Evaluation of a real time PCR to simultaneously detect and differentiate virulent and non-virulent Newcastle disease virus

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PROJECT DETAILS:

Project: Evaluation of a Real time PCR to simultaneously detect and differentiate virulent and non-virulent Newcastle disease virus.

Project Dates: 1st September 2011 to 30th May 2012

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Project Background

Avian paramyxoviruses (APMV) have been grouped into nine distinct serotypes, APMV-1 to APMV-9 (Huovilainen et al., 2001). The most recognized of these is APMV-1, a virus more commonly known as Newcastle disease virus (NDV). The name 'Newcastle Disease' is exclusively reserved for the disease that results from infection with strains of APMV-1 that are pathogenic for chickens (Leighton & Heckert, 2007). NDV is a significant avian pathogen with worldwide distribution and is a disease listed by the World Health Organisation. Infection with NDV can cause outbreaks of virulent disease in poultry with devastating economic impact as a result of high mortality and slaughter for disease control. NDV strains are traditionally characterized as one of three pathotypes based on the disease severity in chickens. These include highly virulent (velogenic), intermediate (mesogenic) or non-virulent (lentogenic) (Beard & Hanson, 1984).

APMV and avian influenza viruses (AIV) have been obtained frequently from migratory waterfowl and other wild bird populations including in Australian studies (McKenzie *et al.*, 1984; Peruolis & O'Riley, 2004; Haynes *et al.*, 2009; Hansbro *et al.*, 2010). Waterfowl are important reservoirs of these viruses and are considered as vectors for the transfer to poultry, which can lead to outbreaks of disease (Alexander, 1995). Most of the isolates detected in wild birds have been of low virulence for chickens and would be classified as lentogenic. Australia has been free from outbreaks of virulent Newcastle disease since 2002, when one incident in Victoria in 2002, and another in NSW from 1998-2000 were eradicated. As a consequence of those events, the national ND management Plan was developed, which included the formation of a steering committee to oversee further development and implementation of the plan. The goal of the current ND Management Plan, which is due to finish in 2012, is to reduce the spread of viruses that are precursors to virulent ND virus through the application of biosecurity measures in the poultry industry, as well through a vaccination program using live V4 and inactivated vaccine. The current plan is designed to lead to a risk-based exit strategy that may result in minimal or no vaccination in chicken flocks at the end of the program in 2012.

The most common PCR tests currently used in Australia cannot differentiate between virulent and avirulent strains of APMV. Therefore, whilst the tests are adequate for the detection of APMV, a positive result from a chicken sample causes concern until the virulence can be determined. This is generally delayed by at least a couple of days until after sequencing has been completed. Presently an epidemiological assessment is made, but there is some risk of a wrong decision pending the result of follow-up testing. One type of incorrect decision could result in further spread of virulent ND, with significantly greater control and eradication costs, and the second type, imposition of unnecessary control measures. This project seeks to evaluate a new test capable of differentiating virulent from virulent strains of APMV-1. A PCR test that could quickly and easily confirm that an APMV-1 PCR positive sample was caused by a virulent strain of NDV, as opposed to a V4–like avirulent strain, would be a great advantage in veterinary diagnostics.

Objectives

1. To determine an appropriate NDV PCR to use for the simultaneous detection and differentiation of virulent and avirulent NDV strains
2. To screen a selection of NDV positive and negative wild bird samples previously tested in Kim/Wise PCR using new PCR to compare diagnostic sensitivity
3. To technology transfer the newly acquired NDV PCR test to Biosecurity Sciences Laboratory (BSL, Queensland Department of Agriculture, Fisheries and Forestry - DAFF)

4. Undertake phylogenetic analysis of pathotyping sequencing results to show relationships of clades

Results

Objective 1:
To determine an appropriate NDV PCR to use for the simultaneous detection and differentiation of virulent and avirulent NDV strains

A desktop study was undertaken to determine the best PCR available for the simultaneous detection and differentiation of virulent and avirulent Avian Paramyxovirus 1 (APMV-1) strains. The most appropriate PCR for the differentiation of virulent from avirulent APMV-1 strains was determined to be the real-time reverse-transcriptase PCR developed by Fuller *et al.* (2009). This PCR employs two probes; one to detect virulent APMV-1 (probe VRP2) and one to detect avirulent APMV-1 (Probe ARP2). The test is designed to work for Class II NDV's (such as with the Wise PCR test) but not Class I NDVs (such as those samples positive in the Kim PCR test).

The probes are designed to cover the cleavage site sequence of the F-gene. They incorporate inosine in positions where the nucleotide ambiguity is greatest and locked nucleic acids (LNAs) at nucleotide positions that encode the amino acids which are determinate for virulence. LNAs increase the thermal stability of probe/template duplexes and improve selectivity compared to the corresponding unmodified reference strands. Thus LNAs act to improve the ability of the probe to hybridise with sequence variants that have multiple mismatches between the probe and the target. All further work was conducted with this test, and is referred to in this report as "virulent/avirulent multiplex qPCR".

To confirm the ability of the new virulent/avirulent multiplex qPCR to correctly identify a virus as virulent or avirulent, a range of known virulent and avirulent samples were tested including recently obtained virulent APMV-1 strain from pigeons, a sample containing the avirulent vaccine strain (V4) and a range of reference organisms supplied by the national NDV reference lab (CSIRO AAHL). All of the avirulent samples were correctly identified using this PCR (Refer to Table 1). However, one known Class II virulent strain of NDV (sample 20 in Table 1) that was positive in the Wise PCR test, was not able to be detected in the virulent/avirulent multiplex qPCR (highlighted in green in Table 1). It is not known why this sample was not detected in the virulent/avirulent multiplex qPCR given that it gave a strong positive PCR result in the Wise PCR test. One explanation may be that the degree of mismatch in the probe was more than able to be tolerated in the PCR and therefore the probe was unable to bind to the target DNA. One other sample that did not give a clear result in the virulent/avirulent multiplex qPCR was sample 11 (a mesogenic chicken sample from Komarov). This sample gave a clear strong positive result in the Wise PCR and then gave a clear and strong result with both virulent and avirulent probes in the multiplex qPCR, rather than with just one or the other probe. This may have something to do with the fact that this sample is classified as mesogenic and may contain sequence similarities with both the velogenic and lentogenic probes.

Despite the fact that two samples that did not give clear results, the virulent/avirulent multiplex qPCR appears to be an appropriate choice of test to use to determine whether a Class II APMV-1 sample is virulent or avirulent.

Table 1: Comparison of results for the virulent/avirulent APMV-1 qPCR and the Wise and Kim qPCR tests for APMV-1. Green shading indicates where the virulent/avirulent PCR test did not give the expected result.

Sample Number		Species – place of origin	Virulent/Avirulent APMV-1 qPCR		APMV-1 Multiplex qPCR	
			Ct* ARP2 Probe	Ct VRP2 Primer	Ct APMV Wise Class II	Ct APMV Kim Class I
1	L	Chicken V4	28.50	>40	24.67	>40
2	L	Chicken V4	38.56	>40	30.74	>40
3	V	Pigeon	>40	24.56	NT	NT
4	V	Pigeon	>40	29.98[#]	NT	NT
5	V	Pigeon	>40	29.69[#]	NT	NT
6	V	Chicken - Beaudette	>40	14.82	10.68	>40
7	M	Chicken - Eaves	16.68	>40	9.71	>40
8	V	Chicken - Essex	>40	16.34	14.60	>40
9	V	Chicken - Texas	>40	13.80	10.78	>40
10	L	Chicken	17.91	>40	11.83	>40
11	M	Chicken - Komarov	18.39	18.17	12.07	>40
12	V	Chicken - Indonesia	>40	17.95	13.73	>40
13	V	Chicken - Indonesia	>40	13.63	12.79	>40
14	V	Chicken	>40	13.24	12.48	>40
15	L	Chicken	22.88	>40	15.54	>40
16	V	Chicken - Singapore	>40	13.29	10.93	>40
17	V	Chicken - NSW	>40	13.69	12.69	>40
18	?	Chicken - NSW	>40	14.40	13.75	>40
19	V	Chicken - Vietnam	>40	>40	>40	10.11
20	V	Chicken - PNG	>40	>40	9.46	>40
21	V	Chicken - Ciamis	>40	16.57	11.24	>40
22	V	Chicken - Hertz	>40	31.70	18.42	>40
23	V	Chicken – Bengkulu 2006	>40	14.08	12.38	>40
24	V	Chicken - Jhapa 2007	>40	10.72	18.14[a]	>40
25	V	Chicken – Meredith 2002	>40	12.79	13.46	>40
26	-	APMV-7 Negative control	>40	>40	>40	>40

[#] Confirmed as being virulent using a specific PCR for the APMV-1 strain infecting pigeon in Victoria in 2012 at the CSIRO AAHL laboratories (J. Wang Pers. Comm.)

* Ct refers to the crossing threshold value in Real time PCR. Typically a value >40 is negative, and Ct values < 36-40 are positive (this value can vary depending on the test).

[a] Sample tested negative in Kim/Wise PCR and so was repeated on CSIRO AAHL NDV PCR

NT Not tested

Objective 2:
To screen a selection of NDV positive and negative wild bird samples previously tested in Kim/Wise PCR using the new PCR to compare diagnostic sensitivity

A total of 62 diagnostic and wild bird samples of known APMV-1 status were screened in the new virulent/avirulent multiplex qPCR, and the Wise and Kim qPCR tests (the Kim/Wise multiplex qPCR was evaluated by BSL (DAFF) and co-workers as part of a previous WEDPP project funded in 2010/2011). The samples were selected to represent a range of positive and negative APMV-1 samples and included samples from ducks (10), chickens (49) and pigeons (3)(Refer to Table 2).

All samples that produced a negative result (Ct value of >40) in the Kim/Wise multiplex qPCR test also produced a negative result (Ct value of >40) in the virulent/avirulent multiplex qPCR test. However, 12 samples that produced a positive result in the Wise qPCR test did not produce a positive test result (Ct value of <40) in the virulent/avirulent multiplex qPCR test (For example Table 2; sample numbers 1, 2, 4, 5 , 7, 8 and 10; highlighted in green). In other words, the new PCR gave some false negative results. In all cases where the new virulent/avirulent multiplex qPCR failed to detect the APV-1 genome, the Ct value in the Wise test was greater than 27-28 (weaker positive result). These results suggest that the new virulent/avirulent multiplex qPCR is not as sensitive as the Kim/Wise qPCR and consequently this test would not be recommended as the screening test, but instead be used as a follow up test on Wise qPCR positives.

Ct values for samples which produced positive results for both tests were, in general, 1.5-3 Ct values higher in the virulent/avirulent multiplex qPCR (For example Table 2; sample numbers 15-22) than in the Wise qPCR test. This again supports the fact that the virluent/avirulent multiplex qPCR is not as sensitive as the Kim/Wise multiplex qPCR.

To further test the diagnostic sensitivity of the new virulent/avirulent multiplex qPCR, an additional 460 wild bird samples were screened for the presence of APMV-1 using the Kim/Wise qPCR (refer to Table 3). All wild bird samples were from ducks and included the following species: Pacific black duck, grey teal and unidentified duck species. All samples originated from cloacal samples collected from individual birds. Of the 460 samples screened in the Kim/Wise qPCR, a total of 31 were positive for APMV-1, and 429 tested negative for APMV-1. Surprisingly, all 31 APMV-1 Wise PCR positives tested negative in the new virulent/avirulent multiplex qPCR. This was repeated several times to check for error, however the result remained the same at each testing. The most likely explanation is that the samples were below the limit of detection for the virulent/avirulent multiplex qPCR given that all samples produced a Ct value above 28.60 in the Wise PCR. When stronger positives are encountered in the Wise PCR, the virulent/avirulent multiplex qPCR appears to give more reliable results. Based on these results, we would not recommend the new virulent/avirulent multiplex qPCR for wild bird samples with a low positive result (eg Ct >28) in the Wise PCR.

It was decided to try and further improve the diagnostic sensitivity of the virulent/avirulent multiplex qPCR, given that testing of samples with a high Ct using the Wise/Kim multiplex qPCR yielded negative results using the virulent/avirulent multiplex qPCR. All class II APMV-1 samples with a Ct value of approx >28 using the Wise/Kim multiplex PCR gave no amplification (Ct >40) in the virulent/avirulent qPCR. To improve the sensitivity of the virulent/avirulent qPCR, the concept of a nested PCR was introduced. The first round of the nested PCR used the F-gene primers described by (Collins *et al.*, 1993) which were previously used to characterise Australian

NDV isolates (Peroulis-Kourtis *et al.*, 2002; Peroulis & O'Riley, 2004). For the second round of the PCR, the same virulent/avirulent multiplex qPCR as described in this report (Fuller et al, 2009) was employed. As a proof of concept, the nested qPCR protocol was tested on strong and weak positive avirulent control samples as well as the LEADDR network quality control sample which is derived from a virulent NDV strain (Table 4).

The Ct values for nested virulent/avirulent APMV-1 qPCR on the strong and weak positive avirulent samples improved markedly from those obtained with just the standard virulent/avirulent multiplex qPCR (Table 4). The LEADDR network quality control samples that tested negative in the standard virulent/avirulent APMV-1 qPCR test, tested positive in the nested virulent/avirulent APMV-1 qPCR test although the Ct values for the three replicates varied (Table 4). Further testing of this protocol is required to confirm that it is able to consistently produce amplification products from samples which produce Ct values >28 using the Wise/Kim multiplex PCR. It is also suggested that further work needs to be done to identify if the primer pair used for the first round PCR in the nested virulent/avirulent APMV-1 qPCR are able to amplify the F-gene of all known variants of the virus circulating at present.

Table 2: Virulent/avirulent APMV-1 qPCR and Wise/Kim qPCR results for a range of bird samples. Yellow shading indicates where the different PCR test results correlate; green shading highlights where the virulent/avirulent PCR did not give the expected result.

Sample Number	Species	Virulent/Avirulent APMV-1 qPCR		APMV-1 Multiplex qPCR	
		Ct# ARP2 Probe	Ct VRP2 Primer	Ct APMV Wise	Ct APMV Kim
1	Black duck/Teal	>40	>40	31.6	>40
2	Black duck/Teal	>40	>40	38.41	>40
3	Black duck/Teal	37.78	>40	34.35	>40
4	Black duck/Teal	>40	>40	38.65	>40
5	Black duck/Teal	>40	>40	34.93	>40
6	Black duck/Teal	>40	>40	>40	>40
7	Grey Teal	>40	>40	37.58	>40
8	Grey Teal	>40	>40	30.46	>40
9	Duck	29.71*	>40	28.17	>40
10	Chestnut teal	>40	>40	30.53	>40
11	Chicken	>40	>40	>40	>40
12	Chicken	>40	>40	>40	>40
13	Chicken	>40	>40	>40	>40
14	Chicken	36.72*	>40	29.86	>40
15	Chicken	22.31	>40	20.71	>40
16	Chicken	23.02	>40	20.79	>40
17	Chicken	24.52	>40	21.47	>40
18	Chicken	23.92	>40	20.95	>40
19	Chicken	25.37	>40	23.13	>40
20	Chicken	23.37	>40	20.97	>40
21	Chicken	30.92	>40	28.39	>40
22	Chicken	30.92*	>40	26.45	>40
23	Chicken	>40	>40	>40	>40
24	Chicken	>40	>40	>40	>40
25	Chicken	>40	>40	>40	>40
26	Chicken	>40	>40	>40	>40
27	Pigeon	>40	>40	ND	ND
28	Chicken	>40	>40	>40	>40
29	Chicken	>40	>40	>40	>40
30	Chicken	>40	>40	>40	>40
31	Chicken	>40	>40	>40	>40
32	Chicken	>40	>40	>40	>40
33	Chicken	>40	>40	>40	>40
34	Chicken	>40	>40	>40	>40
35	Chicken	>40	>40	>40	>40
36	Chicken	>40	>40	>40	>40
37	Chicken	>40	>40	>40	>40
38	Chicken	>40	>40	29.34	>40
39	Chicken	>40	>40	28	>40
40	Chicken	>40	>40	>40	>40
41	Chicken	>40	>40	>40	>40
42	Chicken	>40	>40	>40	>40
43	Chicken	>40	>40	30.82	>40
44	Chicken	>40	>40	27.11	>40
45	Chicken	>40	>40	>40	>40

Table 2 continued.....

Sample Number	Species	Virulent/Avirulent APMV-1 qPCR		APMV-1 Multiplex qPCR	
		Ct# ARP2 Probe	Ct VRP2 Primer	Ct APMV Wise	Ct APMV Kim
46	Chicken	28.29*	>40	28.13	>40
47	Chicken	>40	>40	>40	>40
48	Pigeon	>40	>40	>40	>40
49	Chicken	>40	>40	>40	>40
50	Chicken	>40	>40	30.3	>40
51	Chicken	28.88	>40	26.39	>40
52	Chicken	>40	>40	>40	>40
53	Chicken	>40	>40	>40	>40
54	Chicken	>40	>40	>40	>40
55	Chicken	>40	>40	29.4	>40
56	Chicken	>40	>40	>40	>40
57	Chicken	>40	>40	>40	>40
58	Chicken	>40	>40	>40	>40
59	Chicken	33.68	>40	27.73	>40
60	Chicken	>40	>40	>40	>40
61	Chicken	>40	>40	>40	>40
62	Chicken	>40	>40	>40	>40

* Confirmed as being avirulent based upon sequence analysis of the fusion gene

\# Ct refers to the crossing threshold value in Real time PCR. Typically a value >40 is negative, and Ct values < 36-40 are positive (this value can vary depending on the test).

Table 3: Virulent/avirulent APMV-1 qPCR results for a range of wild bird samples that tested positive in the Wise/Kim qPCR test used to pre-screen the samples for the presence of APMV-1.

Sample Number#	Species	APMV-1 Multiplex qPCR		Virulent/Avirulent APMV-1 qPCR	
		APMV Kim qPCR Ct	APMV Wise qPCR Ct	Ct ARP2 Probe	Ct VRP2 Primer
578	Grey Teal	>40	32.18	>40	>40
589	Wood Duck	>40	37.89	>40	>40
595	Wood Duck	23.52	>40	>40	>40
596	Grey Teal	>40	37.37	>40	>40
642	Chestnut Teal	30.45	33.80	>40	>40
657	Chestnut Teal	30.91	38.59	>40	>40
800	Pacific black duck	28.12	>40	>40	>40
805	Pacific black duck	32.56	>40	>40	>40
816	Pacific black duck	35.56	>40	>40	>40
848	Duck	>40	30.97	>40	>40
856	Duck	33.66	31.87	>40	>40
858	Duck	>40	29.31	>40	>40
859	Duck	>40	33.10	>40	>40
866	Duck	33.10	29.74	>40	>40
873	Duck	>40	32.89	>40	>40
875	Duck	25.07	31.58	>40	>40
896	Duck	>40	29.36	>40	>40
897	Duck	34.68	30.30	>40	>40
898	Duck	>40	35.10	>40	>40
902	Duck	>40	31.02	>40	>40
909	Duck	>40	31.02	>40	>40
912	Duck	>40	33.10	>40	>40
921	Duck	32.89	28.60	>40	>40
923	Duck	>40	31.57	>40	>40
930	Duck	34.41	>40	>40	>40
931	Duck	>40	30.80	>40	>40
975	Pink ear	34.12	>40	>40	>40
986	Grey Teal	27.92	>40	>40	>40
995	Pink ear	>40	36.51	>40	>40
1001	Grey Teal	30.83	>40	>40	>40
1008	Grey Teal	23.02	>40	>40	>40
429 samples	Mixed duck	>40	>40	NT	NT

Sample numbers are the same as used in the Avian influenza project and have not been renumbered for this project to avoid confusion.

NT Not tested

Red Also Positive in AIV PCR test

Table 4: Virulent/avirulent APMV-1 qPCR and nested Virulent/avirulent APMV-1 qPCR results for a set of control samples positive in the Wise/Kim qPCR test used to pre-screen the samples for the presence of APMV-1.

Samples	Class	Vir/Avir	Virulent/Avirulent APMV-1 qPCR		Nested Virulent/Avirulent APMV-1 qPCR		APMV-1 Multiplex qPCR	
			ARP	VRP	ARP	VRP	APMV Kim qPCR	APMV Wise qPCR
Strong Positive control	II	A	28.50	>40	5.19	>40	>40	24.67
Weak Positive control	II	A	38.56	>40	17.53	>40	>40	30.74
NDV NQC replicate 1	II	V	>40	>40	>40	16.47	>40	32.55
NDV NQC replicate 2	II	V	>40	>40	>40	15.70	>40	32.39
NDV NQC replicate 3	II	V	>40	>40	>40	35.86	>40	32.42

Objective 3:
To technology transfer the newly acquired NDV PCR test to Biosecurity Sciences Laboratory (BSL, Queensland Department of Agriculture, Fisheries and Forestry, DAFF)

All information regarding the test of choice and the conditions for use were provided to BSL. A small change in the running conditions were needed compared with the DPI Vic laboratory given that the QLD laboratory use a different Real time PCR platform (Corbett compared to Applied Systems at DPI Vic). Therefore the appropriate reagents were used to optimise the test at the QLD laboratory.

There are long delays in receiving the probe after ordering due to the locked nucleic acid requirement which complicates the manufacture. Therefore, DPI Vic sent primers and probes to BSL whilst their stocks are on order. This had the added advantage of standardising an additional component of the testing given that identical batches of primers and probes were used at both laboratories.

BSL tested a total of 26 samples in the newly transferred virulent/avirulent NDV PCR test (refer to Table 5). This included a range of virulent (n=8), avirulent (n=15) and negative (n=3) NDV samples. As expected, none of the three Class I NDV or three negative samples were detected in the virulent/avirulent NDV PCR. Of the 20 Class II APMV-1 samples tested, 18 samples were correctly detected in the virulent/avirulent multiplex qPCR, and two samples were not detected in the virulent/avirulent multiplex qPCR. These results very much reflect the results obtained by the DPI Victoria group, where the virulent/avirulent multiplex qPCR was able to correctly identify the majority of the reference strains, however those with high Ct values (low viral load) were missed. This reflects the lack of sensitivity of the multiplex qRT-PCR on the Corbett instrument. This is in agreement with the observations made on the Applied Biosystem instrument. The lack of sensitivity was also illustrated by the inability of both PCRs to detect the NQC-1, a highly dilute RNA from the Deans Park isolate, a virulent NDV.

Objective 4:
Undertake phylogenetic analysis of pathotyping sequencing results to show relationships of clades

It was planned to undertake phylogenetic analysis for any virulent strains detected during the course of this study. However, despite testing only avirulent viruses were detected and therefore the phylogenetic comparisons could not be completed.

Table 5: BSL (Queensland DAFF) Fuller Real time PCR (ARP/VRP) results using a standard panel of APMV-1 positive samples. (Note that the same batch of primers and probe were used for both DAFF and DPI Vic, however a Corbett Rotor-Gene PCR machine was used at BSL)

Samples	Class	Vir/Avir	Virulent/Avirulent APMV-1 qPCR	
			ARP	VRP
Nepal Kaski	II	V		31.07
Nepal Kailali	II	A	32.48	
Nepal 1111	II	V		23.16
Nepal 1114	II	V	ND	ND
VN 1116	II	V		21.21
F Strain (vaccine)	II	A	29.95	
WA 1886	I	A	ND	ND
WA 4317	II	A	24.65	
WA 5332	II	A	22.34	
WA 4359	II	A	24.90	
WA 4386	II	A	22.31	
WA 4405	II	A	27.21	
5760	II	A	43.17/-	
APMV1 Shep 1109-14-4303	II	V?		31.17
Ibis/Qld 0702-14-1242	I	A	ND	ND
Ck/Nepal/Kaski 0804-18-0210	II	V		39.14
F strain	II	A	29.64	
Negative	-	ND	ND	
Negative	-	ND	ND	
V4	II	A	28.89	
Deans Park	II	V		38.66
V4	II	A	28.09	
A/Ch/NSW/Aust/CV10-1004-3/2010/H10N7	-	ND	ND	
NDV NQC-1	II	V	ND	ND
116603	I	A	ND	ND
NDV V4	II	A	31.87	

ND Not detected

Conclusions

This project has seen the successful introduction of a new qPCR test at DPI Victoria capable of differentiating virulent from avirulent APMV-1. A test such as this is invaluable for use with diagnostic avian samples because the current variety of tests available for screening for APMV-1 can only provide a positive or negative result for the presence of APMV-1 regardless of whether or not the strain of APMV-1 is virulent or avirulent. This is further complicated by the extensive use of the NDV vaccine in which case samples from vaccinated birds appear positive in the screening tests due to the avirulent V4-like strain of NDV used in the vaccine. A test capable of confirming that those screening test positives are from avirulent APMV-1 would therefore be a huge advantage.

Whilst duplicate samples were not tested at both laboratories, the transfer of technology to the Biosecurity Sciences laboratory in Queensland (DAFF) was successful. Both the Corbett and Applied Biosystems instruments appear to have performed similarly, however identical samples would need to be compared to confirm any differences in the detection limits between the platforms employed. Furthermore, on both machines two virulent APMV-1 were not detected, underlining the lower sensitivity of the multiplex avirulent/virulent qRT-PCR compared to the Kim/Wise multiplex qRT-PCR.

The virulent/avirulent multiplex qPCR developed by Fuller *et al.* (2009), was chosen as the best alternative of a test capable of differentiating APMV-1 strains. In our hands, the virulent/avirulent multiplex qPCR worked well with laboratory strains of APMV-1, however it is not as sensitive as other tests available for the detection of APMV-1. Direct comparisons of positive samples tested in the Kim/Wise qPCR and the virulent/avirulent multiplex qPCR showed Ct values approximately 1.5-3 cycles lower (detected earlier) in the Kim/Wise test. In addition the virulent/avirulent multiplex qPCR totally missed some of the Kim/Wise qPCR positives which would lead to the wrong assumption of a false negative result if this test was being used as a screening test. The reason for this is likely to be the complexity of the two probe system and the locked nucleic acid requirement all of which are attempting to hybridise pathogen nucleic acid where there are inherent variations within the area of the genome that the test is designed to.

The virulent/avirulent multiplex qPCR proved to be robust enough to use on infected chicken and pigeon samples with relatively high viral loads. However it did not perform well with wild bird samples that presumably have a much lower viral load and so consequently had a high Ct value in the Wise qPCR.

Therefore in conclusion, we recommend that the Kim/Wise multiplex qPCR or other such qPCR continue to be used as the screening test to detect APMV-1. The new virulent/avirulent multiplex qPCR should only be used on known PCR positive samples in an attempt to determine whether the Class II APMV-1 virus detected was virulent or avirulent. In the case of a weak positive PCR screening result, there is a high likelihood that the virulent/avirulent multiplex qPCR will not provide a result. Preliminary results at this laboratory have shown that introducing a nested PCR format using the F gene PCR of Collins *et al.*, (1993) as a first round PCR prior to conducting the Fuller *et al.*, (2009) virulent/avirulent multiplex qPCR, may resolve some of the issues with the diagnostic sensitivity on samples with a lower level of nucleic acid.

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References

Alexander DJ. The epidemiology and control of avian influenza and newcastle disease. *J Comp Path* 1995;112:105-126

Beard PD, Spalatin J, Hanson RP. (1970). Strain identification of Newcastle Disease Virus in tissue culture. *Avian Diseases* 14(4):636-45.

Collins, M. S., Bashiruddin, J. B. & Alexander, D. J. (1993). Deduced amino acid sequences at the fusion protein cleavage site of Newcastle disease viruses showing variation in antigenicity and pathogenicity. *Arch Virol* **128**, 363-370.

Fuller C.M., M. S. Collins & Alexander, D. J. (2009). Development of a real-time reverse-transcription PCR for the detection and simultaneous pathotyping of Newcastle disease virus isolates using a novel probe) Arch Virol 154:929–937

Huovilainen A, Ek-Kommonen C, Manvell R, Kinnunen L. Phylogenetic analysis of avian paramyxovirus 1 strains isolated in Finland. *Arch Virol* 2001;146:1775-1785.

Kim LM, Suarez DL, Afonso CL. (2008). Detection of a broad range of class I and II Newcastle disease viruses using multiplex real-time reverse transcription polymerase chain reaction assay. *J Vet Diagn Invest* **20**:414-425.

Mackenzie JS, Edwards EC, Holmes RM, Hinshaw VS. Isolation of ortho- and paramyxoviruses from wild birds in Western Australia and the characterisation of novel influenza A viruses. *Aust J Exp Biol Med Sci* 1984;62:89-99

Peroulis-Kourtis, I., O'Riley, K., Grix, D., Condron, R. J. & Ainsworth, C. (2002). Molecular characterisation of Victorian Newcastle disease virus isolates from 1976 to 1999. *Aust Vet J* **80**, 422-424.

Peroulis, I. & O'Riley, K. (2004). Detection of avian paramyxoviruses and influenza viruses amongst wild bird populations in Victoria. *Aust Vet J* **82**, 79-82.

Wise MG, Suarez DL, Seal BS, Pedersen JC, Senne DA, King DJ, Kapczynski, Spackman E. (2004). Development of a real-time reverse-transcriptase PCR for detection of Newcastle disease virus RNA in clinical samples. *J Clin Microbiology* **42**:329-338.

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