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Submitted by

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Project Title: **Use Of A Multiplex Real-Time Pcr For The Detection And Differentiation Of Class I And Class Ii Newcastle Disease Viruses (NDV) In Wild Bird Species And Poultry**

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Project Summary

A multiplex real-time PCR targeting the polymerase gene of class I Newcastle disease viruses (NDVs) and the matrix gene of class II NDVs evaluated during the course of a WEDPP funded project during 2009-2010 (Diallo *et al.*, 2010) was used routinely to test samples from wild birds and poultry samples submitted for surveillance and routine diagnostic investigations. A total of 161 poultry samples and 1421 wild birds' samples have been tested using the multiplex real-time PCR. The 1421 wild birds included a variety of species roosting in the Moreton Bay and in Northern Queensland. Two samples (one wild bird sample and one poultry sample) were weak positives in the class II NDV real-time PCR. Further testing confirmed that the positive poultry sample was from a broiler vaccinated against NDV using a V4 vaccine. Attempts to isolated NDV from the wild bird sample failed to yield a haemagglutinating virus.

The NDV multiplex real-time PCR was improved further by multiplexing it with a real-time PCR targeting the 18S rRNA described by Heine *et al.* (2007). The incorporation of such real-time PCR allows checking of the PCR competency of tested samples, therefore preventing the reporting of false negatives. Further work undertaken at the Australian Animal Health Laboratory (AAHL, CSIRO) and Biosecurity Sciences Laboratory (BSL, Queensland) have highlighted the need for multiplexing 2 class II NDV real-time PCRs targeting the fusion gene (AAHL real-time PCR) and the matrix gene (Wise *et al.* real-time PCR) to allow the detection of all class II NDVs.

The multiplex real-time PCR has been made available to other state laboratories through the Laboratories for Emerging Diseases and Emergency Response (LEADDR) network.

A published pan-avulavirus PCR was evaluated for the detection of other avian paramyxoviruses (APMV) other than NDV (APMV-1). The PCR was found to be non-specific and its evaluation was discontinued.

The NDV multiplex real-time has been multiplexed with the generic influenza virus type A (IVA) real-time used for the detection of type A influenza viruses (Heine *et al.*, 2007). The new multiplex NDV/IVA was shown to be specific and sensitive.

Introduction

Newcastle disease is a devastating disease of poultry that causes enormous economic losses to the poultry industry worldwide (Alexander, 2001). The disease is caused by avian paramyxovirus type 1 (APMV-1) (commonly known as Newcastle disease virus – NDV), a single-stranded negative sense RNA virus belonging to the *Avulavirus* genus of *Paramyxoviridae.* There are number of classifications of NDVs based on antigenic and genotypic variations of their fusion protein and gene respectively (Alexander *et al.*, 1986, 1992 & 1999; Ballagi-Pordany, *et al.* 1996; Collins *et al.*, 1993 & 1998; Aldous *et al.* 2003). Of special interest is the work by Czegledi *et al.* (2006), where the authors describe 2 classes of NDV (class I and class II) based on genomic data and sequencing of the fusion (F) and RNA-directed RNA polymerase (L) genes. These 2 classes contain the entirety of previously described genotypes and lineages. The work of Aldous *et al.* (2003) grouped all avirulent APMV-1 in lineage 1 and lineage 6, which contain among other groups the H group NDVs as defined by monoclonal antibodies. This particular group H contains mainly isolates from wild birds such as MC110/77 (isolated from a shelduck in France), NZ/1/97 and Fin/97 (isolated from mallard ducks in New Zealand and Finland respectively). Although isolates belonging to this group are usually of low pathogenicity (Alexander, 2001), this group also contains two chicken isolates (APMV-1/chicken/Ireland/34/90 and APMV-1/chicken/Ireland/48/90) that were shown to be highly virulent for poultry and that caused outbreaks in Ireland in 1990 (Alexander *et al.* 1992).

Wild birds are reservoirs of NDV (Alexander, 2001; Takakuwa *et al.*, 1998). Alexander *et al.* (1986) isolated in Western Australia 14 isolates of NDV from wild birds belonging to the orders *Charadriiformes, Passeriformes* and *Anseriformes* and showed that the isolates could be divided in two groups based on their reactivity to monoclonal antibodies: V4-like (class II NDV), similar to APMV-1/chicken/Qld/V4/66 (Simmons, 1967) and MC110-like (class I NDV), similar to APMV-1/shelduck/France/MC110/77. The V4-like isolates were found mainly in *Charadriiformes* and *Passeriformes* and the MC110–like isolates were found in *Charadriiformes* and *Anseriformes*. The authors concluded that the isolation of V4-like NDV in passerine birds was significant as they are more likely to come in contact with poultry. This contrasted with a study carried out by Garnett and Flanagan (1989) in northern Queensland, which failed to detect any haemagglutinating viruses and

antibodies against NDV in 1235 birds surveyed. Similarly, the testing of 257 samples from Anseriform birds (Magpie geese) from the Kakadu National Park in 2007 (Diallo *et al.* 2007) failed to detect any APMV-1. However, in 2006 the testing of an ibis that died of unknown causes resulted in the isolation and identification of an NDV belonging to class I (Gordon and Field, 2006). The virus isolated from the ibis (Qld/116603/06) is a class I NDV and is different from the more widespread and more described Queensland V4 isolated by Simmons in 1966 (Simmons, 1967), which belongs to class II NDVs. Its fusion and matrix genes were not amplified by any of the then published NDV PCRs, which were developed using sequences from poultry isolates and therefore detect only V4-like NDV.

Funding secured from WEDPP in 2006-2007 allowed the development of a real-time PCR that would detect class I NDVs. Further funding, from WEDPP in 2009-2010, allowed the evaluation and validation of other real-time PCRs for the detection and differentiation of class I and II NDVs. As a result a multiplex real-time combining a real-time PCR targeting the polymerase gene of class I NDVs (Kim *et al.,* 2008) and a real-time PCR targeting the matrix gene of class II NDVs (Wise *et al.,* 2004) was validated and adopted for implementation in state laboratories for routine diagnostic and surveillance of class I and II NDVs in both poultry and wild birds.

The aim of the current project was to use the multiplex real-time PCR for Newcastle disease investigation in wild birds and poultry. The use of such real-time PCR was to be extended to all interested state laboratories. The project had also the aim of evaluating a published conventional PCR that can detect all Avulaviruses. This would allow the investigation of other APMVs in wild birds and poultry. Finally, the project would evaluate the multiplexing of the NDV multiplex real-time PCR with the generic IVA real-time PCR, which would allow the simultaneous detection of not only both classes of NDVs but also IVA in wild bird and poultry samples.

The current report describes the use of the multiplex NDV real-time PCR, its combination with the generic IVA real-time PCR used for the detection of IVA and the evaluation of a published Avulavirus PCR.

Objectives

The aims of this project are to:

1. Use the NDV multiplex real-time PCRs targeting the polymerase gene of class I and the matrix gene of class II NDVs routinely for all Newcastle disease investigations in wild birds as well as in domestic poultry.
2. Evaluate a published Avulavirus PCR for its ability to detect other APMV other than APMV-1.
3. Transfer the multiplex NDV real-time PCR to other state laboratories for further evaluation and routine use as diagnostic and investigation tool.
4. Multiplex the NDV multiplex real-time PCR with the generic IVA real-time PCR widely used in Australia for the detection of IVA.

Study design

The validated NDV multiplex real-time was used to routinely test samples from wild birds and from domestic poultry. RNA was extracted using the MagMax (Ambion, Applied Biosystems, Scoresby, Australia) magnetic bead technology (ThermoFisher, Scoresby, Australia). Resulting RNA was evaluated for PCR competency using the 18S rRNA, house-keeping gene real-time PCR. The 18S rRNA real-time PCR was multiplexed with the NDV multiplex real-time PCR to allow a one tube reaction.

A published Avulavirus generic PCR was evaluated for its ability to detect other APMVs. Primers were ordered and tested against NDV strain V4 and an MC-110 like isolate from an ibis.

The NDV multiplex real-time PCR was further multiplexed with the IVA real-time PCR and evaluated. The 4-plex real-time PCR (18S rRNA, class I and II NDV and IVA) was evaluated by testing known positive and negative NDV and IVA samples. The testing of an IVA proficiency panel of 10 samples was also included in this study. The results were then compared to results from monoplex NDV and IVA real-time PCRs to check if there was any loss of sensitivity. The samples used were cloacal and tracheal samples from dead and sick poultry and faecal samples from healthy wild birds. The bird samples were derived from submissions to BSL and other laboratories of the Biosecurity Veterinary Laboratory Network (Queensland) in conjunction with ongoing avian influenza surveillance activities in Queensland.

Positive samples were forwarded to AAHL for confirmation by real-time PCR and characterisation including virus isolation, haemagglutination (HA), haemagglutination inhibition (HI) and electron microscopy (EM).

Materials and Methods

Virus isolates and bird samples:

A total of 161 poultry samples and 1421 wild bird samples were tested using the NDV multiplex real-time PCR. The 1421 wild birds included a variety of species roosting in the Moreton Bay and Northern Queensland.

NDV positives (V4 and APMV-1/Ibis/Qld/116603-2006) and IVA positive (proficiency testing panel and positive wild bird samples) were included in this study for the evaluation of the 4-plex real-time PCR that combines the NDV multiplex real-time PCR with the IVA real-time PCR and the 18S rRNA house-keeping gene real-time PCR.

Nucleic acid extraction and Polymerase Chain Reaction

RNA Extraction

RNA from the 161 poultry samples and 1421 from wild birds was extracted using MagMax . The method was modified to remove PCR inhibitors. A modified MagMax protocol was used, where samples were mixed with the bead mix first and left to incubate for 30 min to an hour (P. Kirkland personal communications) and then the extraction carried out as recommended by the manufacturer. Template addition was performed using the CAS 1200 robot (Qiagen, Australia).

Real-time Polymerase chain reactions

NDV multiplex real-time PCR combined with the 18S rRNA real-time PCR:

The NDV multiplex real-time PCR, which detects class I and class II NDV was multiplexed with the 18S rRNA house-keeping gene real-time PCR. In order to achieve this, the 18S rRNA probe was labelled with Cy5 fluorophore at the 5’ end and had a BHQ-3 quencher at the 3’ end. The choice of Cy5 (red channel) was motivated by the fact that this real-time PCR was multiplexed with the NDV multiplex real-time PCR which already uses the FAM and VIC™ labels. Furthermore Cy5 has an

excitation wavelength of 625 nm and an emission wavelength of 660 nm, which is far removed from the excitation and emission wavelength of 2 channels: 470 and 510 for FAM and 530 and 555 for VIC™. The resulting multiplex real-time PCR was used to test DNA extracts from clinical samples used in this study to show PCR competency of the DNA extracts assayed and NDV reactivity in a single tube assay.

Four-plex (18S rRNA, class I and II NDV and IVA) real-time PCR:

The IVA real-time PCR was described by Heine *et al.* (2007) and has been used in Australia for avian influenza surveillance. During the equine influenza (EI) outbreak in 2007-2008 in Australia, the IVA real-time PCR was also used for the diagnosis of and the proof-of-freedom from EI. This real-time PCR is a fully validated PCR. The multiplexing of the IVA real-time PCR with the NDV multiplex real-time PCR will allow the detection of NDV and IVA simultaneously and would be performed in a single tube. To achieve this, the IVA probe was labelled with the fluorophore Rox at its 5' end and a TAMRA quencher at its 3' end. The resulting multiplex real-time PCR is a 4-plex real-time PCR that can detect and differentiate the 2 classes of NDV, IVA and it contains the 18S rRNA house-keeping gene real-time PCR, which allows the checking of the PCR competency of samples under investigation.

Cycling conditions and platforms

The cycling parameters were those described for the NDV multiplex real-time (Wise *et al.*, 2004 and Kim *et al.*, 2008). This is a three-step real-time PCR consisting of a reverse transcription at 50°C for 15 min and an amplification performed by initial activation of the Taq polymerase at 95°C for 2 min, followed by a denaturation step at 95°C for 10 sec and annealing at 52°C for 30 sec and an extension at 72°C for 15 sec. Fluorescence acquisition was performed at the extension stage either on the green channel (for FAM), yellow channel (for JOE/VIC), red channel (for Cy5) and orange channel (for Rox). The mastermix used for all of the real-time PCRs performed at BSL was the SuperScript® III Platinum One-step qRT-PCR (Invitrogen, Australia).

The platforms used are as follows:

- RotorGene 3000 and 6000 in BSL (Brisbane, Queensland) and Animal Health Laboratory (Perth, WA)
- ABI7500 and 7900 in AAHL (Geelong, VIC) and DPI Victoria (Attwood, VIC)

Evaluation and validation of the 4-plex real-time PCR

The 4-plex real-time PCR was used to test known positive and negative NDV and IVA samples. AN avian influenza (AI) proficiency testing panel comprising 10 samples of unknown reactivity was included in the validation of the 4-plex real-time PCR. Furthermore 10-fold dilutions of NDV and IVA positives were also tested. The results were compared with the monoplex NDV and IVA real-time PCRs to ensure that the multiplexing would not result in loss of sensitivity for any of the 3 main components (NDV class I and II and IVA).

The 4-plex real-time PCR was evaluated on the basis of:

- *Sensitivity:*

The sensitivity was evaluated on the basis of the determination of the cut-off. Ten-fold dilutions of RNA from the positive control were tested in each real-time PCR. The cut-off is determined as the highest dilution at which the real-time PCR gives a Ct value as confirmed by the presence of an amplicon of the right size on a 1.5% gel visualised under UV light using the Quantity one Gel Documentation System (BioRad, Gladsville, Australia). The results were then compared to the results of the monoplex real-time PCR to ensure that there was no loss of sensitivity when the real-time PCRs are multiplexed.

- *Specificity:*

The specificity of all 4 components of the 4-plex real-time PCR was determined previously as these real-time PCRs have been validated previously. Briefly, the specificity of the NDV multiplex real-time PCR was assayed by testing extracted nucleic acids (NA) from avian influenza A virus, infectious bronchitis virus, infectious laryngotracheitis virus, fowlpox virus, *Avibacterium paragallinarum* and *Pasteurella multocida*. Likewise, the IVA real-time specificity was assayed by testing extracted nucleic acids (NA) from class I and II NDVs, infectious bronchitis virus (IBV), infectious laryngotracheitis virus (ILTV), fowlpox virus (FWPV), *Avibacterium paragallinarum* and *Pasteurella multocida*. The evaluated real-time PCR was assessed as specific if the above-listed NA did not react in the real-time PCR.

Evaluation of the NDV multiplex real-time PCR at AAHL and BSL

During the course of the project known positive class I and class II NDVs from clinical samples as well as archival samples were tested using 2 real-time PCRs targeting the fusion gene (AAHL PCR) and the matrix gene (Wise *et al.* PCR). Further evaluation of the 2 real-time PCRs was carried out at AAHL using endemic and exotic NDV strains.

Technology transfer to state laboratories and AAHL

The NDV multiplex real-time PCR was transferred to interested state laboratories under the umbrella of the Laboratory for Emergency Animal Disease Diagnosis and Response (LEADDR) NDV working group. The technology was therefore transferred to Victoria, NSW and Western Australia. Once evaluated by all state laboratories the NDV multiplex real-time PCR will be used in the LEADDR proficiency testing for NDV.

Evaluation of the generic Avulavirus PCR

A conventional PCR described by Tong *et al.* (2008) for the detection of other APMVs that might occur in wild birds and poultry was evaluated. Primers were ordered from Sigma-Aldrich (Sydney, Australia) and known class I and II NDVs were tested in the PCR to evaluate its sensitivity and specificity.

Results

Testing of clinical samples from wild birds and sick poultry

A total of 161 poultry samples and 1421 wild bird samples have been tested using the NDV multiplex real-time PCR. Two samples (one wild bird sample and one poultry sample) were weak positives in the class II NDV real-time PCR. Further testing at AAHL confirmed that the poultry sample, which came from a broiler vaccinated using a V4 NDV vaccine, was positive. Attempts at AAHL to isolate NDV from the wild bird sample failed to yield any haemagglutinating virus. Occasionally high Ct values in class II real-time PCR were observed from faecal samples from wild birds. However, subsequent testing of such samples using conventional RT-PCRs gave no bands or multiple non-specific bands.

Evaluation and validation of the 4-plex real-time PCR

The 4-plex PCR performed relatively well when 10-fold dilutions of positive controls (NDV and IVA) and proficiency testing samples were used. However, there was a

loss of sensitivity of approximately 1 log (Ct = 3-4) for the NDV multiplex real-time PCR.

Sensitivity of the 4-plex real-time PCR

The 4-plex real-time PCR was shown to be sensitive. However, there was a slight loss of sensitivity for both NDV real-time PCRs when compared to the monoplex real-time PCRs, with Ct values approximately 3-4 cycles higher than those obtained with the corresponding monoplex PCRs. This was equivalent to a 10-fold dilution.

On the other hand, the IVA real-time PCR showed a gain of sensitivity. The testing of positive wild bird samples and samples from a proficiency testing panel showed an increased sensitivity marked by lower Ct values, with the highest decrease in Ct value was 9 Ct values, which corresponds to approximately 3 logs. The difference between the monoplex IVA real-time PCR and the IVA in the 4-plex set up was firstly the fluorophore used to label the probe. The IVA probe in the monoplex real-time PCR was labelled with FAM while the IVA probe in the 4-plex real-time PCR was labelled with Rox. Secondly, the difference was the cycling parameters, whereas the IVA monoplex real-time PCR was run as a 2-step while the 4-plex real-time was run as a 3-step real-time as described by Kim *et al.* (2008). Further testing have shown that the difference may lay in the difference in the fluorophore used as the comparison of the cycling parameters of the monoplex real-time PCR showed no difference in Ct values between the 2 step and the 3-step real-time PCR.

Specificity of the 4-plex real-time PCR

The 4-plex real-time PCR was shown to be specific as none of the avian pathogens assayed in the 4-plex real-time PCR was positive in this system while class I and II NDV and the avian influenza positive samples including the positive controls reacted in the 4-plex real-time PCR in their respective systems.

At AAHL, the evaluation of the 4-plex real-time PCR assays for detection of NDV and AIV has been complicated by the finding that three different assays were required for detection of all NDV strains (one class I NDV real-time PCR, 2 class II NDV real-time PCRs). The addition of another assay for detection of IVA in the multiplex reaction and the inclusion of an internal control using 18S rRNA is at the technical limit for multiplex capabilities of real-time PCR instruments. Combinations of NDV

and AIV assays are currently being investigated and will be established by the influenza and NDV working groups of the LEADDR program.

Technology transfer and evaluation of NDV multiplex real-time at AAHL and BSL

Testing of endemic as well as exotic strains of NDV carried out at AAHL has shown that both the AAHL real-time PCR and the Wise *et al.* real-time PCR do not detect all class II NDVs. The AAHL real-time PCR detected some class II and not others and the Wise *et al.* real-time PCR also detected some class II NDV not detected by the AAHL real-time PCR but was unable to detect other class II detected by the AAHL real-time PCR. However, multiplexing of these real-time PCRs allowed the detection of all class II NDVs showed some loss of sensitivity. All class I NDVs were detected by the Kim *et al.* real-time PCR.

Other state laboratories including BSL have not experienced such problems as state laboratories had no access to the range of NDV strains available at AAHL, especially the exotic strains of NDV. In fact, state laboratories, such as the Victorian state laboratory and the Western Australian have used the NDV multiplex real-time PCR and have not reported any issues. The Victorian state laboratory has used the NDV multiplex real-time PCR to detect 1 class I NDV and 9 class II NDVs in wild birds (Warner et al., 2011). This was part of a service agreement funded by WEDPP and carried out at Attwood. The samples have been transferred to AAHL for confirmation and typing.

Evaluation of the Avulavirus PCR

The generic Avulavirus PCR (Tong *et al.* 2008) was tested against known NDVs and was shown to be non-specific and non-sensitive. The class I NDV positive control did not react in this PCR and the class II NDV positive gave higher Ct values than Wise *et al.* real-time PCR, which is characteristic of lack of sensitivity. No further evaluation was carried out on this PCR.

Similar results were obtained at AAHL. When the generic Avulavirus conventional hemi-nested PCR assay described by Tong *et al.* (2008) was investigated for NDV detection it showed low analytical sensitivity for detection of NDV. Modified assays using primers optimised for NDV have not as yet improved the assay sensitivity and investigations are continuing.

Discussion

The NDV multiplex real-time PCR for the detection and differentiation of class I and II NDVs was shown to be sensitive and specific. In the current project, the NDV multiplex real-time PCR was used to test samples from wild birds and poultry samples. The wild bird samples were faecal and cloacal samples obtained in the Moreton Bay Redland area of Queensland and Northern Queensland, while the poultry samples were samples submitted to BSL for routine exclusion of either avian influenza or Newcastle disease or both. All poultry samples were made of tracheal and cloacal samples collected either on live sick birds or dead birds. The use of the NDV multiplex real-time PCR for the testing of faecal samples from wild bird highlighted the presence of inhibitors in RNA extracted from those samples. It is well established that faecal samples contain PCR inhibitors, especially if the samples are from an animal with a plant based diet (Das *et al.*, 2009). In the current project, steps were taken to alleviate this problem. In the first instance the RNA extraction method was modified to remove PCR inhibitors. This was done by modifying the MagMax magnetic bead method, where the samples were first mixed with the bead mixture and subsequently lysed and RNA extracted. Additional modification consisted of extra washes during the extraction procedure. Furthermore, the NDV multiplex real-time PCR was improved by multiplexing it with a house-keeping gene real-time PCR targeting the 18S rRNA (Heine *et al.* 2007). This was necessary as the majority of samples tested were faecal/environmental samples. In order to increase confidence in the results reported it was necessary to make this change.

Evaluation of the NDV multiplex real-time PCR at AAHL using endemic and exotic strains of NDV has revealed that some exotic strains of NDV were not detected by the Wise *et al.* real-time PCR while they were detected by the AAHL real-time and some NDV strains detected by the Wise *et al.* real-time PCR were not detected by the AAHL real-time PCR. A similar observation was described also by Fuller *et al.* (2010) who showed that the Wise *et al.* and Kim *et al.* real-time PCRs could not detect some class I NDVs (lineage 6) as well as some class II NDVs (lineages 1, 4b and 5d). To overcome this problem they designed a real-time PCR that uses 2 FAM-labelled probes for the detection of both class I and class II NDVs. Within the LEADDR NDV working group another approach was contemplated. This would

consist of multiplexing the class II NDV real-time PCRs (AAHL and Wise *et al.*). However, preliminary work at AAHL suggested that there was a loss of sensitivity when the class II NDV multiplex real-time PCR was used. Further evaluation of this multiplex will be undertaken at BSL.

Despite the numerous samples tested only 2 samples (one wild bird sample and one poultry sample) returned weak positive results. Attempts to isolate NDV from these samples at AAHL were unsuccessful. These results agree with the results reported by Garrnett and Flanagan (1989). These authors tested 1235 samples from 130 wild bird species from Northern Queensland and they found no haemagglutinating virus.

The 4-plex real-time PCR would be a good surveillance tool as it detects and differentiates simultaneously class I and II NDV and IVA. In the case of surveillance of these viruses in wild birds the use of such a technique will allow a single-tube testing of samples, which would help in high throughput situations. The loss of sensitivity of both NDV real-time PCRs in the 4-plex set up was minimal (3-4 Ct values). This is not an issue as during an active infection with any of these two pathogens the titres of the virus would be very high and therefore easy to detect. On the other hand, the use of the 4-plex real-time PCR resulted in increased sensitivity for the IVA real-time PCR. This was probably due to the fluorophore used to label the IVA probe as the comparison of varying cycling parameters yielded similar results. The IVA probe used in the 4-plex real-time PCR was labelled with Rox, while the probe used in the IVA monoplex real-time PCR was labelled with FAM. The use of the Rox-labelled probe resulted in an improved analytical sensitivity even in difficult samples such as faecal or environmental samples from wild birds.

Other projects complementary to WEDDP have been conducted by research and diagnostic groups at AAHL to improve diagnostic capabilities for NDV and IVA.

A Luminex bead array assay system has been investigated to complement real-time PCR assays and increase the capabilities for multiplex detection of viral RNA. A 10-plex assay panel has been developed that can detect and differentiate IVA, NDV class 2 and class 1 as well as West Nile virus and infectious bursal disease virus. The Luminex assays will be further developed and evaluated for application in routine diagnostic operation.

Summary and Recommendations

1. Although the NDV multiplex real-time PCR was shown to be a sensitive and specific tool, very few NDVs were detected during the course of this project. Only 2 samples returned weak positive results, one of which confirmed to be of vaccine origin and the second did not yield any haemagglutinating virus in virus isolation. These results show the low prevalence of both classes of NDV in wild birds and poultry. According to these results, class I NDVs are not widespread unlike class II. However, there is still a necessity to use the NDV multiplex real-time PCR, as other studies have shown that class I NDV can be found in poultry and although there are usually of low virulence, they can mutate and spark outbreaks in poultry (Collins *et al.*, 1998)
2. The 4-plex real-time PCR combining the class I and II NDV, the IVA and 18S rRNA real-time PCRs was shown to be sensitive and specific. The 4-plex real-time PCR would be a good surveillance and screening tool. The use of such a tool will allow high throughput testing as the testing would be carried out in a single tube.
3. During the course of this project the NDV multiplex real-time PCR has been successfully transferred to other state laboratories (DPI Attwood, VIC; Department of Agriculture, Perth, WA and Elizabeth Macarthur Agriculture Institute, NSW) and the Australian Animal Health Laboratory (AAHL, Geelong, VIC).
4. Based on the results obtained at AAHL, there is a further need to improve the NDV multiplex real-time PCR to allow the detection of all class II NDVs in clinical samples. Attempts to multiplex the 2 class II NDV real-time PCRs (AAHL and Wise et al. PCRs), although allowing the detection of all class II NDVs, has resulted in loss of analytical sensitivity. Furthermore, the combination of these 2 NDV real-time PCRs for the detection of all class II NDVs complicates the multiplexing of NDV assays with IVA real-time PCR as current real-time PCR machines have only a 4-plex capability and will not handle 5-plex real-time PCRs.
5. The evaluation of the generic Avulavirus PCR showed that the PCR was not sensitive enough to be used in the course of this project.

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