemic pathogen (Y/N)</th>
<th>Endemic (Y/N)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit</td>
<td><em>Francisella tularensis</em></td>
<td>Tularaemia/rabbit fever</td>
<td>Y</td>
<td>Y</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>Rabbit</td>
<td>Rabbit haemorrhagic disease virus (rabbit calicivirus)</td>
<td>Rabbit hemorrhagic disease</td>
<td>Y</td>
<td>N</td>
<td>N</td>
<td>Y&lt;sup&gt;51&lt;/sup&gt;</td>
</tr>
<tr>
<td>Rabbit</td>
<td>Rabbit fibroma virus (Shope fibroma virus)</td>
<td>Shope fibromas</td>
<td>N</td>
<td>Y&lt;sup&gt;52&lt;/sup&gt;</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>Rabies virus</td>
<td>Rabies</td>
<td>Rabies</td>
<td>Y</td>
<td>Y</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td><em>Treponema paraluisnuniculi</em></td>
<td>Treponematosis</td>
<td>N</td>
<td>Y</td>
<td>N</td>
<td>N</td>
<td></td>
</tr>
<tr>
<td>Rodents - all</td>
<td>Hantaan virus (Korean haemorrhagic fever virus)</td>
<td>Haemorrhagic fever with renal syndrome/Korean haemorrhagic fever</td>
<td>N</td>
<td>Y</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>Lymphocytic choriomeningitis virus (Arenavirus)</td>
<td>Lymphocytic choriomeningitis</td>
<td>N</td>
<td>N</td>
<td>Y</td>
<td>Y</td>
<td></td>
</tr>
<tr>
<td>Murine adenovirus</td>
<td>Subclinical infection</td>
<td>N</td>
<td>N</td>
<td>Y&lt;sup&gt;53&lt;/sup&gt;</td>
<td>Y</td>
<td></td>
</tr>
<tr>
<td>Rabies virus</td>
<td>Rabies</td>
<td>Rabies</td>
<td>Y</td>
<td>Y</td>
<td>N</td>
<td>N</td>
</tr>
</tbody>
</table>

<sup>51</sup> Biological control agent for rabbits. Contamination with less virulent strains has the potential to undermine the effectiveness of the introduced strain.

<sup>52</sup> Confers immunity to myxomatosis. Escape of the virus into wild rabbits could undermine the effectiveness of myxoma virus as a biological control agent.

<sup>53</sup> Serological evidence of MAV2 in south-eastern Australia but no evidence of MAV1.

<sup>54</sup> As determined by AQIS on application.