WILDLIFE AND EXOTIC DISEASE PREPAREDNESS PROGRAM

FINAL REPORT FOR PROJECT 4WEDPP-04

AUGUST 2005

Development of rapid molecular surveillance tools for the detection of Avian Influenza Virus within Victorian wild bird populations

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PROJECT DETAILS:

Project: Development of rapid molecular surveillance tools for the detection of Avian Influenza Virus within Victorian wild bird populations
Project number 4WEDPP-04

Project Dates: 1st July 2004 to 30th June 2005

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PROJECT AIM:

To develop a PCR test to detect avian influenza virus directly from bird cloacal samples and use the test in a targeted surveillance program of Victorian wild birds.

PROJECT OBJECTIVES:

1. To design and undertake a targeted surveillance program for the detection of AIV in Victorian water birds – in particular migratory wading birds and appropriate species of wild ducks.
2. To develop a rapid screening PCR to detect avian influenza from wild bird cloacal samples.
3. To validate the AIV screening PCR against traditional viral culture techniques.
4. To characterise the H protein cleavage site of AIV isolates isolated from wild birds.
5. To generate a reference genetic database for comparison of existing and new AIV strains.
6. To further understand the molecular epidemiology of AIV within Victorian wild bird populations and possible implications to Victorian poultry industries.
7. To present results in a written report and a scientific article.

ABBREVIATIONS:

AIV	Avian influenza virus
APMV	Avian paramyxovirus
H	Haemagglutinin subtype
HA	Haemagglutination assay
HI	Haemagglutination inhibition assay
N	Neuraminidase subtype
NDV	Newcastle Disease virus
NE	North East (Victoria)
NSW	New South Wales
NW	North West (Victoria)
PCR	Polymerase Chain Reaction
PP	Port Phillip region (Victoria)
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SE	North East (Victoria)
SW	North East (Victoria)
VTM	Viral Transport Medium

INTRODUCTION:

Avian influenza is not a new disease, however the widespread infection of production birds around the world is emerging as a significant and increasing problem. This has been highlighted by the most recent outbreaks of highly pathogenic avian influenza (HPAI) throughout South-East (SE) Asia from 2003-5. This outbreak has been unprecedented in terms of the size of the area effected, the high pathogenicity of the virus and the length of time it has persisted. Over 120 million poultry have been killed or culled during these outbreaks.

Influenza viruses are subtyped based on their haemagglutinin (H1 to H16) and neuraminidase (N1 to N9) surface proteins (Rohm *et al.*, 1996; Pouchier *et al.*, 2005). The haemagglutinin glycoprotein for influenza viruses is produced as a precursor, Ho, which requires cleavage by host proteases to become functional and render the virus infectious. The H protein of mild pathogenic (MPAI) subtypes have two basic amino acids at their cleavage site (Wood *et al.*, 1993). These viruses are limited to cleavage by host proteases and are therefore restricted to replication at sites in the host such as the respiratory and intestinal tract where large amounts of virus are generated usually without producing clinical signs (Kida *et al.*, 1980; Webster *et al.*, 1978). In contrast, highly pathogenic (HPAI) subtypes possess multiple basic amino acids at the cleavage site and can be cleaved by a ubiquitous protease present throughout the bird, resulting in pathogenic damage to vital organs and tissues, disease and death (Rott, 1992, Wood *et al.*, 1993). Many combinations of H and N proteins are possible and they can be prone to reassortment, however it appears that the H and N proteins remain stable in wild bird hosts and do not mutate as readily as documented in domestic poultry (Sharp *et al.*, 1997).

Traditionally, AIV subtypes H5 and H7 have generally been considered to be mildly pathogenic, however all HPAI viruses that have been characterised to date are of the subtype profile H5 or H7 (and possibly H9). The increasing occurrences of AIV outbreaks are creating confusion as to whether all H5 and H7 isolates are in fact HPAI. The H5N1 virus responsible for the most recent outbreak causing widespread disease in several countries now appears to be highly pathogenic for chickens, ducks and migratory birds. In addition, there is always the potential for LPAI and MPAI viruses to undergo reassortment/mutation and lead to the emergence of other threatening HPAI viral subtypes. For example, MPAI were shown to have mutated to HPAI during the 1983, 1994 and 1999 outbreaks.

Until recently, only low pathogenic AIV subtypes were common in wild birds (Alexander, 2000), however the highly pathogenic Asian H5N1 virus has now also been isolated from wild birds in several countries. In Australia by contrast, despite available information from surveillance and other studies, the role of wild birds in the transmission of avian influenza virus to poultry remains uncertain (Arzey 2004a, Bunn 2004, Turner 2004). Australian surveillance studies have detected a range of non-pathogenic avian influenza virus including H1, H3, H4, H6, H7 and H12 (Mackenzie *et al*, 1984; Mackenzie *et al.*, 1985, Peroulis *et al.*, 2004). All five past Australian outbreaks have been due to H7N7, H7N3 or H7N4 subtypes (Barr *et al.*, 1986; Selleck *et al.*, 1997, Selleck *et al.*, 2003, Turner, 1976 & Westbury, 1998), and yet interestingly these subtypes have never been isolated from wild bird and duck surveillance (Arzey, 2004b).

It is known that many migratory birds and ducks travel to or pass through countries currently infected with H5N1 where they may acquire the virus. A recent review identified shorebirds (Charadriiformes) as the group most likely to introduce avian influenza viruses from SE Asia (Tracey *et al.*, 2004). Shorebirds such as red-necked stints, curlew sandpipers, sharp-tailed sandpipers and red

knots congregate in large numbers, often interact with terrestrial species and migrate to the northern hemisphere each year (Alexander, 2000; Tracey *et al.*, 2004). Suss *et al.*, (1994) however, reported that it is more effective to target ducks (Anatidae) for detecting AI virus than shorebirds.

Based on the available information for avian influenza, it was decided that this project would focus on sampling both Charadriiformes and Anatidae, particularly where the two can co-mingle. To date, surveillance programs in Australia for AIV have been generally limited in scope and geographic area, and occurred with little collaboration. This project was designed with the aim of establishing the groundwork for a more long term and coordinated avian influenza surveillance approach to be implemented, initially for Victoria, but ultimately for Australia. Part of the credit for the success of this program was due to the excellent links made with an extensive range of individuals and groups. These include:

Paul Selleck	CSIRO Australian Animal Health Laboratories: AIV Reference Laboratory
Alan Hampson	WHO Collaborating Centre for Reference and Research on Influenza
Pete Collins	Victorian Waders Study Group (VWSG)
George Grossek	Department of Sustainability and Environment (DSE), VIC
Brent Waldron	Game Council, NSW
John Tracey	Department of Primary Industries, NSW
Tim Buick	Northern Australian Quarantine Strategy (NAQS)
Rupert Woods	Australian Wildlife Health Network
Chris Bunn	Department of Agriculture, Fisheries & Forestry
Brendan Rodoni	Department Primary Industries (DPI), Knoxfield, VIC
Jason Ferris	Migratory and marine species, Department of the Environment & Heritage

METHODOLOGIES:

Wild Bird Sample Collection

Appropriate collaborations were formed to provide access to migratory bird and duck samples. These included the WHO Collaborative Centre for Reference and Research on Influenza and the Victorian Waders Study Group for collection of wading bird samples. Wading bird samples were collected from various sites around Victoria including Werribee (SW Vic), Queenscliff (SW Vic), Yallock creek (SE Vic) and Gippsland (SE Vic). Collaborations with the DSE, the VWSG and the Game Council of NSW enabled collection of duck samples from a wide range of locations including the Victorian SW, NW, Port Phillip and SE regions, and from southern NSW. Where possible, areas were strategically targeted to where ducks were known to co-inhabit wetlands with wading birds. A number of samples however, were opportunistically collected during duck and quail shooting season from a variety of locations.

DPI Attwood hosts an Arbovirus surveillance program for the state of Victoria. Access to this program allowed testing of the sentinel chickens that are positioned in a total of five sites along the length of the Murray River (Kerang, Echuca, Wodonga, Tatura and Tallangatta) and one site in Gippsland. Cloacal samples of the sentinel chickens at each of these sites were taken for AIV testing.

Dacron or paediatric swabs were used to collect cloacal samples from each bird. Swabs were placed into 2mls of VTM (3.7% w/v brain heart infusion powder, 5000units/mL penicillin, 5 mg/mL streptomycin, 0.1 mg/mL gentamicin and 4 ml/mL amphotericin B) and stored chilled for transport back to the laboratory.

Virus culture

Samples were pooled into groups of five for viral culture. That is, 200µl of each VTM sample was mixed and then 200µl of the mixture inoculated into the allantoic cavity of an embryonated egg. Allantoic fluid was collected from each dead egg as they were detected and screened by HA and HI for APMVs 1, 4 and 6 and AIV using standard protocols. Antiserums for the HI assays were kindly provided by Paul Selleck, CSIRO Australian Animal Health Laboratory.

Any pooled samples giving a positive HA titre were then reinoculated from original VTM samples and the same procedure followed except that 200µl for each sample was inoculated into each of five eggs. At the completion of the second passage, the five eggs were harvested, pooled and screened by HA whether or not there was any egg deaths. This allows for the detection of less virulent strains of haemaglutinating virus that may not cause egg death.

PCR design

International literature was obtained and Australian colleagues contacted to determine the most appropriate PCR methodology to trial. After much consideration, it was decided that the Pouchier *et al.*, (2000) primers be obtained for use in this study.

Additional primers based on the redesign of the Pouchier primers by Hans Heine at CSIRO AAHL (personal communication) were also obtained. The difference however, was that the CSIRO group use a probe in their PCR and the test was optimised at this laboratory without the probe included. This aids transfer of the test from one laboratory to another and also reduces the cost of the test, an

important consideration when considering the large numbers of samples that are processed in surveillance work. Unfortunately, this modified test was also unable to detect virus directly from cloacal swabs, but were good for detection after preliminary egg culture. Primers reported in an article published by Spackman *et al.*, (2002) were also considered, however on the basis of the negative feedback obtained from our WHO collaborators on their reliability, these primers were omitted from further testing.

Since none of the single round PCR tests were able to reliably detect AIV from bird swabs, it was decided to modify the Fouchier primers to allow a nested PCR to be employed. This had the anticipated benefit of increasing the sensitivity and detecting the virus directly from cloacal swabs. All further PCR experimental work was performed with these primers as shown below:

PCR Primers omitted from this document by request of Author.

PCR conditions and optimisation

In order to perform the PCR, it was necessary to first isolate RNA from the VTM, which was reverse transcribed and amplified in a RT-PCR. This was then followed by a nested PCR.

Viral RNA was isolated from 140µl VTM using the QIAgen RNeasy kit and eluting the RNA with 60µl of water. An aliquot of 5µl of purified RNA was then transcribed to cDNA using AMV reverse transcriptase (Promega) and T*th* DNA polymerase (Promega) in a RT-PCR of 30 cycles, 2µl of the final product was then used as template DNA for the nested PCR.

The nested PCR was performed on a Roche Lightcycler Real Time PCR machine. Optimisation included varying the magnesium chloride concentration, primer concentration, annealing temperature, number of PCR cycles and DNA concentration. The final optimised PCR conditions were:

Magnesium concentration	3mM
Primer concentration	0.5µM
Annealing temperature	62°C
Number of PCR cycles	30 cycles
DNA concentration	2µl of RT-PCR product

These conditions produced a specific, single product of 87°C melting temperature, with no primer dimers or other non-specific products.

Sample collection

During the period Feb 04 to April 05, a total of 1390 bird cloacal samples were collected. This included 785 duck samples, 504 samples for migratory and other birds and 101 samples from sentinel chickens. Most of the wading bird samples were collected during late spring and summer, whereas most of the duck samples were collected during summer and autumn. Table 1 provides a breakdown of the numbers collected of duck and other bird species. The first year of sampling had an emphasis on wading birds, however as information was gathered, the focus for the second year turned more toward duck sampling. A significant effort was also made to collect samples from a variety of locations around Victoria (refer to Table 2). Due to the presence of a collaborator in NSW, 170 of the total duck samples were collected from southern NSW.

Viral culture

All 1390 bird and duck samples collected were processed and propogated in embryonated eggs for avian viruses. Though the primary focus for this project was detection of AI virus, this testing procedure also detects other haemagglutinating viruses such as Newcastle disease virus (NDV) and other avian paramyxoviruses (APMV).

A total of 9 viral pools had haemagglutinating viruses detected. The individual samples comprising these pools were then inoculated individually into eggs to determine the individual number of birds that were contributing to the positive result. In total, there were three isolates of AIV, one isolate of APMV-1, one isolate of APMV-4 and four isolates of APMV-6 cultured in eggs (Refer to Table 3 for a full summary of the viruses detected using this method). The three isolates of AIV came from two pools and these were subsequently typed AIV H4 using the haemagglutination inhibition assay. All of these AIV samples were from ducks caught during summer (Dec 2004) in Werribee (Port Phillip region). Two of the APMV-6 samples came from pools of three quail that were pooled in the field and were subsequently unable to be tested individually.

Nested Real Time PCR

After screening the 1390 samples for the presence of virus using viral culture in eggs, all of the samples were subjected individually to the nested Real Time PCR (Refer to table 3 for a full list of culture and PCR results).

All seven samples that were positive for APMV in culture were negative by PCR. All three AIV H4 samples detected by culture were positive in the nested PCR. Of the remaining 1380 egg culture negative samples, 1368 were negative and 12 positive by PCR. The twelve samples that tested positive by PCR, but that were negative by pooled culture were sequenced. The sequence of all twelve PCR products matched the published AIV sequences in Genbank, confirming that the are true AIV sequences. This suggests that there is avian influenza virus in these samples but for some reason the virus cannot be cultured, but that the viral RNA is being detected. The most likely reason for this is that the virus did not survive transport to the laboratory and subsequent storage.

Specificity of Nested PCR

The Real Time PCR test developed is a test designed to detect all 15 subtypes of avian influenza. This project aimed to test as many different subtypes as possible, however due to the limited availability of some of the subtypes, and the quarantine restrictions surrounding the handling of exotic subtypes, the conduct of a more thorough investigation was hampered. It was however, possible to get at least one veterinary representative isolate from each H subtype other than H2 and H14 (H14 is not available in Australia). The isolates are from a range of species including birds, pigs and horses (Refer to Figure 2). In addition, a total of four human avian influenza isolates of the H1, H2 and H3 subtypes were obtained from the WHO Reference laboratory. These samples also tested positive in the nested Real Time PCR test (data not shown). Listed below is the full list of AIV isolates that were included in the nested PCR and tested positive:

H1N9	-	A/shelduck/WA/1757/78
H3N8	-	A/black duck/WA/699/83
H3N2	-	A/teal/VIC/76C/2001
H4N4	-	A/grey teal/WA/1840/79
H5N1	-	A/chicken/Vietnam/8/2004
H5N3	-	A/shearwater/Australia/75
H7N7	-	A/chicken/Victoria/85
H7N3	-	A/chicken/Queensland/94
H6N5	-	A/shearwater/Australia/72
H8N4	-	A/turkey/Ontario/6118/67
H9N2	-	A/turkey/Wisconsin/66
H10N7	-	A/chicken/Germany/N/49
H11N6	-	A/tern/Australia/75
H12N9	-	A/red-necked stint/WA/5745/84
H13N6	-	A/gull/Maryland/704/77
H15N9	-	A/shelduck/WA/1762/79

H1	-	A/Brisbane/193/2004
H1	-	A/Victoria/500/2005
H2	-	A/SAT
H3	-	B/Brisbane/3/2005

A number of other veterinary viruses have also been tested to ensure that they do not cross react in the AIV test including Newcastle disease virus and other avian paramyxoviruses, akabane virus, aino virus, ephemeral fever virus and bovine parainfluenza virus (results not shown). All of these viruses were negative in the nested Real Time PCR test.

Sensitivity of Nested PCR

More experimental work is required to determine the analytical sensitivity of the nested Real time PCR test. Testing to date however, has shown that avian influenza virus is directly detected from cloacal swabs without prior amplification in culture, which was the primary aim of the test design. Experiments are also in progress to determine how many cloacal samples can be pooled without losing the ability to detect virus directly from the VTM samples.

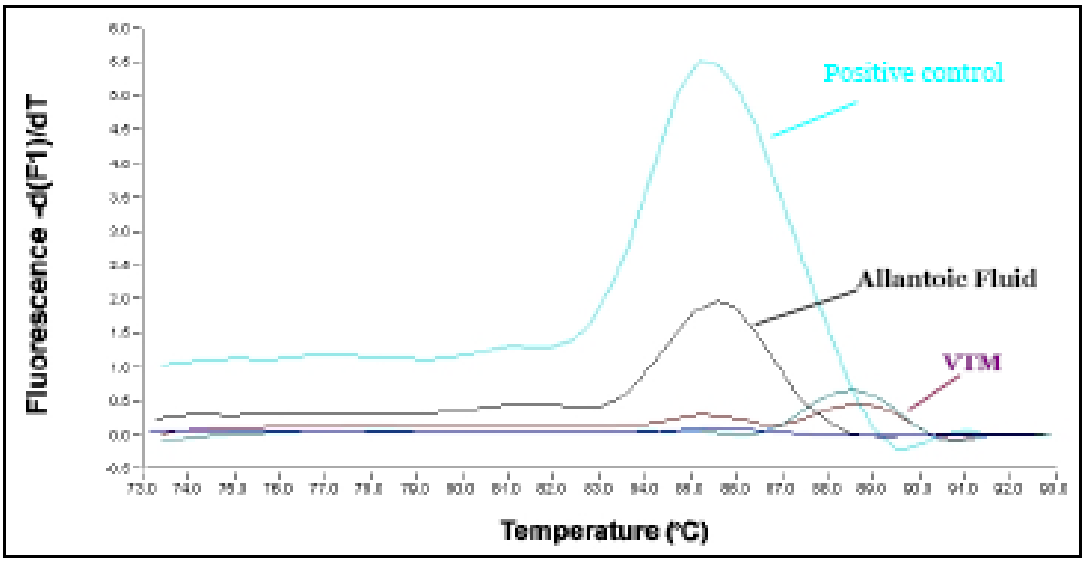
Table 1: Number of wild bird samples collected at various sites within Victoria between Feb 2004 and April 2005.

Bird Species	Common Name	Number of samples		TOTAL
		2004	2005	
Ducks	Black duck	65	127	785
	Grey Teal duck	55	249	
	Chestnut Teal duck	53	57	
	Wood duck	35	103	
	Pink ear duck	9	2	
	Shelduck	19	0	
	Hardhead duck	1	4	
	Mountain duck	0	3	
	Blue-wing Shovel duck	2	1	
Migratory and other birds	Red-necked stints	296	0	504
	Curlew sandpipers	16	0	
	Sharp-tailed sandpipers	46	0	
	Red knots	39	0	
	Bar Tailed Godwit	0	40	
	Green Shank	2	0	
	Masked lapwing	2	0	
	Swans	4	0	
	Ruddy turnstone	1	0	
	Quail	58	0	
Chickens	Sentinel Chickens	0	101	101
				1390

Table 2: Summary of sample collection by region

BIRD	VICTORIA					NSW	TOTAL
	PP	NE	SE	NW	SW		
Ducks	160	99	131	192	33	170	785
Shorebirds & other	286	0	160	0	58	0	504
Sentinel Chickens	0	82	0	19	0	0	101

(a) Standard RT-PCR



(b) Nested RT-PCR

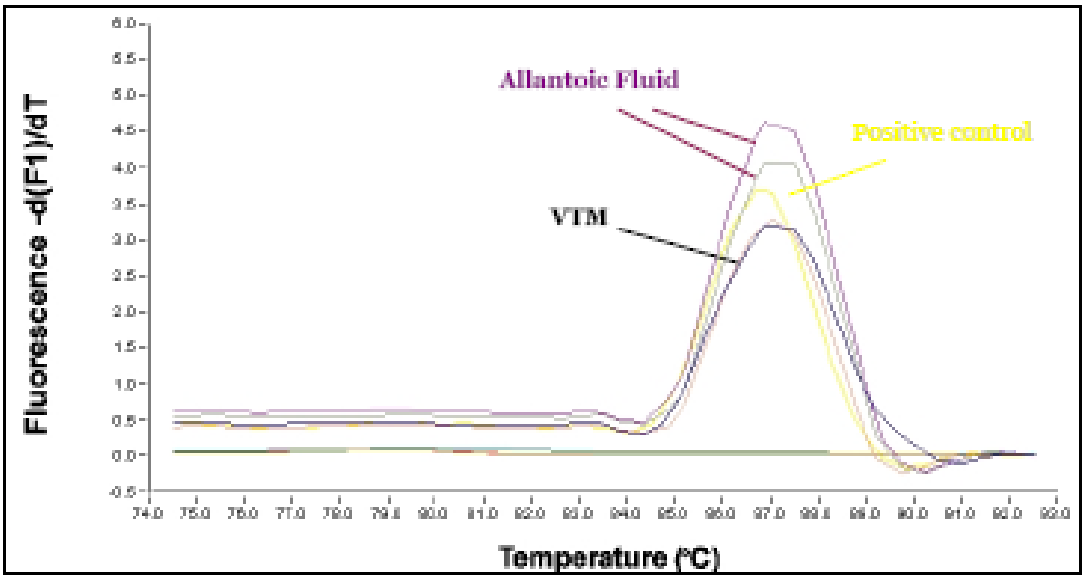
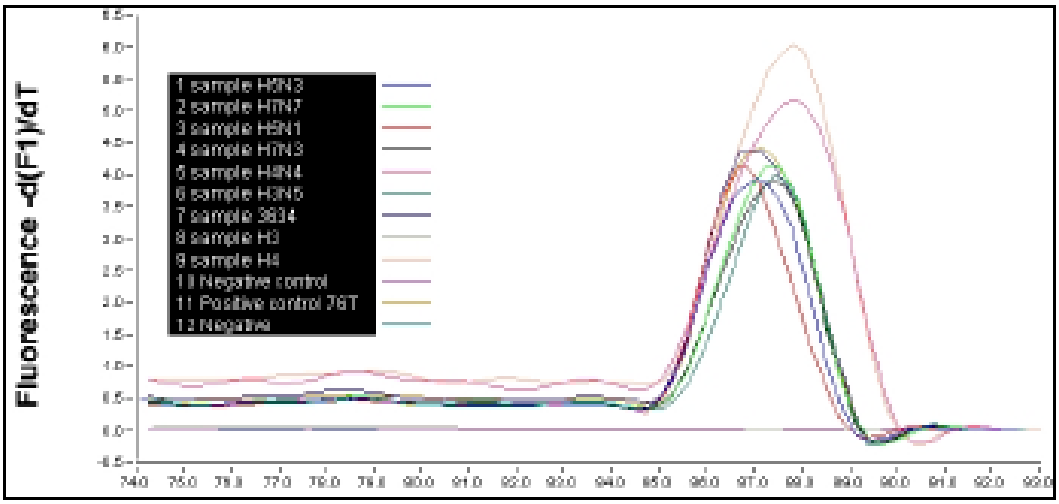


Figure 1: Standard RT-PCR compared with nested RT-PCR. (a) A field sample that was positive for AIV by culture in embryonated eggs was unable to be detected using published Fouchier *et al.*, (2000) primers. (b) A modification of the protocol to allow a nested PCR to be employed, resulted in the AIV H_4 samples as detected by egg culture, to be clearly detectable directly from the VTM sample.

(a)



(b)

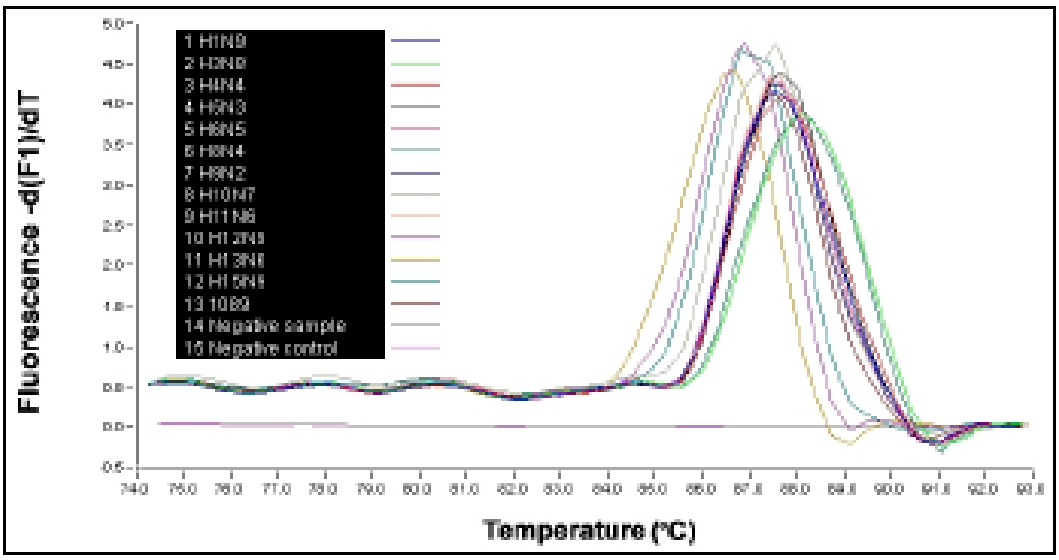


Figure 2: Nested Real Time PCR melting curve analysis for a range of AIV H subtypes. The full range of AIV subtypes tested were positive by nested Real Time PCR (a) H3, H4, H5 and H7 subtypes (Note: the H5N1 isolate is the recent Asian strain). (b) H1, H2, H6, H8, H9, H10, H11, H12, H13 and H15 subtypes.

Table 3: Summary of viral culture in embryonated eggs and AIV nested Real Time PCR results for all 1390 cloacal samples.

Sampling details					Test result	
Isolate Ref	Bird Type	Age	Region	Date	Egg Culture	AIV PCR
5C	Grey teal	ND	SW	Mar 04	APMV 4	Negative
7B	Black Duck	ND	PP	Mar 04	APMV 6	Negative
7Q	Quail	ND	SW	Mar 04	APMV 6	Negative
10A	Black Duck	ND	NW	Mar 04	APMV 6	Negative
13Q	Quail	ND	SW	Mar 04	APMV 6	Negative
13B	Black Duck	ND	PP	Mar 04	APMV 6	Negative
66	Black Duck	Adult	NSW	Jan 05	APMV 1	Negative
11	Chestnut Teal	ND	PP	Dec 04	AIV H4	Positive
20	Chestnut Teal	ND	PP	Dec 04	AIV H4	Positive
22	Chestnut Teal	ND	PP	Dec 04	AIV H4	Positive
21	Chestnut Teal	ND	PP	Dec 04	Negative	Positive
23	Chestnut Teal	ND	PP	Dec 04	Negative	Positive
24	Chestnut Teal	ND	PP	Dec 04	Negative	Positive
280	Grey teal	Adult	PP	Mar 05	Negative	Positive
281	Grey teal	Adult	PP	Mar 05	Negative	Positive
286	Black Duck	Adult	PP	Mar 05	Negative	Positive
292	Black Duck	Adult	PP	Mar 05	Negative	Positive
295	Black Duck	Adult	PP	Mar 05	Negative	Positive
303	Black Duck	Adult	PP	Mar 05	Negative	Positive
314	Grey teal	Adult	NE	Mar 05	Negative	Positive
323	Grey teal	Adult	NE	Mar 05	Negative	Positive
332	Grey teal	Adult	NE	Mar 05	Negative	Positive
All 1368 remaining duck / wading bird / sentinel chicken samples					Negative	Negative

ND Not determined

Table 4: Summary of percentages of samples positive for various cultured viruses and PCR amplified AIV. For both ducks and quail samples, the exact percentage of APMV-6 found is unable to be calculated due to some samples being pooled in the field.

Species	Total Number	No of Pos min – max (AIV PCR)	% Pos min – max (AIV PCR)	Virus cultured
Duck	785	1	0.13%	APMV-1
		1	0.13%	APMV-4
		4 – 12	0.5 – 1.5 %	APMV-6
		3 (15)	0.4% (1.9%)	AIV H4
Quail	58	2 -6	3.5 – 10.4%	APMV-6
Sentinel chickens	101	-	-	-
Wading birds	446	-	-	-

DISCUSSION:

This project sought to undertake "targeted" surveillance of wild birds or the detection of avian influenza virus. Without the presence of a formal risk assessment for Victoria, an approach of careful planning was employed. The targeted sampling program was designed to sample the bird species most likely to carry AIV, that is specific species of wading birds and ducks identified as likely carriers from published literature (Alexander, 2000; Tracey *et al.*, 2004). Other factors included accounting for seasonal variations in virus prevalence where possible, choosing locations where interactions between wading birds and ducks were likely, and proximity to the poultry industry. In addition to the considerations above, it has also been suggested that juvenile ducks are likely to have a higher prevalence of AIV. Unfortunately however, our limited access to sampling of wild ducks during their juvenile phase meant that only small numbers of juveniles were sampled.

Several collaborations were formed during this project that led to the acquisition of 1390 bird samples. This was a cost-effective means of obtaining samples from a wide range of locations in Victoria, as well as 170 duck samples from NSW. All of the samples were subjected to the traditional "gold standard" method for virus detection, culture in embryonated eggs, in pools of five. Using this technique two pools were positive for AIV and when cultured as individual samples, a total of three AIV isolates were detected, which were confirmed as the H4 subtype using the haemagglutination inhibition assay. In addition, one APMV-1 (non-virulent NDV: V4-like), one APMV-4 and five APMV-6 isolates were also detected. No haemagglutinating viruses were cultured for the remaining 1380 samples.

Several single round PCR tests were trialed, however despite optimisation, each of the single round RT-PCR assays tested were only able to detect AIV if bird samples were first grown in eggs. That is, these PCR tests failed to reliably detect virus directly from the cloacal swabs. The most promising of the primer sets (Fouchier *et al.*, 2002) were then modified to allow nested PCR to be employed. Nested PCR is a two-step PCR that has the advantage over single-round PCR of higher sensitivity and a confirmation of sequence within the first-round PCR product. The newly developed nested RT-PCR was able to reliably detect virus directly from the bird cloacal swab in viral transport medium. Validation experiments were also extremely promising in that all of the AIV subtypes tested were positive and all non-AIV viruses tested were negative, without exception.

All 1390 bird samples were subjected individually to the AIV nested RT-PCR. Using this technique there was a total of 15 PCR positives. Three of these positives were the three egg culture positives, the other 12 were PCR positive only and were not able to be cultured in eggs despite an additional blind passage. Sequencing of these PCR products revealed that they were type A AIV isolates, and not spurious PCR products. The most likely conclusion is that these are samples where the virus has died during transport and subsequent storage, however it is also possible that the virus is in too low a concentration to be detected by culture. It is highly unlikely that these are false positives due to contamination as numerous precautions are followed to prevent such occurrences, and the fact that the sequences do not exactly match that of the positive control or each other.

This study has demonstrated that AIV is only present at a very low prevalence in wild birds in Australia. Based on egg culture results the prevalence was found to be 0.2%, whereas based on the PCR results the prevalence was 1.1%. Either way, both figures are low. Interestingly, all of the positives from both culture and PCR were from ducks; six

from chestnut teal, five from grey teal and four from black duck. This makes the prevalence of AIV in ducks 0.2% by culture and 1.9% by PCR.

In summary, this study found that there is only a low prevalence of AIV in wild birds and ducks in Australia. The only subtype of AIV detected was H4, which is considered LPAI. No H5 or H7 isolates were found. Future studies will ideally encompass a range of factors including:

- sampling the appropriate species and age of birds, particularly ducks
- identification of priority areas
- seasonal fluctuations
- proximity of wild birds to poultry establishments
- a yearly sampling regime contributing to a longer-term longitudinal study

ACKNOWLEDGMENTS:

The collection of field samples would not have been possible without the valuable help of many people particularly Pete Collins, Roz Jessop and Clive Minton of the Victorian Waders Study Group, and George Grossek, Craig Whiteford, Michael Severns, Charles Franken, Steve McDougall, Murray Rohde, Nik Chapman, Richard Boekel and Rolf Webber of the Department of Sustainability and Environment, Victoria. Thanks also to the following DPI Victoria employees for participating in sample collection: Keng Teo, Kari Bunce, Andrea Howse, Janine Muller, Ian Holmes, Jeff Cave, Sam Ellis, Alan Semminer and Lachlan King.

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