

WILDLIFE AND EXOTIC DISEASE PREPAREDNESS PROGRAM



Application of rapid diagnostic tests in the targeted surveillance of Avian Influenza Virus within Victorian wild bird populations

FINAL REPORT

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PROJECT DETAILS:

Project: Application of rapid diagnostic tests in the targeted surveillance of Avian Influenza Virus within Victorian wild bird populations
Project number 4WEDPP-04 (continuation)

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1. PROJECT AIM

To undertake a targeted surveillance program for avian influenza. This will be done by using a geographic information system to identify the key risk areas for the transmission of avian influenza between wild ducks and domestic poultry.

2. PROJECT OBJECTIVES

1. Development of a GIS for Victoria for identifying key areas for wild bird surveillance, including mapping of poultry establishments with increased potential for interaction with wild birds, and mapping the abundance of bird species using Birds Australia data.
2. Field sampling of targeted species undertaken at locations identified as being the biggest risk for the Victorian poultry industry.
3. Recommendation of a PCR screening test for use in Avian influenza surveillance identified by participation of various laboratories in a coordinated PCR testing program.
4. Current PCR methodologies adapted for high throughput testing.
5. Presentation of surveillance data in a format that may be incorporated within a national surveillance database and publication in a scientific journal if applicable.

3. ABBREVIATIONS

AIV	Avian influenza virus
BHI	Brain heart infusion medium
Ct	Crossing threshold (Real Time PCR)
GIS	Geographic information system
H	Haemagglutinin subtype
HPAI	Highly pathogenic avian influenza
N	Neuraminidase subtype
NSW	New South Wales
PCR	Polymerase Chain Reaction
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SE	South East
VTM	Viral Transport Medium
VWSG	Victorian Waders Study Group
WHO	World Health Organisation

4. INTRODUCTION

Avian Influenza (AI) is emerging as significant problem around the world due to the widespread infection of production and wild birds. Bird flu is a devastating disease in the poultry industry because the existence of large numbers of infected birds increases the potential for AI viruses to mutate from low pathogenic forms into the much deadlier highly pathogenic forms. 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.

All birds are believed to be susceptible to avian influenza, but ducks and other wild birds may carry the virus without any disease symptoms. 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, H11 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 involved collaboration between the Victorian and NSW Departments of Primary industries. The NSW group performed the GIS mapping and risk assessments at the start of the project, and this was followed by wild bird sampling based on their results by the Victorian DPI team. The 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.

Cloacal samples were collected from a range of wild birds and ducks across Victoria as part of a National surveillance program for AIV. Valuable links for sample collection have been made with the WHO Collaborating Centre for Reference & Research on Influenza, the Department of Sustainability and the Environment, and the Victorian Waders Study Group.

5. Results

5.1 Development of a GIS for Victoria for identifying key areas for wild bird surveillance, including mapping of poultry establishments with increased potential for interaction with birds, and mapping the abundance of bird species using Birds Australia data.

A geographic information system was used to identify priority areas for surveillance in Victoria following the methods used for pilot trials in NSW (Tracey, 2005). Two models for surveillance were investigated: (1) to assess the risk of endemic low pathogenic avian influenza viruses in wild birds becoming highly pathogenic through interactions with poultry and (2) to assess the risk of wild birds introducing foreign subtypes of avian influenza. Data on all bird surveys conducted and the number of Anseriformes observed has been received from Birds Australia; up-dated poultry locations have been sourced from the Department of Agriculture Fisheries and Forestry and through negotiations with the Australian Poultry Association. Wetlands data for Victoria has been sourced from Geoscience Australia. Further improved wetlands information including criteria for waterbird abundance, and suitable waterbird habitat has been sourced from negotiations with the Department of Sustainability and Environment Victoria, Wetlands Research and Development Program, the Wetlands and Waterbirds Taskforce and Australian Wetlands database.

5.1.1 Commercial Poultry Operations

Victoria has just over 20% (156 of 751) of Australia's major commercial poultry operations. The major centres for poultry in Victoria are just east of Melbourne surrounding Cranbourne and in the south-west surrounding Geelong. Secondary poultry farms with large numbers of birds also occur in Bendigo (up to 200 000 birds), Traralgon (86 000 birds) and near Colac (150 000 birds) (Figure 1). A minimum number of poultry of around 10 000 birds and a threshold of 40-50 000 has been suggested before avian influenza viruses rapidly mutate into highly pathogenic forms (Woods *et al.* 2005). Biosecurity measures are fundamental to reducing the risks of virus transfer, and the type (free-range, broilers, layers) and size of the operation may also influence the risk of an outbreak of HPAI. Targeting surveillance of Anseriformes in these regions will improve our understanding of the risks associated with naturally circulating low pathogenic avian influenza and potential transfer to poultry.

There are many small-scale "backyard" poultry sheds in Victoria not presented in Figure 1. These sheds may play a role in the epidemiology of avian influenza as they are likely to have few biosecurity measures in place and interactions with wild birds may be higher. However there is uncertainty of the ability of a small population of poultry to initiate an outbreak of HPAI. Some researchers have suggested (Ito *et al.* 2001; Turner 2004) that extensive passaging and selection is required to generate a HPAI virus. Conversely, other studies indicate that mutation into HPAI can occur with as few as 1 or 2 passages (Arzey, 2005). This uncertainty highlights the absolute requirement for surveillance.

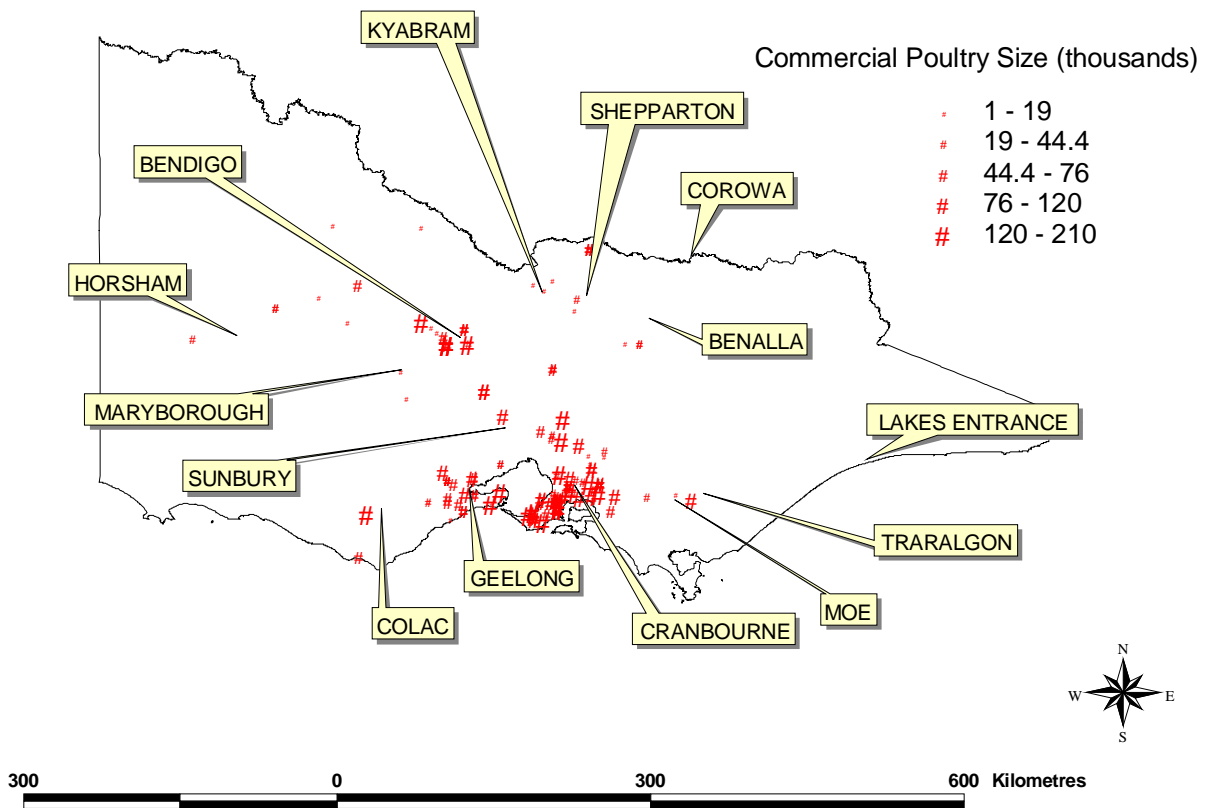


Figure 1 : Locations of commercial poultry operations in Victoria

5.1.2 Occurrence of Anseriformes

The Anseriformes order includes members of the Anatidae (Ducks and Geese) and Anseranatidae families (Magpie Geese). Movements of Anseriformes in Australia are less predictable than their counterparts in the northern hemisphere and many populations are nomadic (Lawler and Briggs 1991). Their movements and distribution in Australia is largely determined by available water (Briggs 1992; Lawler *et al.* 1993; Kingsford, 1995; Roshier *et al.* 2001; Roshier *et al.* 2002). Anseriformes are widespread and abundant in Victoria (Figure 2).

There is a need to determine Anseriformes density with particular focus in and around poultry farms. To measure Anseriformes density throughout Victoria a value called the "Reporting rate" was calculated. In the current investigation, the reporting rate was calculated using the number of species observed of the Anseriformes order and the number of surveys conducted at 10' localities as follows:

$$\text{Reporting Rate (RR)} = \frac{\text{Number of species of Anseriformes}}{\text{Number of surveys}} \quad (2)$$

The estimated reporting rate used here differs from the species specific reporting rate used by Birds Australia (Barrett *et al.* 2003) as multiple species of the same family can be observed on each survey, hence the values are displayed as a proportion rather than a percentage and can be greater than 1. The mean number of surveys per 10' (0.167 degree) area was 30 (S.E = 2.0, range 1-1986, n=2414).

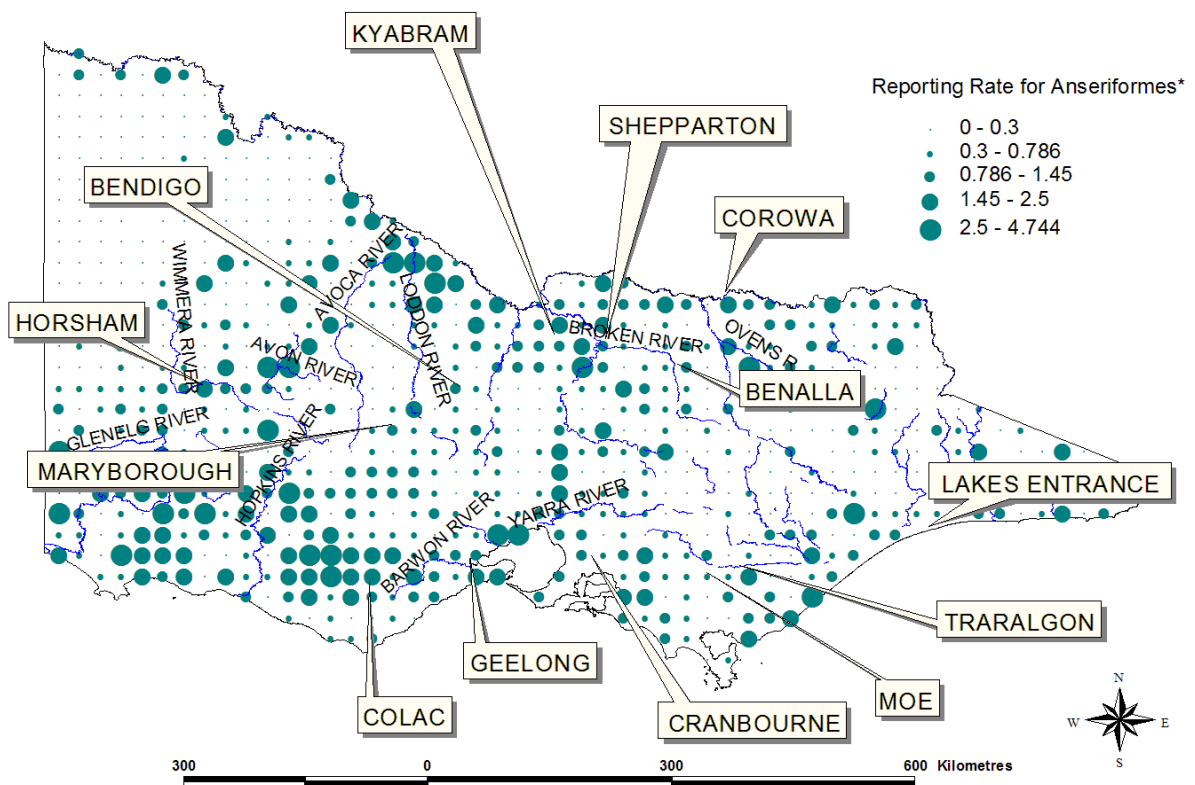


Figure 2: The occurrence of Anseriformes superimposed over important wetlands for Charadriiformes in Victoria. Reporting Rate was calculated using the number of species observed and the number of surveys conducted within a 10' (0.167 degree) area.

Using Birds Australia data, the highest number of Anseriformes were observed on wetlands of the south-west including Lake Corangamite, Lake Colongulac, Lake Bullen Merri, Lake Purrumbete, Lake Linlithgow and along the Glenelg and Wannon Rivers and waterbodies adjacent to Mount Emu Creek. A large number of species were also observed along the Avoca River and Loddon River near the junction of the Murray River in the North. Other wetlands including Waranga Basin and along the Goulbourn, Lake Denisen south-west coast and the inlet to the Werribee River near Melbourne also had high numbers of Anseriformes.

Internationally important wetlands have also been identified, which fulfil the Ramsar criteria relating to waterbirds: 5, 6, 3a, 3b and 3c (refer to Appendix 1). This has allowed a more accurate identification of wetlands that regularly support high numbers of Charadriiformes and Anseriformes.

5.1.3 Identifying Wetlands for Surveillance

To identify the regions and wetlands of highest priority for surveillance the reporting rate was calculated using formula (2), but included all observations within 15km of commercial poultry operations. The mean number of surveys per 15km buffer area was 774 (S.E = 393, range 4-9241, n=29).

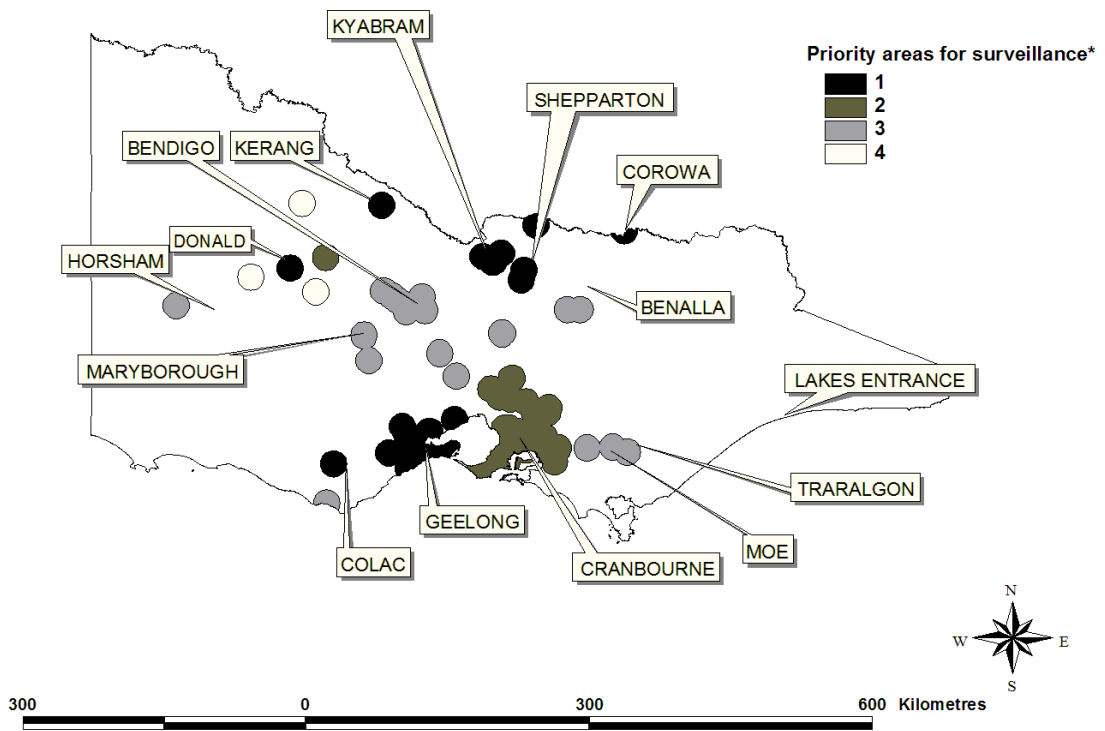


Figure 3: Priority areas for surveillance in Victoria.


*Rankings are based on the proximity (within 15km buffer) of major wetlands to commercial poultry operations, and the occurrence of Anseriformes, where RR is the reporting rate for Anseriformes (average number of Anseriformes observed per survey); n is the number of surveys within 15km of a commercial poultry operator; and Rank 1= $RR >1.25$; Rank 2= $RR 1 - 1.25$; Rank 3= $RR 0.5-1$; Rank 4= $RR <0.5$.

Large numbers of Anseriformes are evident in the wetlands around Geelong, Kyabram, Shepparton and Colac which occur in close proximity to many commercial poultry operations (Figure 3). Major wetlands in these areas are priorities for surveillance for addressing Objective 1.

5.2 Field sampling of targeted species undertaken at locations identified as being the biggest risk for the Victorian poultry industry.

A total of 2223 samples were collected from a range of ducks and shorebirds between September 2005 and April 2006. The majority of samples were collected from Chestnut Teal, Grey Teal, Pink-eared Ducks, Australian Wood Ducks, Red-necked Stints, Red Knots, and Bar-tailed Godwits (Refer to table 1 for a complete listing of samples).

The aim of this project was to collect as many samples as possible from areas identified in Objective 1 as being a high priority area for AIV surveillance. The major wetlands in the areas around Geelong, Kyabram, Shepparton, Donald, Kerang and Colac were identified as the highest priority areas. Due to the difficulties in obtaining live wild bird samples, several collaborations were used in the collection of samples. The VWSG were instrumental in the collection of shorebird samples, and our team accompanied them to selected sampling sites in line with the outcomes of the risk assessment where possible. The main source of duck samples are those obtained on the opening day of the Victorian duck shooting season in collaboration with the Department of Sustainability and Environment and Parks Victoria staff. Inspections of duck bags occurs in many regions of Victoria, and our group targeted the Kerang, Lismore, Geelong and Ballarat regions which were identified as high priority areas for surveillance. With this in mind, cloacal samples were collected from a total of 723 anatids (refer to table 2) and 1500 shorebirds and other waterbirds (refer to table 3) from regions as close as possible to the target areas.

Immediately after collection, cloacal samples were placed into a BHI-broth based viral transport medium and stored chilled in eskys for transport back to the laboratory. In some cases, the transport time from the field to the laboratory was 2-3 days. The samples were then stored at -80°C until ready for processing using the PCR test. All samples were processed individually during the course of all testing. RNA was extracted using high throughput methods using Qiagen RNeasy 96 kits (refer to Section 5.4). The RNA was then run through the DPI AIV Type A PCR which is a nested PCR test designed to detect any of the 16 subtypes of Type A AIV. 

Using the AIV Type A PCR test, 45 samples of the total 2223 collected tested positive by PCR, 40 from anatids and 5 from shorebirds and other waterbirds. This equates to an overall prevalence of 5.5% (40 of 723) in anatids and 0.33% (5 of 1500) in shorebirds and other waterbirds (refer to Table 4). The birds that had a higher prevalence included black swans, Australasian Shovelers, Chestnut Teal and Grey Teal. Interestingly, 3 of the total of 18 black swans tested positive, equating to a prevalence of 21.43% in our study for that species. All three samples were collected on different days, 18th Sept, 9th Oct and 20th Feb, from the South-West region. This region was classed as a “medium priority area” using the GIS mapping (refer to figure 3). Also of higher prevalence in this study were the three positive Australasian Shoveler samples from the 14 samples collected (21.43%). Unlike the black swans, the Australasian Shoveler samples were all collected from the Gippsland region on the opening day of the duck shooting season, 18th March 2006. This area was not identified as being a high priority region from the GIS mapping work. Likewise, there were 17 positive Chestnut Teal samples, from the same region on the same day. Overall, there were 19 positive Chestnut Teal samples from a total of 204, one of which was from a high-priority area, which equates to a prevalence of 9.31%. Lastly, there were 7 Grey Teal PCR positive samples, 5 of which came from the high-priority areas of Geelong, Kerang and Lismore. The overall prevalence in Grey Teal was 4.45%. Interestingly, the majority of positive duck samples came from the Gippsland region which was not identified as a priority area from the GIS mapping and subsequent risk assessment.

Unfortunately, the 45 PCR positives could not be confirmed by viral culture as the attempted egg culture gave negative results for all samples. It is generally accepted that not all PCR detected viruses can be grown in culture, with some reports suggesting that less than 30% of samples can be confirmed. Even with this in mind however, the reasons for not growing any viruses from the 45 PCR positives are not clear, and disappointing. Some of the samples were also provided to the WHO Centre for culture, and they were unable to grow any viruses either. The most probable explanations are that the transport and/or storage conditions were not favourable for the long-term survival of live viruses, the level of virus was below the detection limit for this test, or that the viruses were not alive in the birds at the time of sampling. Therefore, a much more extensive regime for confirming the PCR positives was undertaken. This involved several tests including:

1. Repeating the DPI Type A PCR from the original samples not using high-throughput techniques
2. Sequencing the DPI Type A PCR products
3. Using the CSIRO AAHL Type A PCR on all 45 positive samples
4. Sending a selection of samples to the WHO Centre for use in their range of PCR tests

In addition, all precautions were taken to reduce the likelihood of the positive control contaminating the samples, including the use of multiple rooms for each step of the PCR, and limiting the positive control to the later steps of the procedure and not being included in the RNA extraction steps. In steps where it is desirable to include a positive control, the positive control DNA is only included after all of the sample RNA or DNA have been processed and put away. This provides a level of assurance that the original samples can never be contaminated with positive control DNA, which gives an extra level of confidence if repeat testing is required. In addition, a H5N3 subtype is used for the positive control as this is a more unlikely subtype to be obtained from wild bird samples.

All 45 samples that tested positive using high-throughput methods were repeated using manual methods in the DPI Type A PCR test and confirmed as positive. Sequencing of the resulting PCR products confirmed that the sequences were from AI viruses. Analysis of the sequence showed the great majority of samples had at least one mutation from the positive control sample, indicating that it is unlikely that the sequenced product was positive control DNA. The total number of mutations within the PCR product has been summarised and documented in table 5. For example, one Chestnut Teal from Lake Watt Watt (Ct 3.01) actually had no sequence mutations, meaning that the sequence of the PCR product matched exactly that of the H5N3 positive control. However, further analysis of this sample at the WHO Centre confirmed it as the H3 subtype. The CSIRO AAHL Type A Taqman PCR was also employed in the confirmation process. This test was able to confirm 10 of the 45 PCR positive results, with a further 12 having Ct values in the ambiguous range, and 23 not having a detectable PCR product. This seemed more than reasonable, as the AAHL test is designed for application in AIV outbreak investigation rather than for surveillance of healthy wild birds. The DPI Type A PCR on the other hand, was designed specifically for surveillance work and therefore appears to have a higher diagnostic sensitivity which results in the detection of more “lower-positive” samples (demonstrated in the lower Ct values in table 5). Nine of the ten samples that tested positive in the AAHL Type A Taqman PCR did have very low Ct values in the DPI Type A PCR, suggesting that those samples were likely to contain more genetic material. Despite only 10 of the 45 samples testing positive in the alternative PCR, the confirmed AIV sequence of the DPI PCR products, and the sufficient differences from the positive control, has led to all 45 PCR positives being considered valid results.

As mentioned previously, several PCR positive samples were sent to the WHO Centre for verification testing as they have a range of PCR tests for use with surveillance samples. They were able to confirm an additional 4 of the positive results (refer to table 5). In addition, they were also able to subtype two of the samples by sequencing the product of a haemagglutinin gene PCR. The two samples were subtyped as H3 and H12 (see table 5, red text). Another two samples are currently awaiting subtyping results (see table 5, blue text).

Table 1: Summary of the total number of wild bird samples collected at various sites primarily within Victoria between September 2005 and March 2006.

Species Group	Order	Family	Common Name	TOTALS	
Ducks, Swans, Geese.	Anseriiformes	Anatidae	Pacific Black Duck	70	723
			Grey Teal	154	
			Chestnut Teal	204	
			Australian Wood Duck	96	
			Pink-eared Duck	144	
			Hardhead	1	
			Musk Duck	3	
			Freckled Duck	1	
			Australian Shelduck	18	
			Australasian Shoveler	14	
			Black Swan	18	
Shorebirds	Charadriiformes	Scolopacidae	Red-necked Stint	667	1469
			Little Stint	1	
			Curlew Sandpiper	78	
			Sharp-tailed Sandpiper	3	
			Red Knot	420	
			Great Knot	35	
		Charadriidae	Bar-tailed Godwit	248	
			Masked Lapwing	1	
			Red-kneed Dotterel	1	
			Black-fronted Dotterel	2	
			Grey Plover	2	
Laridae	Caspian Tern	1			
Haematopodidae	Oystercatcher	10			
Other Waterbird Species	Ciconiiformes	Ardeidae	Nankeen Night Heron	3	12
			White-faced Heron	6	
		White-necked Heron	1		
	Threskiornithidae	Australian White Ibis	2		
		Gruiformes	Rallidae	Dusky Moorhen	8
	Eurasian Coot			1	
	Purple Swamphen			3	
Pelecaniformes	Phalacrocoracidae	Great Cormorant	5	7	
		Little Pied Cormorant	1		
	Anhingidae	Darter	1		
TOTAL				2223	

Table 2: Summary of the total number of duck and swan samples collected for each region and the AIV Type A screening PCR result.

Region	Date collected	Species	Type A PCR		Total
			Negative	Positive	
Gippsland Region	18/03/2006	Pacific Black Duck	46	1	47
		Chestnut Teal	153	17	170
		Grey Teal	74	1	75
		Hardhead	1		1
		Australian Shelduck	7		7
		Australasian Shoveler	7	3	10
Port Phillip Region	18/03/2006	Pacific Black Duck	1		1
		Chestnut Teal	13		13
		Grey Teal	21	1	23
North East Region	18/03/2006	Pacific Black Duck	1		1
		Grey Teal	21	1	22
		Pink-eared Duck	130	3	133
		Australian Shelduck	2		2
		Freckled Duck	1		1
		Black Swan	1		1
South East Region	18/03/2006	Pacific Black Duck	2		2
		Australian Shelduck	3		3
		Pink-eared Duck	1		1
		Australian Wood Duck	82	2	84
South West Region	18/09/2005	Musk Duck	2		2
		Black Swan	2	1	3
	9/10/2005	Black Swan	4	1	5
	20/02/2006	Black Swan	8	1	9
		Pacific Black Duck	2		2
	18/03/2006 19/03/2006	Pacific Black Duck	1		1
		Australasian Shoveler	3		3
		Chestnut Teal		1	1
		Grey Teal	15	3	18
		Australian Shelduck	6		6
Pink-eared Duck		8	2	10	
Murray River, NSW	23/11/2005 24/11/2005	Australian Wood Duck	6		6
		Pacific Black Duck	6		6
		Australasian Shoveler	1		1
		Grey Teal	6		6
Tasmania	11/03/2006 12/03/2006	Chestnut Teal	19	1	20
		Grey/Chestnut Teal	9	1	10
		Australian Wood Duck	6		6
		Pacific Black Duck	10		10
TOTAL			683	40	723

Table 3: Summary of the total number of shorebird samples collected for each region and the AIV Type A screening PCR result.

Region	Date	Bird	PCR Screen		
			Negative	Positive	Total
Gippsland Region	5/02/2006 6/02/2006 7/02/2006 8/02/2006	Bar-tailed Godwit	184	1	185
		Curlew Sandpiper	32		32
		Great Knot	34		34
		Red Knot	145		145
		Red-necked Stint	400	2	402
	18/03/06	Muster	1		1
Murray River, NSW	23/11/2005 24/11/2005 25/11/2005	Dusky Moorhen	8		8
		Purple Swampphen	3		3
		White-necked Heron	1		1
		White-faced Heron	6		6
		Nankeen Night Heron	3		3
		Australian White Ibis	2		2
		Darter	1		1
		Great Cormorant	5		5
		Little Pied Cormorant	1		1
		Masked Lapwing	1		1
		Black-fronted Dotterel	2		2
		Red-kneed Dotterel	1		1
North-East Region	18/03/2006	Coot	1		1
	22/10/2005	Bar-tailed Godwit	60		60
		Red Knot	144		144
	6/11/2005 7/11/2005	Curlew Sandpiper	6		6
		Red-necked Stint	196	1	197
		Bar-tailed Godwit	3		3
		Caspian Tern	1		1
		Great Knot	1		1
		Grey Plover	2		2
		Little Stint	1		1
		Red Knot	129	1	130
	Sharp-tailed Sandpiper	1		1	
	27/12/2005	Curlew Sandpiper	4		4
		Red-necked Stint	65		65
	18/02/2006	Oystercatcher	10		10
	21/02/2006	Curlew Sandpiper	36		36
		Red-necked Stint	3		3
		Sharp-tailed Sandpiper	2		2
	TOTAL			1495	5

Table 4: Prevalence of AIV in ducks and shorebirds as determined by AIV Type A PCR. Only species where positives were detected are presented. Overall prevalence was calculated using all species sampled.

Bird Type	Common Name	PCR Positive	Total Number	Prev.	Overall Prevalence
Ducks, swans, geese	Pacific Black Duck	1	70	1.43%	5.53%
	Grey Teal	7	154	4.55%	
	Chestnut Teal	19	204	9.31%	
	Australian Wood duck	2	96	2.08%	
	Pink-eared Duck	5	144	3.47%	
	Australasian Shoveler	3	14	21.43%	
	Black Swan	3	18	16.67%	
Shorebirds and other waterbirds	Red-necked Stint	3	667	0.30%	0.33%
	Bar-tailed Godwit	1	248	0.40%	
	Red Knot	1	420	0.24%	



Table 5: Verification of the positive PCR results for each of the priority areas.

Location	Bird	Age (A/J)	DPI Type A PCR			AAHL Type A PCR		WHO Centre Type A PCR
			Ct value	Result	Number of mutations	Result	Ct value	Result
HIGH PRIORITY AREA								
Swan Island, Queenscliff	Red Knot	A	3.79	Positive	ND	Negative	-	ND
Swan Island, Queenscliff	Red-necked Stint		3.01	Positive	ND	Negative	-	ND
Kellys Lake, Kerang	Grey Teal	A	6.12	Positive	8	Negative	-	ND
Kellys Lake, Kerang	Pink-eared Duck	A	3.38	Positive	4	Positive	26.49	ND
Kellys Lake, Kerang	Pink-eared Duck	A	3.63	Positive	8	Ambiguous	36.92	ND
Lake Struan, Lismore	Chestnut Teal	A	4.09	Positive	0	Ambiguous	36.08	ND
Murpheys Lake, Kerang	Pink-eared Duck	A	8.74	Positive	9	Negative	-	ND
Point Henry, Geelong	Grey Teal	A	3.06	Positive	55	Ambiguous	33.42	ND
Lake Struan, Lismore	Grey Teal	A	2.81	Positive	0	Positive	28.62	ND
Lake Struan, Lismore	Grey Teal	A	2.91	Positive	8	Positive	28.08	ND
Lake Struan, Lismore	Grey Teal	A	29.29	Positive	5	Negative	-	ND
Lake Struan, Lismore	Pink-eared Duck	A	8.42	Positive	8	Negative	-	ND
Lake Struan, Lismore	Pink-eared Duck	A	9.32	Positive	ND	Negative	-	ND
MEDIUM PRIORITY AREA								
Lake Wendouree, Ballarat	Black Swan	A	3.71	Positive	6	Ambiguous	33.10	ND
Lake Wendouree, Ballarat	Black Swan	ND	13.77	Positive	4	Negative	-	ND
Paul's Wetland, Ballarat	Black Swan	ND	5.09	Positive	4	Ambiguous	37.29	ND
Taylor's Lake, Horsham	Australian Wood Duck	A	4.07	Positive	2	Negative	38.42	ND
Taylor's Lake, Horsham	Australian Wood Duck	A	13.80	Positive	1	Negative	-	ND

NON PRIORITY AREA									
Manns Beach Corner Inlet	Bar-tailed Godwit	A	18.54	Positive	ND	Positive	32.06	ND	
Manns Beach Corner Inlet	Red-necked Stint	A	9.96	Positive	ND	Negative	-	ND	
Manns Beach Corner Inlet	Red-necked Stint	A	8.57	Positive	ND	Negative	-	ND	
Lake Watt Watt, Orbost	Pacific Black Duck	A	12.34	Positive	3	Negative	-	ND	
Lake Watt Watt, Orbost	Chestnut Teal	A	3.50	Positive	2	Ambiguous	33.24	ND	
Lake Watt Watt, Orbost	Chestnut Teal	A	3.82	Positive	1	Ambiguous	37.14	ND	
Lake Watt Watt, Orbost	Chestnut Teal	A	2.84	Positive	2	Positive	29.26	ND	
Lake Watt Watt, Orbost	Chestnut Teal	A	4.24	Positive	2	Negative	-	ND	
Lake Watt Watt, Orbost	Chestnut Teal	A	7.57	Positive	0	Negative	-	ND	
Lake Watt Watt, Orbost	Chestnut Teal	A	11.78	Positive	7	Negative	-	ND	
Lake Watt Watt, Orbost	Chestnut Teal	ND	4.20	Positive	2	Negative	-	ND	
Lake Watt Watt, Orbost	Chestnut Teal	A	10.31	Positive	2	Negative	-	ND	
Lake Watt Watt, Orbost	Chestnut Teal	A	4.36	Positive	2	Ambiguous	34.00	Positive (**)	
Lake Watt Watt, Orbost	Chestnut Teal	A	26.03	Positive	6	Negative	-	(**)	
Lake Watt Watt, Orbost	Australasian Shoveler	A	9.16	Positive	0	Negative	-	ND	
Lake Watt Watt, Orbost	Australasian Shoveler	A	4.48	Positive	2	Ambiguous	35.31	Positive (**)	
Lake Watt Watt, Orbost	Australasian Shoveler	A	3.72	Positive	4	Ambiguous	36.31	ND	
McClouds Moras, Bairnsdale	Chestnut Teal	A	3.01	Positive	0	Ambiguous	35.84	Positive (H3)	
McClouds Moras, Bairnsdale	Chestnut Teal	A	3.16	Positive	4	Ambiguous	35.14	ND	
McClouds Moras, Bairnsdale	Chestnut Teal	A	9.97	Positive	2	Negative	-	ND	
McClouds Moras, Bairnsdale	Grey Teal	A	2.83	Positive	1	Positive	32.36	ND	
Sale	Chestnut Teal	A	3.61	Positive	3	Negative	-	ND	
Sale	Chestnut Teal	A	2.90	Positive	1	Positive	28.89	Positive (H12)	
Sale	Chestnut Teal	A	3.00	Positive	2	Positive	31.02	ND	
Sale	Chestnut Teal	J	2.89	Positive	4	Positive	29.88	ND	
Tasmania	Chestnut Teal	ND	7.97	Positive	4	Positive	28.00	ND	
Tasmania	Grey/Chestnut Teal	ND	2.60	Positive	5	Negative	-	ND	

ND Not determined

A Adult

J Juvenile

(**) Subtyping in progress

3. Recommendation of a PCR screening test for use in Avian influenza surveillance identified by participation of various laboratories in a coordinated, Australia-wide PCR testing program.

The CSIRO AAHL group obtained funding from the Biosecurity CRC to coordinate a program to evaluate the AAHL developed AIV Type A Real Time PCR test. Several laboratories from all around Australia participated in this program including our laboratory. It is not the intention of this laboratory to compete with other Australian laboratories, and we see that a further standardisation attempt is not necessary.

Based on the results of this exercise however, it has become evident to us that our nested test is in the several orders of magnitude more sensitive than the AAHL Real Time PCR test and so we choose to use our PCR for any further surveillance work.

4. Current PCR methodologies adapted for high throughput testing.

Our previous WEDPP project (4WEDPP-04) used Real Time PCR to test 1390 wild bird samples for avian influenza virus. The technique used followed standard protocols of RNA extraction, RT-PCR and finally Real Time PCR on a Roche Lightcycler machine. The Roche machine holds 32 individual glass capillaries and the process cannot be adapted for processing large numbers of samples. Since then, DPI Attwood has purchased an Applied Biosystems 7500 Real Time PCR machine, which is the machine of choice in many Australian laboratories thus lending itself more readily to standardisation of tests between laboratories. This machine also has the advantage of using a 96 well plastic plate for holding samples instead of individual capillaries. Samples can then be added to the plate using high throughput devices.

DPI Attwood is also in possession of other devices that can be used for processing large numbers of samples. These machines have all been successfully used in the AIV Type A Real Time PCR. Therefore, each step of the Real Time PCR procedure can now be processed on the following machines:

RNA extraction	Qiagen Biorobot 3000 or Corbett X-tractor-Gene 1820
Real Time PCR	Applied Biosystems 7500

The combination of these machines allows the processing of hundreds of individual samples in a day if necessary.

5. Presentation of surveillance data in a format that may be incorporated within a national surveillance database.

The results of this project and the previous WEDPP project have been provided to Franz Zikesch for incorporation within the eWHIS National AIV surveillance database.

Further characterisation of the PCR positives is still in progress. It is hoped that it will be possible to subtype more isolates using PCR techniques under development at the WHO Centre. Any results obtained from this point on will be submitted to the National eWHIS database.

CONCLUSIONS:

In summary, this project led to the successful collaboration between the Victorian and NSW Departments of Primary industries. The NSW group performed the GIS mapping and risk assessments at the start of the project, and this was followed by wild bird sampling based on their results. The mapping work led to the identification of several priority areas including the wetland areas surrounding Geelong, Kerang, Kyabram, Shepparton, Donald and Colac. Due to the inherent difficulties in collecting wild bird samples, particularly from live birds, there was a proportion of samples that were sampled from “non priority areas” since the opportunity to collect samples presented itself.

The overall prevalence of avian influenza virus in anatids and in shorebirds and other waterbirds was 5.5% and 0.33% respectively. Some species, such as black swans and Australasian Shovelers, appeared to have a higher prevalence than average and this could possibly be targeted further in future surveillance work. Given the overall low prevalence of AIV in shorebirds, it would be wise to pool samples in future work to reduce the testing costs associated with this type of work. This is possible with the DPI Type A PCR since the diagnostic sensitivity of this test appears to be more than enough to detect weak positive samples (data not shown). Given the apparent higher prevalence in some ducks and black swans, it would probably not be worth pooling these species, and all samples should be just be processed individually.

The lack of confirmation by viral culture in eggs of the 45 AIV PCR positive samples was disappointing. This highlights the care that must be taken in sample collection, transport and subsequent storage. More work is needed to determine the optimal conditions to address this issue for future surveillance work. The lack of confirmation of PCR results using culture served to highlight the issue of validation for PCR positives. This was an important issue that was addressed after consultation with several people. It was decided that the best way to verify the 45 PCR positive samples was by testing in multiple different AIV PCR tests and sequencing of the PCR products where possible. This was done, and all 45 PCR positives were confirmed and determined to be true results. Two of the PCR positives were able to be subtyped at the WHO Centre by sequencing the PCR product of a HA-2 PCR. This identified genetic material one H3 and one H12. It may be possible to determine the subtype of more of the positive samples, however this is very time consuming and costly and is outside the scope of this project. Future projects should make an effort to include this subtyping analysis wherever possible, particularly in the absence of live viruses, as this genetic analysis provides meaningful information on the ecology of endemic AI viruses circulating in Australia to help inform risk management.

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Appendix 1: Ramsar Criteria for Internationally Important Wetlands

These criteria were revised in 1999, many sites still use the old criteria. A wetland should be considered internationally important if:

1. it contains a representative, rare, or unique example of a natural or near-natural wetland type found within the appropriate biogeographic region.
2. it supports vulnerable, endangered, or critically endangered species or threatened ecological communities.
3. it supports populations of plant and/or animal species important for maintaining the biological diversity of a particular biogeographic region.
4. it supports plant and/or animal species at a critical stage in their life cycles, or provides refuge during adverse conditions.
- 5. it regularly supports 20,000 or more waterbirds.**
- 6. it regularly supports 1% of the individuals in a population of one species or subspecies of waterbird.**
7. it supports a significant proportion of indigenous fish subspecies, species or families, life-history stages, species interactions and/or populations that are representative of wetland benefits and/or values and thereby contributes to global biological diversity.
8. is an important source of food for fishes, spawning ground, nursery and/or migration path on which fish stocks depend (either within the wetland or elsewhere).

Old Ramsar Criteria

A wetland should be considered internationally important if:

- 1a. It is a particularly good representative example of a natural or near-natural wetland, characteristic of the appropriate biogeographical region.
- 1b. It is a particularly good representative example of a natural or near-natural wetland, common to more than one biogeographical region.
- 1c. It is a particularly good representative example of a wetland which plays a substantial hydrological, biological or ecological role in the natural functioning of a major river basin or coastal system, especially where it is located in a trans-border position.
- 1d. It is an example of a specific type of wetland, rare or unusual in the appropriate biogeographical region.
- 2a. It supports an appreciable assemblage of rare, vulnerable or endangered species or subspecies of plant or animal, or an appreciable number of individuals of any one or more of these species.
- 2b. It is of special value for maintaining the genetic and ecological diversity of a region because of the quality and peculiarities of its flora and fauna.
- 2c. It is of special value as the habitat of plants or animals at a critical stage of their biological cycle.
- 2d. It is of special value for one or more endemic plant or animal species or communities.
- 3a. It regularly supports 20,000 waterfowl.**
- 3b. It regularly supports substantial numbers of individuals from particular groups of waterfowl, indicative of wetland values, productivity or diversity.**
- 3c. Where data on populations are available, it regularly supports 1% of the individuals in a population of one species or subspecies of waterfowl.**
- 4a. It supports a significant proportion of indigenous fish subspecies, species or families, life-history stages, species interactions and/or populations that are representative of wetland benefits and/or values and thereby contributes to global biological diversity.
- 4b. It is an important source of food for fishes, spawning ground, nursery and/or migration path on which fish stocks depend (either within the wetland or elsewhere).

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