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Rapid identification and pathotyping of virulent IBDV, NDV and AIV isolates

The development and implementation of laboratory tests for rapid detection and differentiation of viruses

A report for the Rural Industries Research and Development Corporation

by Hans Heine & Lee Trinidad

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Researcher Contact Details
Hans Heine
CSIRO Livestock Industries
Australian Animal Health Laboratory
Private Bag 24 (5 Portarlington Road)
Geelong VIC 3220

Phone: 03 5227 5278
Fax: 03 5227 5555
Email: hans.heine@csiro.au

In submitting this report, the researcher has agreed to RIRDC publishing this material in its edited form.

RIRDC Contact Details
Rural Industries Research and Development Corporation
Level 2, 15 National Circuit
BARTON ACT 2600
PO Box 4776
KINGSTON ACT 2604

Phone: 02 6272 4819
Fax: 02 6272 5877
Email: rirdc@rirdc.gov.au.
Web: http://www.rirdc.gov.au

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Foreword

Emergency animal disease outbreaks result from the incursion into Australia of dangerous animal disease from other countries, or from unusually severe diseases that can emerge in Australia from endemic pathogens. Diagnostic and disease surveillance tests able to be used for rapid detection of these diseases are obvious pre-requisites for preparedness to combat outbreaks of these diseases. Virulent forms of Newcastle disease virus (NDV), infectious bursal disease virus (IBDV) and avian influenza virus (AIV) of poultry have the potential to cause these types of outbreaks and emergency outbreaks of NDV and AIV have occurred in Australia in recent years. The ability to rapidly diagnose index cases and to conduct prompt epidemiological surveys is crucial in an outbreak to support the swift implementation of disease control measures. The same diagnostic capabilities are required to confirm freedom from disease after an outbreak in order to lift trade restrictions and restore consumer confidence. Many of the long-established laboratory diagnostic tests are time consuming, lack sensitivity or cannot differentiate between closely related virus types. The aim of this project was the development, evaluation and implementation of real-time PCR based molecular diagnostic tests for the rapid identification of important avian viral pathogens and their differentiation from circulating endemic non/low-pathogenic strains.

This project was funded from industry revenue which is matched by funds provided by the Australian Government.

This report, an addition to RIRDC's diverse range of over 1500 research publications, forms part of our Chicken Meat R&D program, which aims to support increased sustainability and profitability in the chicken meat industry by focusing research and development on those areas that will enable the industry to become more efficient and globally competitive and that will assist in the development of good industry and product images. Funding was also provided by the RIRDC Egg Program, now the Australian Egg Corporation Limited (AECL).

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Peter O'Brien
Managing Director
Rural Industries Research and Development Corporation

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In addition to the funds received from RIRDC and AECL, the project was supported by a grant from the Australian Biosecurity Cooperative Research Centre for Emerging Infectious Disease for a project on the development of influenza virus type A and subtype H5-specific real-time RT-PCR tests for detection of Asian H5N1 isolates. Additional support from CSIRO Livestock Industries, Australian Animal Health Laboratory is acknowledged.

Abbreviations

AAHL	Australian Animal Health Laboratory
AIV	avian influenza virus
CT	cycle threshold
EID_{50}	median egg infectious doses
H5N1	subtype of HP AIV that emerged from Asia
HI	haemagglutination inhibition
HP	highly pathogenic
HVR	hypervariable region (in VP2 of IBDV)
IBDV	infectious bursal disease virus
LP	low pathogenicity
NDV	Newcastle disease virus
ORF	open reading frame
PCR	polymerase chain reaction
RFLP	restriction fragment length polymorphism
RT-PCR	reverse transcription polymerase chain reaction
RRT-PCR	real-time reverse-transcription PCR
SPF	specific pathogen free
vvIBDV	very virulent IBDV

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Summary

Aims

The project consists of the development and implementation of rapid and highly sensitive molecular tests for detection and identification of important poultry viral pathogens. The objectives for this project were the development, evaluation and implementation of molecular diagnostic tests based on real-time PCR for the rapid identification of three important viral pathogens, infectious bursal disease virus (IBDV), Newcastle disease virus (NDV) and avian influenza virus (AIV). These tests enabled the detection and differentiation of virulent strains from harmless non-pathogenic isolates that may circulate in the Australian poultry population.

Benefits

The new tests will result in substantial time saving for index case diagnosis and confirmation of specific pathogens by diagnostic laboratories. This will assist government authorities and the affected industries in the implementation of rapid response and control measures to minimise further spread of the disease and also provide better diagnostic tools for disease surveillance.

Background

The incursion or outbreaks of very virulent IBDV, virulent NDV or highly pathogenic AI are major threats to the Australian poultry industry. Tests for rapid index case diagnosis are essential for the swift implementation of disease control measures. Current molecular diagnostic tests for differentiation between highly virulent and non-virulent isolates are time consuming and take approximately two days. New test methods based on the intrinsic specificity of probes in real-time PCR (TaqMan fluorescent hydrolysis probes) are fast, highly sensitive and able to differentiate between closely related isolates. These tests can be performed in a few hours without the need for agarose gel electrophoresis and DNA sequencing. The short assay times and the capability to test a large number of samples simultaneously for different viruses make new real-time PCR tests invaluable for rapid identification of suspected index cases and for epidemiological surveys after outbreaks to confirm freedom from disease.

Methodology

DNA sequences were assembled from each of the virus families (IBDV, NDV and AIV) to identify regions conserved within the virus families and sequences characteristic of subtypes or virulent and non-virulent isolates in each family. Sets of PCR primers and fluorescently-labelled probes specific for virus families or virus patho- or sub-types were designed for TaqMan real-time RT-PCR assays. The analytical specificity of the TaqMan assays were evaluated on archived virus strains and the limits of detection determined with serially diluted viral nucleic acid. All work with virulent/ pathogenic and exotic strains of IBDV, NDV and AI was conducted in the microbiologically secure laboratories at AAHL.

Outcomes

Rapid and sensitive TaqMan RT-PCR assays have been developed for the laboratory-based diagnosis of important poultry viruses and are implemented in the AAHL Diagnostic Quality Assurance Manual. These tests can:

- Detect all serotype 1 IBDV strains and differentiate very virulent infectious bursal disease virus (vvIBDV) from classical overseas or Australian field and vaccine strains
- Detect Newcastle disease virus (NDV) and differentiate highly pathogenic strains from endemic low/non-pathogenic and vaccine strains
- Detect of avian influenza virus (AIV) Type A strains and identify H5 (including HPAI H5N1) and Australian H7 subtypes.

Implications

- The TaqMan RT-PCR assays are suitable for the rapid and sensitive detection of very virulent IBDV, virulent NDV and highly pathogenic AIV including H5N1 strains.
- The tests can reduce the time for index case diagnosis facilitating the rapid implementation of control measures in an outbreak.
- Knowledge of circulating virus strains and associated sequences is critical to the successful application of highly specific molecular assays as Australian viruses that differ significantly from those in other parts of the world and may not be detected by tests developed elsewhere.
- TaqMan RT-PCR assays are highly specific and are affected by genetic changes in the target virus population. Subtype- or strain-specific tests should be performed in conjunction with a generic test for the virus family.
- TaqMan RT-PCR assays are highly sensitive allowing the detection of traces of genetic material in samples from which virus cannot be isolated. The diagnostic implications of TaqMan results, especially near the limit of detection, should be considered carefully together with other clinical observations and laboratory results.

Recommendations

The TaqMan RT-PCR assays developed here should be used in qualified, accredited diagnostic laboratories for early and sensitive detection of virus and to recognize the potential emergence or outbreak of virulent or highly pathogenic strains. Strict quality assured procedures through all stages from the field to the lab should be maintained to ensure sample integrity and minimise contamination events.

Introduction

Although the Australian poultry industry is currently free from important diseases such as very virulent infectious bursal disease virus (vvIBDV), virulent Newcastle disease virus (NDV) or highly pathogenic avian influenza virus (HPAI), this disease-free status is under threat from potential incursions of exotic virulent strains or the emergence of pathogenic strains from circulating endemic viruses of low pathogenicity. The capability to carry out rapid diagnosis of index cases is crucial in an outbreak to support the swift implementation of disease control measures. The same diagnostic capabilities are required to conduct prompt epidemiological surveys after an outbreak to confirm freedom from disease and restore consumer confidence. Many of the long-established laboratory diagnostic tests are time consuming, lack sensitivity or cannot differentiate between closely related virus types. Molecular diagnostic tests are increasingly employed to improve diagnosis and support differentiation between highly virulent and non-virulent isolates. These existing tests rely on gene amplification by PCR and require post-amplification steps such as restriction fragment length polymorphism (RFLP), agarose gel electrophoresis or DNA sequence analysis. New real-time PCR test methods, based on the intrinsic specificity of probes such as TaqMan probes (Holland, 1991), are fast and highly sensitive, enabling the differentiation of closely related isolates in a few hours without the need for agarose gel electrophoresis and DNA sequencing. These tests can be automated for screening large numbers of samples for different viruses to support epidemiological surveys following an outbreak. The aim of this project was the development, evaluation and implementation of real-time PCR based molecular diagnostic tests for the rapid identification of important avian viral pathogens and their differentiation from circulating endemic non/low-pathogenic strains.

Infectious bursal disease virus (IBDV)

Infectious bursal disease (IBD) or Gumboro disease is caused by a virus from the family Birnaviridae. IBDV is distributed worldwide and has two serotypes. Only serotype 1 strains cause clinical disease in chickens and neither IBDV type 1 nor type 2 has been reported to cause disease in wild birds. Serotype 1 comprises three groups based on pathogenicity and antigenicity; the classical strains, the antigenic variants, and very virulent IBDV (van den Berg 1991). There is no antigenic difference

between the classical form and very virulent forms. Several strains of the classical form exist in Australia. IBD is primarily a disease of young chickens and can range from an acute disease to subclinical effects on the immune system and impairment of the rate of growth and development. The immunosuppression is due to destruction of the cells in the bursa of Fabricius, resulting in impairment of humoral immunity. The morbidity rate is usually high and mortality rates can reach up to 70% depending on the pathogenicity of the strain of IBDV (McFerran, 1993). Transmission of the virus is via the faecal-oral route. IBDV is highly resistant to environmental conditions and chemical and heat inactivation and the virus can persist for a long time. The emergence and rapid spread of vvIBDV strains in chickens throughout most parts of the world since the late 1980's, has resulted in an increase in vaccination failures, mortality rates and subsequent economic losses. Australia has remained free from vvIBDV and an incursion would adversely affect the poultry industry. Highly sensitive diagnostic assays for detection of IBDV in chickens and rapid differentiation between exotic vvIBDV strains and Australian endemic or vaccine strains are crucial for control of disease in an outbreak.

Newcastle disease virus (NDV)

Newcastle disease (ND) of chickens is caused by avian paramyxovirus type 1 (APMV-1), known as Newcastle disease virus (NDV) and is classified with the other avian paramyxoviruses in the genus Avulavirus, sub-family Paramyxovirinae, family Paramyxoviridae, order Mononegavirales. All birds are susceptible to infection with NDV but the pathogenic effects vary greatly between species with chickens being highly susceptible and water birds the least affected (Kaleta and Baldauf, 1988). NDV has a worldwide distribution and strains have been classified, according to the clinical symptoms, as velogenic (highly virulent), mesogenic (moderately virulent) or lentogenic (mildly virulent). Virulent NDV are defined on the basis of intracerebral pathogenicity index (ICPI) and the structure of the N- and C-termini of the F1 and F2 proteins of the virus (Aldous et al., 2003). NDV is transmitted by aerosol and the faecal-oral route and the virus can survive readily in the environment. Newcastle disease is characterised by gastrointestinal, respiratory and neurological signs and the strains can be broadly classified on pathological and clinical descriptions (viscerotropic velogenic, neurotropic velogenic, etc). Australia was free of pathogenic forms of ND until 1998 when outbreaks of virulent ND occurred as a result of mutation of endemic strains. Exotic strains of NDV are a significant threat to Australia's poultry industries, aviculture and on the environment through its impact on wild birds.

Highly pathogenic avian influenza virus (HPAI)

Avian influenza is caused by viruses of the Orthomyxoviridae family. There are three antigenic types of influenza viruses, A, B, and C; only influenza A viruses are known to infect birds. Influenza A viruses are further divided into subtypes on the basis of the haemagglutinin (H) and neuraminidase (N) antigens. At present, there are 16 recognised H subtypes and nine N subtypes. Each virus possesses one H and one N subtype. Highly pathogenic avian influenza (HPAI) is defined on the basis of *in vivo* and *in vitro* tests, and on amino acid sequence of the cleavage site of the haemagglutinin. HPAI has been associated only with subtypes H5 and H7, however, not all H5 and H7 viruses cause HPAI. Wild birds are generally believed to be reservoir hosts for low virulence H5 and H7 viruses that can mutate to high virulence after introduction into poultry (Alexander, 2000). Avian influenza has worldwide distribution. Five outbreaks of HPAI have occurred in Australia, in 1976, 1985, 1992, 1994 and 1997. These outbreaks were all of the H7 subtype and are believed to have been associated with migratory waterfowl. Many strains of non-pathogenic AI viruses have been reported in native and migratory birds in Australia. Clinical signs in chickens range from sudden death to respiratory distress, oedema of the head, cyanosis, and diarrhoea. Mortality rates vary from low, to 100% for HPAI,and are influenced by the host's immune status, age, presence of other disease agents and environmental conditions. Virus is shed in the faeces and respiratory tract and horizontal transmission occurs readily. HPAI virus is sensitive to extremes of environmental conditions, however its survival in organic matter and water forms an important source of virus in outbreaks of disease in poultry. The HPAI H5N1 strains (Li et al., 2004) first detected in live bird markets in Hong Kong in 1997 have spread throughout Asia into Europe and parts of Africa, infecting domestic poultry and wild birds.

H5N1 strains have also shown the potential for infecting other species and have been associated with human fatalities, raising fears of a potential pandemic.

Objectives

The objective for this project was the development and implementation of rapid and highly sensitive molecular tests for detection and identification of three important poultry virus pathogens, infectious bursal disease virus (IBDV), Newcastle disease virus (NDV) and avian influenza virus (AIV). The aim was to establish diagnostic tests to detect all strains in the virus family and to differentiate emerging or exotic virulent and pathogenic strains from harmless non-pathogenic isolates that may circulate in the poultry population. The new tests will be considerably faster than current tests for index case diagnosis and assist authorities and the affected industries in the swift implementation of control measures to minimise further spread of the disease.

To develop and implement TaqMan RT-PCR assays for:

- Detection of all serotype 1 IBDV strains and differentiation of very virulent infectious bursal disease virus (vvIBDV) from classical overseas or Australian field and vaccine strains
- Detection of Newcastle disease virus (NDV) and identification of endemic low/non-pathogenic and vaccine strains to discriminate from highly pathogenic strains
- Detection of avian influenza virus (AIV) Type A strains and identification of subtypes H5 (including HPAI H5N1) and Australian H7.

Methodology

Research strategy and methodology

Different strains from each of the virus families (IBDV, NDV and AIV) were identified and assembled according to place of origin and biological and phylogenetic characteristics. Nucleotide sequence data for the strains were obtained from literature searches, GenBank database and our own sequence data for viruses held at AAHL. Complete genome sequences were only available for relatively few strains in each virus family as most of the strains contained only partial sequence data and often limited to only a small region or single gene. Within each virus family genome, regions were identified with the most sequence data available that were relevant for diagnostic assays. For each of the virus families, DNA sequences from different genome regions were aligned to identify regions conserved within the virus families or characteristic of specific groups or types. Based on these sequence alignments, sets of PCR primers and fluorescently-labelled probes specific for virus families or virus types were designed for TaqMan real-time RT-PCR assays. Optimal reaction conditions and analytical specificity of the TaqMan assays were evaluated on archived virus strains. The limits of detection were determined with serially diluted viral nucleic acid. All work with virulent, pathogenic and exotic strains of IBDV, NDV and AI was conducted in the microbiologically secure laboratories at AAHL. The assays were evaluated on fresh clinical material whenever these were available from research and training activities and from diagnostic specimens sent to AAHL. Assay standard operating procedures were implemented in the Molecular Diagnosis program of the AAHL QA manual under NATA/ISO 17025 standards and form part of AAHL's emergency disease preparedness. Some of the tests have been transferred to state veterinary diagnostic laboratories.

Viruses

IBDV classical virulent strain F52/70 and vv strain CS88 were obtained from Dr. N. Chettle, Central Veterinary Laboratories, Weybridge, UK. Classical virulent strain APHIS and antigenic variant strains GLS and variant E were obtained from Prof. J. J. Giambrone, Auburn University, Auburn, Alabama, USA. The origin of Australian classical isolates 002/73, 06/95, N1/99 and N2/99, Australian variant isolates 02/95 and 05-5, and Indonesian vv isolate Tasik94 have been described previously (Sapats and Ignjatovic, 2000, 2002; Ignjatovic and Sapats, 2002; Rudd et al., 2002). NDV strains were V4 vaccine strain, avirulent Peats Ridge 98 and virulent Deans Park 98 (all held at

AAHL) (Gould, 2001, 2004). HPAI H5N1 isolates were derived from samples submitted to AAHL in 2004 from Vietnam (Regional Veterinary Center, Ho Chi Minh City, Vietnam) and Cambodia (National Animal Health and Production Investigation Centre, Phnom Penh, Cambodia). Strains HPAI A/chicken/Vietnam/008/2004 (H5N1), A/Shearwater/Australia/75 (H5N3) and HPAI A/chicken/NSW/1/97 (H7N4) were used as positive controls in the real-time RT-PCR tests. IBDV stocks were prepared from the bursae of experimentally infected specific-pathogen-free (SPF) chickens as described previously (Sapats and Ignjatovic, 2000). NDV and AIV were propagated in embryonating eggs and in chickens and were processed in approved isolation rooms or biosafety cabinets in the microbiological containment facility at the Australian Animal Health Laboratory (AAHL). Viruses were inactivated in 1% SDS.

Nucleic acid extraction

RNA was extracted from 100-200 µl samples of 10% tissue homogenates (bursal homogenate for IBDV), tracheal swabs, cloacal swabs or allantoic fluid using either an RNeasy kit (Qiagen) for manual extraction or an automated MagNA Pure LC workstation (Roche) with a MagNA Pure LC total nucleic acid isolation kit (Roche). The MagNA Pure system is based on binding of nucleic acid in lysis buffer, containing guanidine isothiocyanate, to magnetic glass particles under chaotropic conditions. This is followed by a wash to remove unbound substances and elution of RNA from the magnetic particles under conditions of low salt concentration and elevated temperature. RNA or total nucleic acid was eluted in 50 µl of water.

Primers and probes

IBDV: The nucleotide sequences of 12 serotype 1 strains representing classical, vv, antigenic variant and Australian isolates were aligned across the entire segment A polyprotein coding region (3,036 bp) using the computer program Align Plus 4, version 4.1 (Sci Ed Central). Sequences in the VP5-VP2 overlapping reading frame were chosen for the design of PCR primers and hybridisation probe for a test to detect all IBDV strains of serotype 1 (IBDV-all) (Table 1). The design of primers and probes to discriminate between vvIBDV and classical strains was based on an alignment of the VP2 hypervariable region (Heine et al., 1991) of 105 serotype 1 IBDV isolates including 60 vv, 6 classical, 15 antigenic variant, 22 attenuated and 2 atypical sequences from 20 countries (Rudd et al., 2002). Non-fluorescent quenching (NFQ) minor groove binder (MGB) probes were chosen in preference over conventional fluorescent quenching probes as this enabled the design of shorter specific probes. In each case, probes and primers were designed using the Primer Express software (Applied Biosystems) and synthesised by Applied Biosystems (Foster City, USA) and GeneWorks (Adelaide, Australia), respectively (Table 1). Very virulent-specific (IBDV-vv) and classical-specific (IBDV-cla) probes were identified in a homologous region where both types differed by 3 silent nucleotide substitutions. Multiple primer sets were utilised to accommodate sequence variations within different virus groups (Table 2; Figure 1). The expected amplicon size for the conserved TaqMan test was 90 bp with all strains, and for the group specific TaqMan tests 128 bp for vv, 132 bp for overseas classical and 138 bp for Australian classical strains.

NDV: The design of TaqMan PCR primers and probes was based on nucleotide sequence alignment of Australian poultry isolates obtained following the national NDV survey (Kite, 2000) and a number of pathogenically diverse Australian NDV strains, including V4 vaccine strains, avirulent Peat’s Ridge ’98 and virulent Deans Park ’99 field strains. The TaqMan test to detect all NDV strains (NDV-all) was based on a conserved sequence region in the F1 portion of the fusion protein. The type-specific TaqMan tests, NDV-V4, NDV-avir and NDV-vir were designed to discriminate between the V4 vaccine and Peats Ridge strains respectively, utilising conserved forward and reverse primers flanking the fusion protein cleavage region and specific TaqMan probes to bind over the cleavage region (Table 4). The V4 vaccine specific TaqMan probe (NDV-F-V4) differed by three nucleotide substitutions from the Peats Ridge strain specific probe (NDV-F-avir), allowing for differentiation of vaccine strain from Peats Ridge strain when all three TaqMan tests were performed on the same sample. The region for NDV-vir probe sequence overlapped with NDV-avir and NDV-V4 and

differed by two nucleotide substitutions with avirulent NDV and four substitutions with NDV V4 vaccine sequences (Figure 3).

AIV: PCR primer and probe sequences of avian influenza Type A-specific (matrix gene) and H5 subtype-specific TaqMan assays designed to detect North American type A influenza virus strains (Spackman et al., 2002), were aligned against Asian H5 (H5N1) sequences in GenBank from 1997 to 2004. Characteristic sequence differences between the primers and the Asian H5N1 strains were identified and new primers (Table 6) designed to improve the sensitivity of detection for the highly pathogenic H5N1 strains currently circulating in Asia (Heine et al., 2005). Redundant nucleotides were incorporated into the primers for the influenza Type A-specific TaqMan assay to increase the sequence multiplicity and sequence homology for H5N1 strains, whilst maintaining the specificity of the original primers (Spackman et al., 2002). New primers were designed for strains of the Eurasian H5 lineage, as the primer sequences for the North American H5 viruses were not suitable for Asian H5N1. The sequences of the Type A-specific (matrix gene) and H5-specific TaqMan probes (Spackman et al. 2002) were identical in Asian H5N1 and were used without changes. The Australian H7 viruses form a genetic lineage which is quite distinct from the Eurasian isolates (Banks et al., 2000). Primers and probe for detection of Australian H7 strains were designed, based on sequences from the previous five outbreaks of HPAI H7: A/chicken/Victoria/76 (H7N7) [GenBank Z47199]; A/chicken/Victoria/1/85 (H7N7) [GenBank M17735]; A/chicken/Victoria/1/92 (H7N3) [GenBank AF202227]; A/chicken/Queensland/667/95 (H7N3) [GenBank AF202231]. The complete coding sequence for the HA of HPAI A/chicken/NSW/1/97 (H7N4) from the last outbreak in Australia was determined in this study [GenBank AY943924].

TaqMan RT-PCR

TaqMan tests were initially carried out in a two-step format with separate reactions for cDNA synthesis (using TaqMan reverse transcription reagents and random hexamer cDNA primers) followed by TaqMan PCR reaction (using TaqMan Universal master mix). Subsequently, one step RT-PCR reactions were performed in triplicate using the TaqMan one step RT-PCR kit (Applied Biosystems, Foster City, CA, USA) in a 25 µl total volume containing the following components: 12.5 µl 2X universal PCR master mix reagent (no AmpErase UNG), 0.625 µl, 40X Multiscribe and RNase inhibitor mix, virus-specific primer-probe mix as specified for IBDV (Table 2), NDV (Table 5) or AIV (Table 7), 0.125 µl each 18S rRNA control reagent forward primer, reverse primer and VIC labeled probe (Applied Biosystems; TaqMan Ribosomal RNA Control Reagents) and 2 µl of RNA template. The TaqMan Ribosomal RNA Control Reagents are designed to detect the 18S ribosomal RNA (rRNA) gene. Ribosomal RNA levels provide an endogenous control for PCR quantitation studies. A master mix containing all reagents except the template was prepared for each set of reactions and 23 µl of master mix was aliquoted onto a 96 well optical reaction plate. Two microlitres of sample template was then added to the plate. No template was added to negative controls, and RNA extracted from specific virus was added to positive controls. Thermocycling parameters for each reaction were 30 min at 48 °C (reverse transcription), 10 min at 95 °C (hot-start Taq polymerase activation), and 45 cycles of 15 sec at 95 °C and 1 min at 60 °C (PCR). Assays were performed and analysed using the ABI 7700 Sequence Detection System and software (Applied Biosystems). The results of a TaqMan assay were expressed in the form of software generated characteristic amplification curves. Amplification curves from positive and negative (No Template Controls) were compared to the test sample and the results expressed as cycle threshold (CT) values representing the number of cycles necessary for a statistically significant rise in reporter dye emission. Threshold bars used to determine these CT values were set to a consistent ΔRn value (fluorescence signal), specific to the assay about half way along the linear portion of an amplification plot. Microsoft Excel-based software was used for the calculation and plotting of standard curves.

DNA sequence analysis

Sequence contigs were created using SeqMan program (DNA star Inc.). Sequence alignments were performed using CLUSTAL W program (Thompson et al., 1994) in BioManager by ANGIS

(http://www.angis.org.au). Primer and probe sequences were checked by Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1997) using "Search for short nearly exact matches" on NCBI http://www.ncbi.nlm.nih.gov/BLAST/ . The haemagglutinin gene complete coding sequence of A/chicken/NSW/1/97(H7N4) was submitted to GenBank, accession number AY943924.

Results

Infectious bursal disease virus (IBDV) TaqMan RT-PCR assays

TaqMan RT-PCR assays to detect all IBDV serotype 1 strains, to specifically identify vv-IBDV isolates and to differentiate them from classical including Australian and vaccine strains were developed from alignments of genome segment A and the VP2 hypervariable region (Rudd et al., 2002). A TaqMan RT-PCR assay to detect all serotype 1 strains was based on highly conserved sequences near the 5'-terminus of segment A, where the open reading frames (ORF) for VP2 and VP5 overlap. Tests specific for either vvIBDV or classical strains, including antigenic variants, vaccine strains and Australian IBDV strains, were based on utilising two different probes (IBDV-vv and IBDV-cla) in the VP2 hypervariable region (Figure 1).

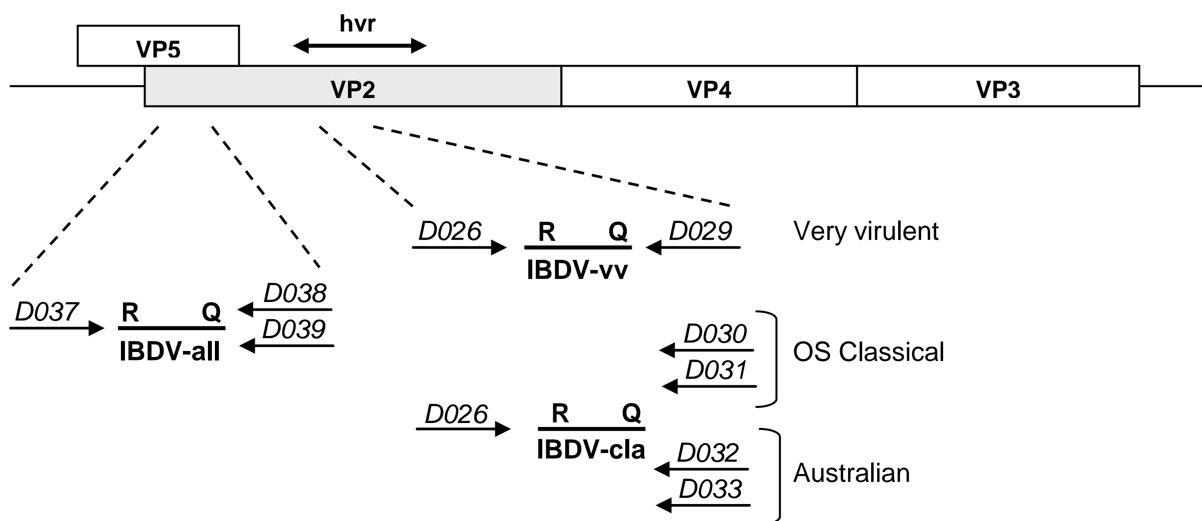


Figure 1. *Design of primer and probe combinations for TaqMan RT-PCR to detect all serotype 1 strains or to differentiate between very virulent and classical (including overseas and Australian) strains. Schematic of primer and probe alignments along the VP2-4-3 coding region in genome segment A is not to scale. The hypervariable region is indicated (hvr) by the arrow above VP2. Primer names are shown in italics and probes in bold. R indicates the fluorescent reporter and Q the quencher in the probe.*

Some of the PCR primers contained multiple nucleotides to accommodate the sequence diversity between different strains and to provide robust primer sets across a large number of strains. The choice of minor groove binding (MGB) non-fluorescent quencher TaqMan probes in preference over TAMRA fluorescent quencher probes enabled the design of shorter vvIBDV-specific or classical IBDV strain-specific probes (Kutyavin et al., 2000). The TaqMan RT-PCR assays were initially performed in a two-step test format comprising a first step RT reaction followed by a separate TaqMan PCR reaction. A one-step method with combined RT and TaqMan PCR reactions was subsequently used in all experiments, as this procedure required less hands-on time and could easily be automated from RNA extraction to TaqMan assay without apparent loss of sensitivity (data not shown).

Table 1. *IBDV primer and probe sequences*

Name	Location [a]	Sequence (5' → 3')
TaqMan Primer		
IBDV-D037	VP5/VP2	CGGAGCCTTCTGATGCCA
IBDV-D038 [c]	VP5/VP2	AGGTCGAGGTCTCTGACCTGAG
IBDV-D039 [c]	VP5/VP2	TGTAGGTTGAGGTCTCTGACCTGAG
IBDV-D026	VP2	GGCCCAGAGTCTACACCATAACTG
IBDV-D029 [c]	VP2	ACGCTTGTTTGAAACACGAGTTC
IBDV-D030 [c]	VP2	GCTCTCCCCCAACGCTG
IBDV-D031 [c]	VP2	AGCTCTCCCCCAATGCTGA
IBDV-D032 [c]	VP2	AACACGAGCTCTCCTCCAACA
IBDV-D033 [c]	VP2	ACACGAGCTCCCCTCCAAC
TaqMan Probe [b]		
IBDV-all	VP5/VP2	(FAM)-CAACCGGACCGGCGT-(MGB-NFQ)
IBDV-vv	VP2	(FAM)-CTCAGCTAATATCGATGCC-(MGB-NFQ)
IBDV-cla	VP2	(FAM)-CAGCCAACATTGATGC-(MGB-NFQ)

[a]) Target gene for PCR amplicon. [b]) TaqMan probes were 6-FAM labelled minor grove binding (MGB) probes containing non-fluorescent quencher (NFQ). [c]) Denotes antisense orientation

Table 2. *IBDV primer and probe combinations (reaction mixes)*

Specificity	Primer fwd	Primer rev	Probe
IBDV type 1	IBDV-D037 (900 nM)	IBDV-D038 (450 nM) IBDV-D039 (450 nM)	IBDV-all (250 nM)
vvIBDV	IBDV-D026 (900 nM)	IBDV-D029 (900 nM)	IBDV-vv (250 nM)
Classical IBDV	IBDV-D026 (900 nM)	IBDV-D030 (450 nM) IBDV-D031 (450 nM) IBDV-D032 (450 nM) IBDV-D033 (450 nM)	IBDV-cla (250 nM)

The ability of the TaqMan RT-PCR tests to detect all IBDV strains and to differentiate between different types of IBDV strain is shown in Table 3 with representative typical strains. Assays were evaluated on a range of strains including vvIBDV from Europe (CS88) and Asia (Tasik94), exotic classical IBDV from Europe and USA (52/70, APHIS), antigenic variants from the US (GLS, variant E), and endemic Australian strains (isolates 002/73, 02/95, 05-5, 06/95, N1/99, N2/99). All of the strains were detected in the conserved test (IBDV-all) and only vvIBDV strains in the vv-specific test and all classical strains in the tests for specific for classical IBDV. Control nucleic acid extracted from other avian pathogenic viruses (NDV, AI, ILT and MDV) or uninfected bursa was negative in the conserved and type-specific IBDV TaqMan RT-PCR tests (data not shown). Virus types could easily be distinguished by their reactivity patterns in the TaqMan tests (Table 3).

Table 3. *Differentiation of IBDV strain types by TaqMan RT-PCR.*

	CT Values from IBDV TaqMan assay [a]		
IBDV strain	**IBDV type 1**	**vvIBDV**	**Classical IBDV**
CS88	21.08 (0.06)	21.78 (0.07)	neg
Tasik94	19.90 (0.11)	20.85 (0.06)	neg
52/70	22.32 (0.06)	neg	25.94 (0.16)
Variant E	16.22 (0.06)	neg	21.07 (0.02)
002/73	18.15 (0.05)	neg	26.20 (0.19)
02/95	16.11 (0.01)	neg	25.92 (0.52)

[a] *Assays were performed simultaneously on six IBDV strains representing vvIBDV (CS88, Tasik94), typical classical (52/70), classical antigenic variant (Variant E) and Australian strains (002/73, 02/95). Viral RNA extracted from bursal homogenate was assayed for 45 cycles. All tests were performed in triplicate and the average CT values are shown with standard deviation in brackets ( ). CT values greater than 40 were considered negative (neg).*

To establish the linear dynamic range of the TaqMan assays, standard curves were generated using *in vitro* transcribed RNA templates derived from the plasmid clones representing Australian, classical and vvIBDV, respectively (p00273AVV shown in Figure 2). RNA template concentrations were converted to genomic equivalent copy number to determine detection limits. The test performance was linear over 5 logs in the assayed range from 10^2 to 10^7 copy numbers of input RNA template. At higher dilutions, using 10 copies or less of input RNA template, the detection of nucleic acid was still possible but not quantitative due to sampling anomalies at low copy numbers.

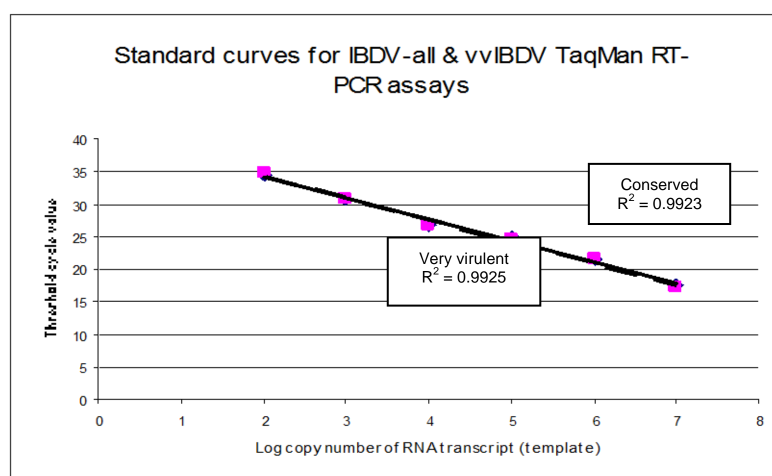


Figure 2. *Standard curves to determine linearity and analytical sensitivity of TaqMan RT-PCR assays. Serial dilutions of quantified RNA transcribed in vitro from plasmid p00273AVV, representing vvIBDV was used as input for each of the TaqMan reactions. Regression analysis of CT values was performed for each data set to construct standard curves and the R^2 values are shown. Each dot represents the mean of triplicate PCR amplifications for each dilution. Data for the conserved TaqMan RT-PCR assay (primer/probe IBDV-D037, D038, D039, IBDV-all) are represented by solid lines and data for the vvIBDV-specific assay (primer/probe IBDV-D026, D029, IBDV-vv) by dashed lines (virtually superimposed in this graph).*

Newcastle disease virus (NDV) TaqMan RT-PCR assays

TaqMan RT-PCR assays were developed to detect Australian NDV strains and to differentiate virulent strains from field strains and V4 vaccine (Figure 3; Table 5). Strain specific probes were based on differences in the fusion protein cleavage sequence which is the primary molecular determinant of pathogenicity. TaqMan tests were evaluated under uniform reaction conditions in a one-step format combining RT and TaqMan PCR reaction containing TaqMan One-step master mix and gene-specific primers and probe (Table 4). The amplification plot of each reaction was generated and the cycle threshold CT and fluorescence signal (ΔRn) values were used to identify the virus(es) present in a sample. The amplification plots of the NDV-all and the type specific TaqMan test were compared for each of the different viruses (Figures 4, 5, 6).

NDV TaqMan assay design based on F cleavage region

```
                     R  R  Q  G  R  L
tctgtgaccacgtccggaggaaggagacaggggcgtcttataggcgccattatcggtggtgtagctc  Avir
..............................a.....t..............................  Vir
.....................g...a......a..................................  V4(W)
.....................g...a......a..c........a......................   (G)
.....................g...a......a..c........a......................   (B)
.....................g...a......a..c........a.................g....   (C)
...............a.....g...a......a..c........a.................g....   (E)
...............a.....g...a......a..c........a......................   (D)
...............a.....g...a......a..c........a......................   (F)
....................gg.........aa...........a......................   (A)
  Forward Primer     TaqMan Probe                Reverse Primer
```

Figure 3. Design of TaqMan based on NDV F cleavage region to distinguish between field and vaccine strains. Reference strains for avirulent (Avir) were Peat's Ridge '98, for virulent (Vir) Deans Park '98 and for vaccine and vaccine-like (V4) V4 vaccine strain (W) and field isolates (A – G) from the 2000 National NDV survey.

Table 4. *NDV primer and probe sequences*

Name	Location [a]	Sequence (5' → 3')
TaqMan Primer		
NDV-D040	F1	TCTGTGACCACGTCCGGA
NDV-D041	F1	TCTGTGACCACGTCCAGA
NDV-D042 [c]	F2	GAGCTACACCACCGATAATGGC
NDV-OzF-D043	F1	GTCAATCATAATCAAGTTACTCCCAAAT
NDV-OzR-D044 [c]	F1	GTAGTCAATGTCCTGTTGTATGCCTC
TaqMan Probe [b]		
NDV-F-allAUS	F1	(FAM)-TTTTGCACACGCCT-(MGB-NFQ)
NDV-V4 [c]	F1-F2	(FAM)-CGTCCCTGTTTCCC-(MGB-NFQ)
NDV-avir [c]	F1-F2	(FAM)-CGCCCCTGTCTCCT-(MGB-NFQ)
NDV-vir [c]	F1-F2	(FAM)-TAAAACGCCTCTGTCT-(MGB-NFQ)

[a]) Target gene for PCR amplicon. [b]) TaqMan probes were 6-FAM labelled minor grove binding (MGB) probes containing non-fluorescent quencher (NFQ). [c]) Denotes antisense orientation

Table 5. *NDV primer and probe combinations (reaction mixes)*

Specificity	Primer fwd	Primer rev	Probe
NDV-all	NDV-OzF-D043 (900 nM)	NDV-OzR-D044 (900 nM)	NDV-F-allAUS (250 nM)
NDV-V4	NDV-D040 (450 nM) NDV-D041 (450 nM)	NDV-D042 (900 nM)	NDV-F-V4 (250 nM)
NDV-avir	NDV-D040 (450 nM) NDV-D041 (450 nM)	NDV-D042 (900 nM)	NDV-avir (250 nM)
NDV-vir	NDV-D040 (450 nM) NDV-D041 (450 nM)	NDV-D042 (900 nM)	NDV-vir (250 nM)

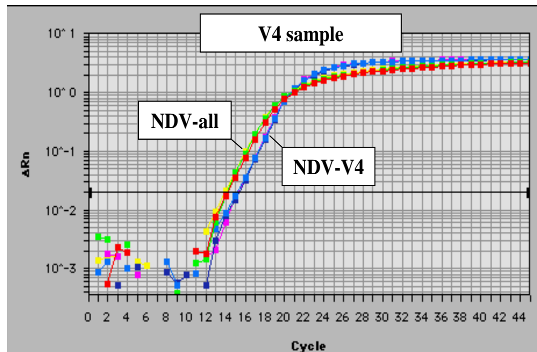


Figure 4. Amplification plots of generic TaqMan test NDV-all together with the strain specific NDV-V4 test performed in triplicates with viral RNA extracted from NDV V4 strain.

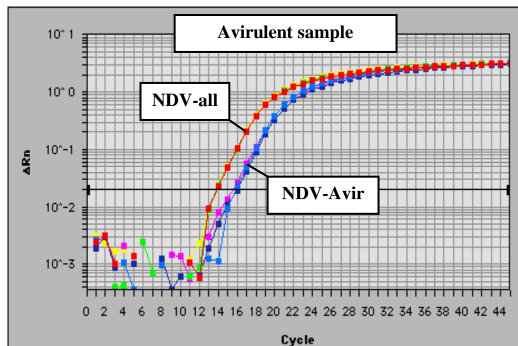


Figure 5. Amplification plots of generic TaqMan test NDV-all together with the strain specific NDV-Avir test performed in triplicates with viral RNA extracted from NDV Peats Ridge '98 strain.

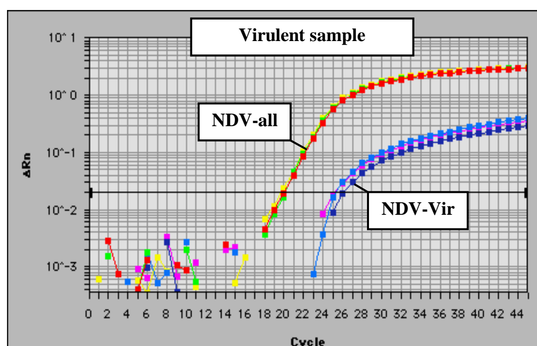


Figure 6. Amplification plots of generic TaqMan test NDV-all together with the strain specific NDV-Vir test performed in triplicates with viral RNA extracted from NDV Deans Park '98 strain.

The reaction efficiencies of the NDV-V4 and the NDV-avir assays were very similar to that of the NDV-all assay, when tested in parallel using the specific target virus. In contrast, the reaction efficiency of NDV-vir was considerably lower than NDV-all when tested in parallel with the virulent Deans Park ’98 virus, resulting in higher CT value and reduced ΔRn. The lower reactivity of the NDV-Vir assay is most likely due to inefficient probe. The probe design guidelines and algorithm are very rigid and difficult to apply when differentiating sequences containing only minor differences and where probe design is confined to a small region, such as the cleavage site. The linear dynamic range and the limit of detection for each assay were determined using serially diluted template, as illustrated in Figure 7 for the NDV-avir and NDV-all assays with cDNA from avirulent Peats Ridge ’98 virus. Each of the virus templates could be detected over at least a log 5 linear range. Examples for the analytical specificity of the assays are shown in Figures 8 and 9. Although there was cross reactivity between the avirulent (NDV-Avir) and the virulent (NDV-Vir) and between the avirulent (NDV-Avir) and the V4 vaccine (NDV-V4) assays, the correct virus was identified when all the assays were performed in parallel for a given template. The reaction pattern consistently showed the CT values of the type-specific assay closest to that of NDV-all, with the non-specific tests always showing higher CT values (indicating lower reactivity) than the specific test.

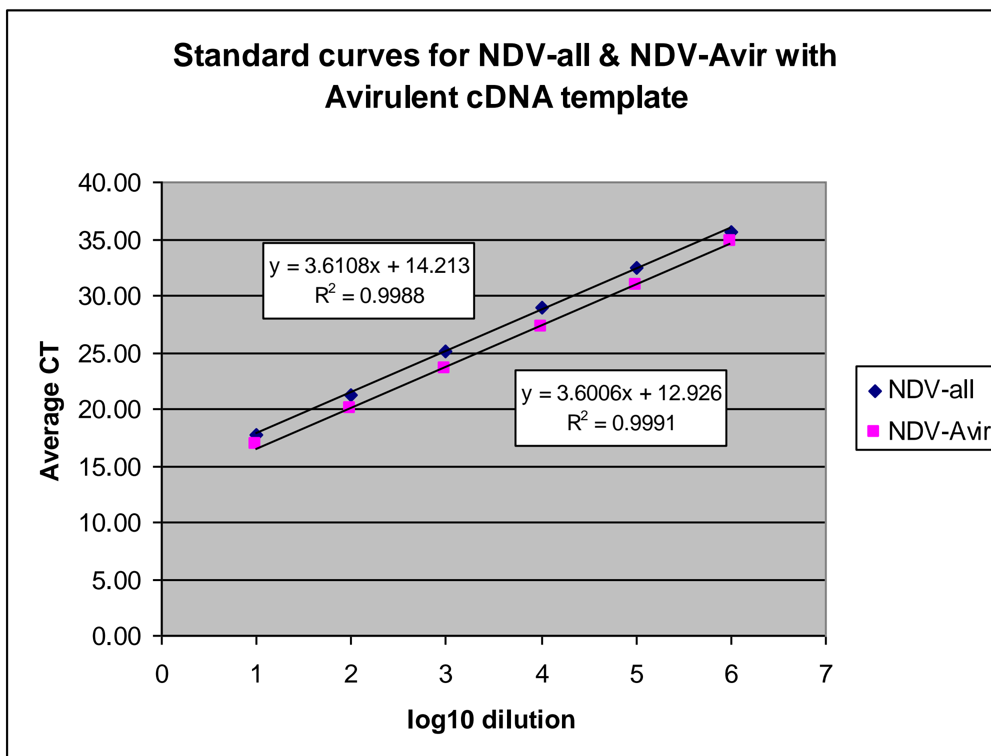


Figure 7. Standard curves for NDV-all and NDV-Avir TaqMan RT-PCR assays performed with serially diluted cDNA from avirulent NDV Peats Ridge ’98 . All CT values are average of triplicates.

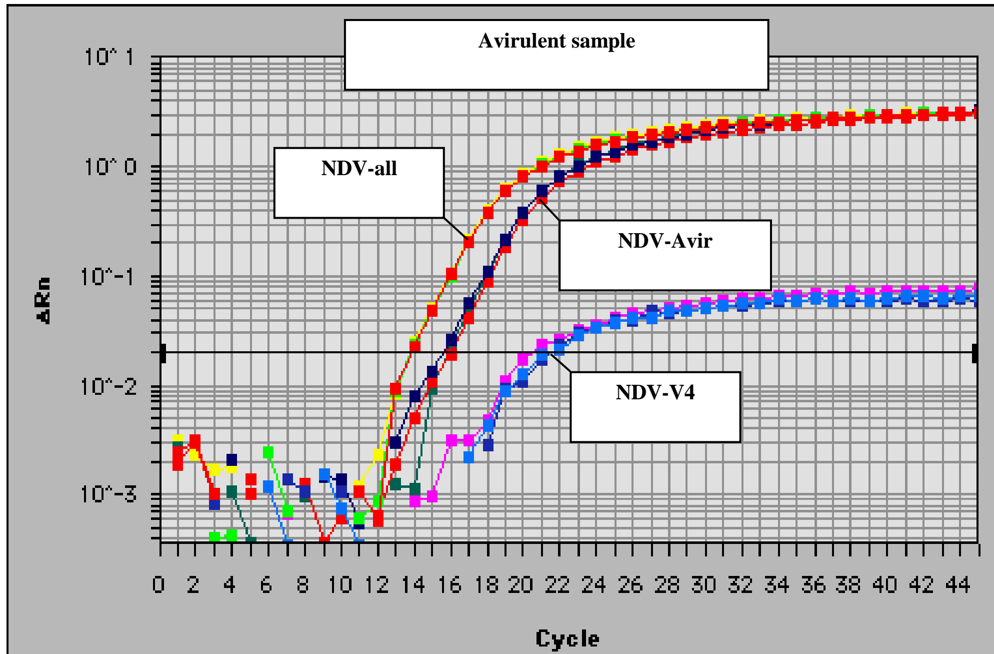
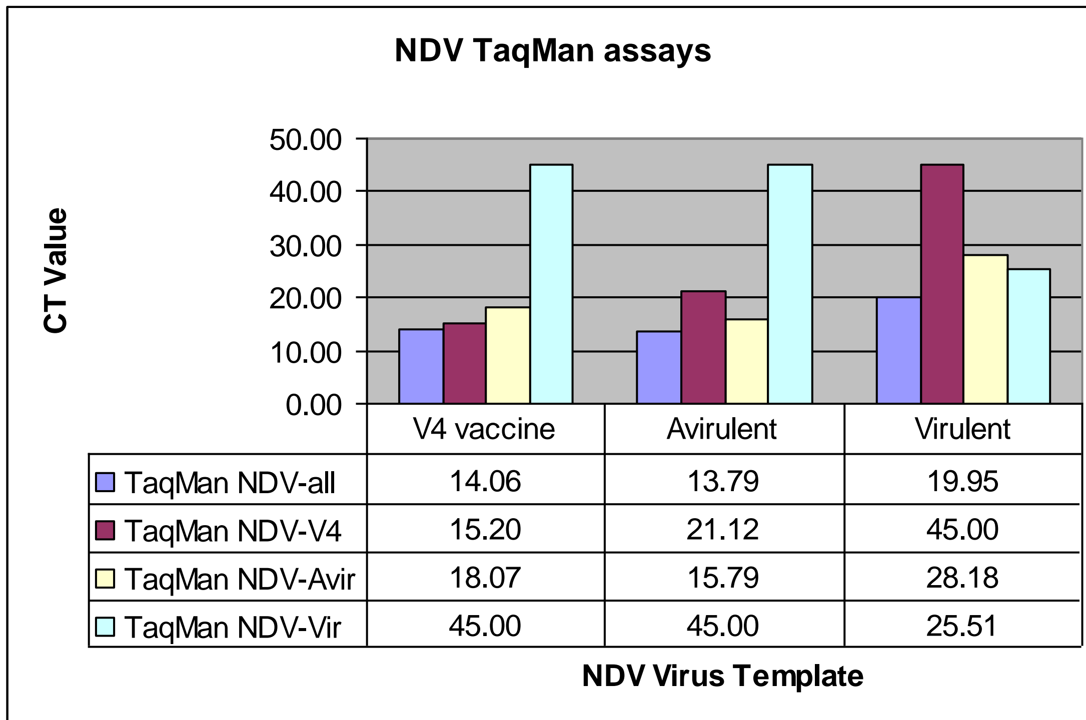


Figure 8. Amplification plots of generic TaqMan test (NDV-all) together with the NDV-V4 and NDV-Avir using viral RNA template extracted from avirulent Peats Ridge ’98 strains.



	V4 vaccine	Avirulent	Virulent
TaqMan NDV-all	14.06	13.79	19.95
TaqMan NDV-V4	15.20	21.12	45.00
TaqMan NDV-Avir	18.07	15.79	28.18
TaqMan NDV-Vir	45.00	45.00	25.51

Figure 9. Characteristic example of CT values when all NDV TaqMan assays were performed simultaneously for each template to determine the analytical specificity of the assays. CT values greater than 40 are considered virus negative.

Avian influenza virus (AIV) TaqMan RT-PCR assays

The primer and probe sequences of TaqMan RT-PCR assays published for detection of influenza Type A and subtyping of North American AI H5 and H7 strains (Spackman et al. 2002) were evaluated for their suitability to detect and subtype strains of the Eurasian linage, especially the current H5N1 strains. Possible deficiencies in the North American assays for detection of Eurasian lineage strains were identified by DNA sequence alignments and BLAST search of GenBank database. Primer and probe sequences were then either modified or newly designed for detection of Eurasian strains (Table 6 and 7).

Table 6. *AIV primer and probe sequences*

Name	Location [a]	Sequence (5' → 3')
TaqMan Primer		
IVA-D161M	Matrix	AGATGAGYCTTCTAACCGAGGTCG
IVA-D162M [c]	Matrix	TGCAAANACATCYTCAAGTCTCTG
IVA-D148H5	HA (H5)	AAACAGAGAGGAAATAAGTGGAGTAAAATT
IVA-D149H5 [c]	HA (H5)	AAAGATAGACCAGCTACCATGATTGC
IVA-D168-H7	HA (H7)	GGATGGGAAGGTYTGGTTGA
IVA-D169-H7 [c]	HA (H7)	CCTCTCCTTGTGMATTTTGATG
TaqMan Probe [b]		
IVA-Ma	Matrix	(FAM)-TCAGGCCCCCTCAAAGCCGA-(TAMRA)
IVA-H5a	HA (H5)	(FAM)-TCAACAGTGGCGAGTTCCCTAGCA-(TAMRA)
IVA-H7-AUS	HA (H7)	(FAM)-TGAAACCATACCACCCA-(MGB-NFQ)

[a]) *Target gene for PCR amplicon.* [b]) *TaqMan probes were 6-FAM labelled minor grove binding (MGB) probes containing either non-fluorescent quencher (NFQ) or TAMRA.* [c]) *Denotes antisense orientation*

Table 7. *AIV primer and probe combinations (reaction mixes)*

Specificity	Primer fwd	Primer rev	Probe
AIV Type A	IVA-D161M (900 nM)	IVA-D162M (900 nM)	IVA-Ma (250 nM)
HA H5	IVA-D148H5 (900 nM)	IVA-D149H5 (900 nM)	IVA-H5a (250 nM)
HA H7 (AUS)	IVA-D168-H7 (900 nM)	IVA-D169-H7 (900 nM)	IVA-H7-AUS (250 nM)

The analytical sensitivity and specificity of the new TaqMan RT-PCR assays for Type A (Table 8) and H5 (Table 9) were compared with the original North American strain specific tests on different representative strains of the Eurasian lineage. The modified Taqman assay for Type A (Table 8) showed a cycle threshold (CT) value about 6.3 lower than the North American specific assay for template A/chicken/Vietnam/008/2004 (HP H5N1), whereas the CT values for A/Shearwater/Aus/75 (LP H5N3) and A/chicken/NSW/1/97 (HP H7N4) were very similar in both assays. The lower CT value of the modified assay for H5N1 indicated a nearly 100-fold increased sensitivity (Figure 10). The modified assay for subtype H5 showed an even greater reduction in CT value of 10.6 for A/chicken/Vietnam/008/2004 (HP H5N1) (Table 9), indicating an approximately 1000-fold increased sensitivity (Figure 11). The modified H5 assay was able to detect the A/Shearwater/Aus/75 (LP H5N3), a LP H5 of the Eurasian lineage that was not detected with the H5 TaqMan assay for North

American strains. As expected, both H5 assays did not detect H7 A/chicken/NSW/1/97 (HP H7N4). Standard curves for each assay (Figure 12) indicated a linear range of detection over 5 - 6 logs for H5N1 virus. The performance of both assays has been evaluated on all diagnostic submissions received by the Australian Animal Health Laboratory (AAHL) as the OIE regional reference laboratory for AI. To date, all H5N1 strains received from various Asian countries including Vietnam, Cambodia and Indonesia have been detected with high sensitivity in both the Type A and the H5 assays.

Table 8. AIV *Type A TaqMan assays. Comparison of CT values obtained using published and modified Type A-specific TaqMan assays.* C_T *values shown are averages of triplicate reactions; standard deviation is shown in brackets.*

	Type A-specific TaqMan assay	
Virus isolate	Modified (A)	Published (B)
A/chicken/Vietnam/008/2004 (HP H5N1)	17.72 (0.27)	24.06 (0.30)
A/Shearwater/Aus/75 (LP H5N3)	18.19 (0.13)	18.00 (0.07)
A/chicken/NSW/1/97 (HP H7N4)	23.12 (0.11)	23.61 (0.14)

(A) *Modified assay optimised for Eurasian type A influenza virus strains (this report).*
(B) *Assay optimised for North American Type A influenza virus strains (Spackman et al., 2002).*

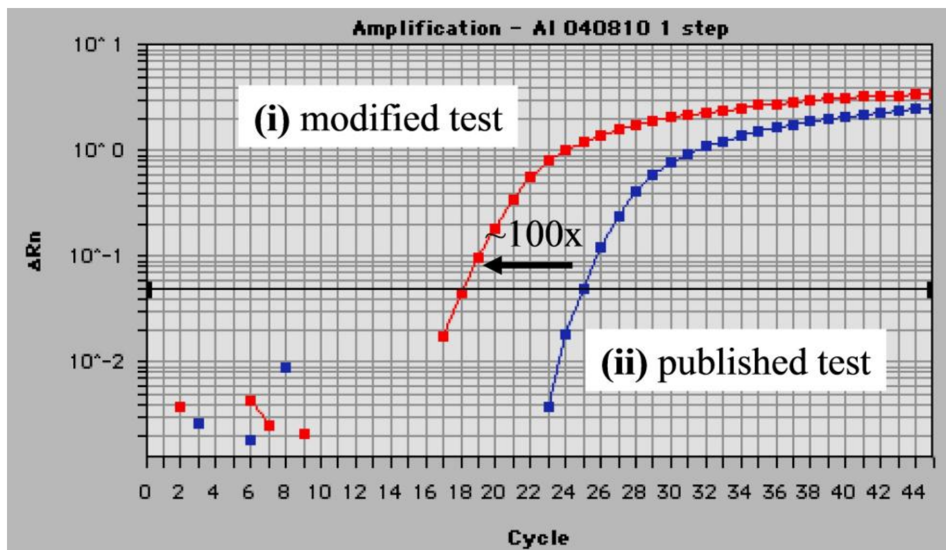


Figure 10. *Amplification plots of TaqMan RT-PCR specific for influenza Type A. Improved sensitivity for detection of Asian H5N1 strains (A/chicken/Vietnam/008/2004) by the modified Type A-specific TaqMan test (this report) compared to the published test (Spackman et al., 2002) for North American isolates.*

Table 9. *Comparison of CT values obtained using published and modified subtype H5-specific TaqMan assays. C_T values shown are averages of triplicate reactions; standard deviation is shown in brackets.*

	H5-specific TaqMan assay	
Virus isolate	Modified [(A)]	Published [(B)]
A/chicken/Vietnam/008/2004 (HP H5N1)	16.92 (0.08)	26.50 (0.11)
A/Shearwater/Aus/75 (LP H5N3)	17.37 (0.05)	>45
A/chicken/NSW/1/97 (HP H7N4)	>45	>45

(A) *Modified assay optimised for Eurasian subtype H5 influenza virus strains (this report).*
(B) *Assay optimised for North American H5 influenza virus strains (Spackman et al., 2002).*

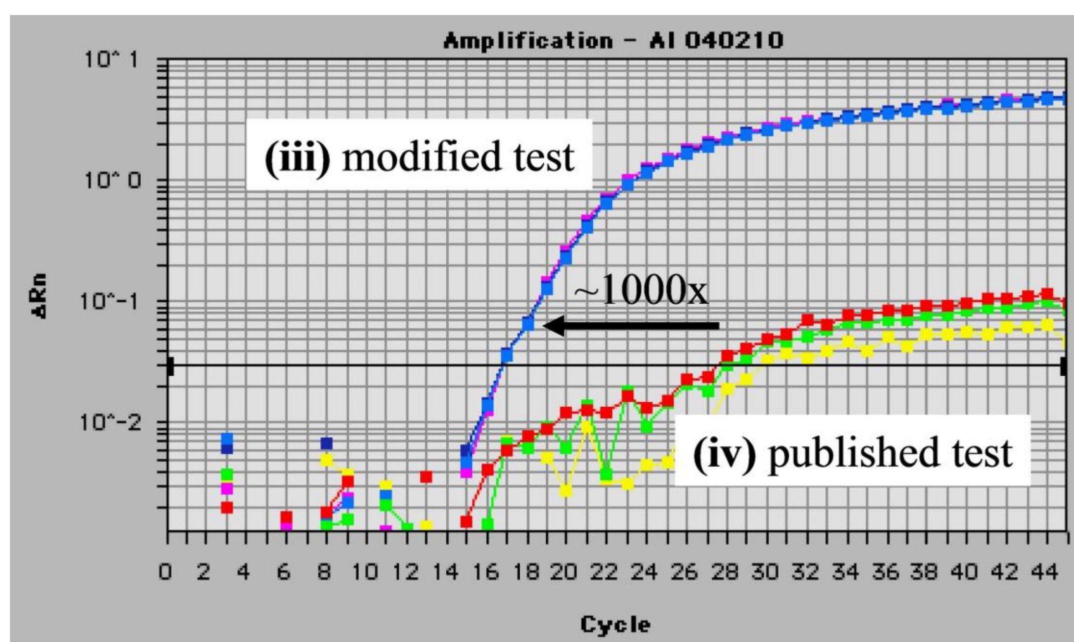


Figure 11. *Amplification plot of TaqMan RT-PCR specific for subtype H5. Improved sensitivity for detection of Asian H5N1 strains (A/chicken/Vietnam/008/2004) by the modified subtype H5-specific TaqMan test (this report) compared to the published test (Spackman et al., 2002) for North American isolates.*

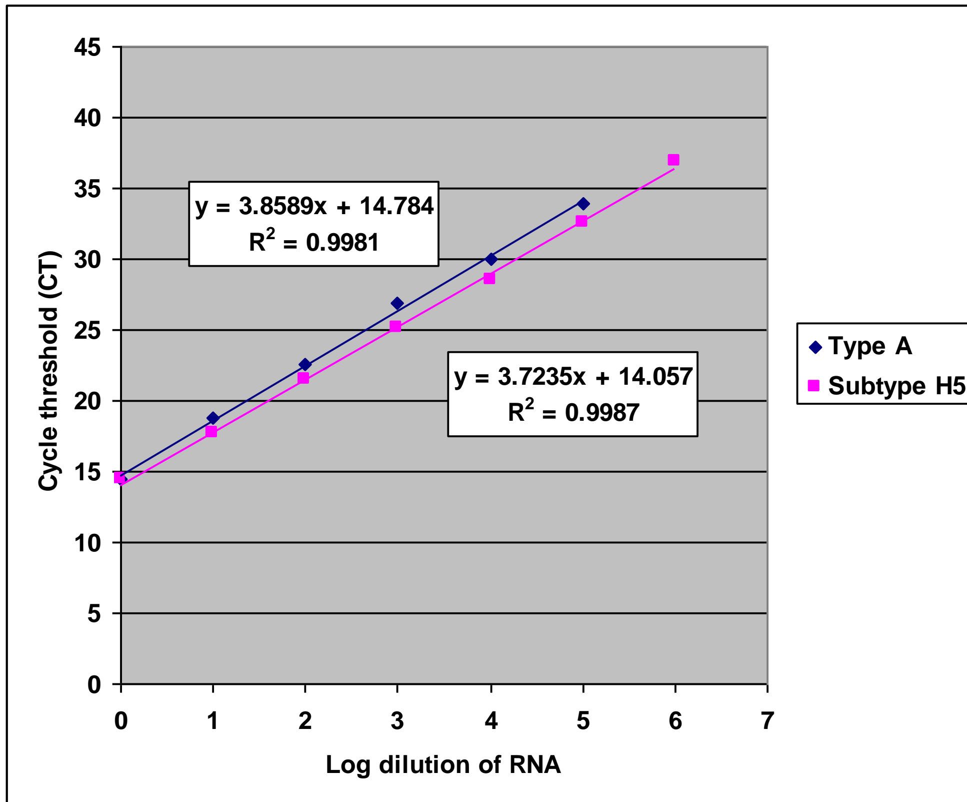


Figure 12. *Standard curves of TaqMan RT-PCR. Influenza Type A and subtype H5-specific TaqMan tests performed on 1/10 serial dilutions of A/chicken/Vietnam/008/2004 (H5N1) RNA template. Average CT values of triplicates are shown.*

The Australian H7 strains form a distinct subgroup in the Eurasian H7 lineage (Banks et al., 2000). We determined the sequence of the complete hemagglutinin gene of the HPAI (H7N4) strain A/chicken/NSW/1/97 from the last Australian outbreak in 1997 [GenBank AY943924]. This latest Australian isolate fell within the same phylogenetic group as the isolates from four previous outbreaks in 1975, 1985, 1992 and 1994. A new TaqMan RT-PCR assay was designed specifically for Australian H7 strains (Table 6 and 7). The analytical specificity of the H7 and H5 TaqMan assays was evaluated on Australian H7 and Asian H5N1 isolates. Amplification plots for A/chicken/Vic/85 (H7N7) using Type A, subtype H7 and subtype H5 specific TaqMan RT-PCR assays are shown in Figure 13 and for A/chicken/Vietnam/008/2004 (H5N1) in Figure 14. Both assays were subtype-specific as the Australian H7 influenza virus was detected by the Type A and subtype H7-specific assays, whilst H5N1 was detected by the Type A and subtype H5-specific assays. TaqMan RT-PCR assays were evaluated in various clinical samples (Table 10) to detect and differentiate virus in tracheal swab, cloacal swab, lung swab, spleen swab, and tissue homogenates. These assays have been used routinely at AAHL for diagnostic samples from outbreaks in Vietnam, Cambodia, Indonesia and other Asian countries and for surveillance studies in Australian bird populations.

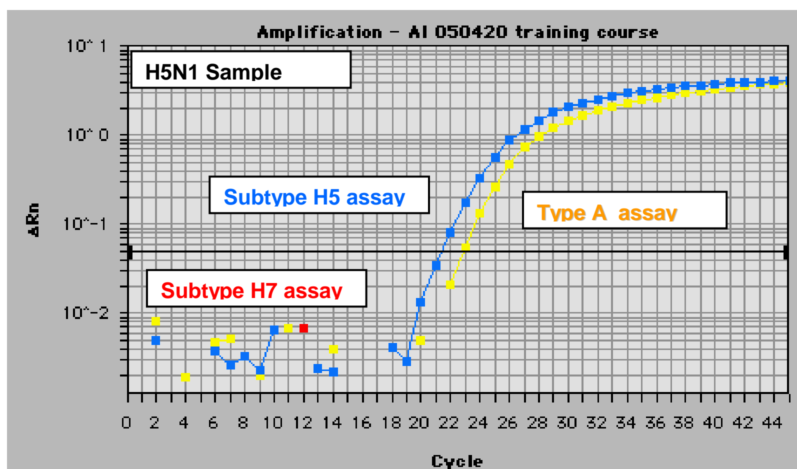


Figure 13. *Amplification plots for A/chicken/Vietnam/008/2004 (H5N1) using Type A, subtype H5 and subtype H7 specific TaqMan RT-PCR assays.*

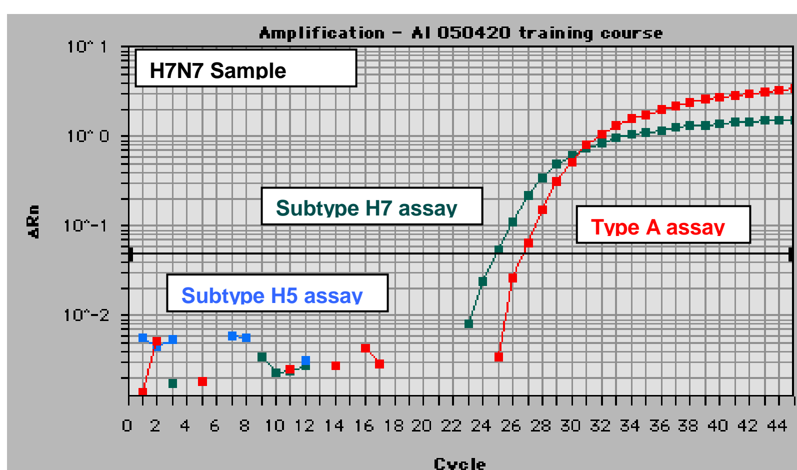


Figure 14. *Amplification plots for A/chicken/Vic/85 (H7N7) using Type A, subtype H7 and subtype H5 specific TaqMan RT-PCR assays.*

Table 10. *TaqMan RT-PCR assays from clinical samples.*

	TaqMan test		
	Type A	**Subtype H5**	**Subtype H7**
A/chicken/Australia/Vic/85 (H7N7)			
tracheal swab	31.48	neg	30.13
cloacal swab	31.87	neg	29.06
tissue homogenate	26.91	neg	25.11
A/chicken/Viet Nam/008/2004 (H5N1)			
lung swab	27.91	24.66	neg
spleen swab	28.19	27.06	neg
tissue homogenate	23.15	21.34	neg
No Template Control	neg	neg	neg

Cycle Threshold (CT) values for A/chicken/Vic/85 (H7N7) and A/chicken/ Vietnam/008/2004 (H5N1) in clinical samples using Type A, subtype H5 and subtype H7 specific assays. Average CT values from triplicates are shown. CT values greater than 40 were considered negative (neg).

Discussion of Results

Diagnostic real-time PCR assays are fast, sensitive and highly specific without the problems of false positives often associated with conventional PCR. The high specificity of TaqMan assays is generated by the use of an intrinsic probe, eliminating the need for DNA sequencing in many molecular diagnostic applications. Characteristic sequence differences between very virulent and non-virulent isolates of a particular virus can be exploited for the development of molecular diagnostic tests. Such virulence factors have been identified for AI and NDV and are under investigation for IBDV. Multiple basic amino acids in the cleavage region of the hemagglutinin gene of AI and the fusion protein of NDV have been shown to influence virulence. Other regions such as glycosylation sites or RNA secondary structure stabilising sequences in AI or the length of HN extension in NDV have been implicated in virulence. Specific amino acid changes in the structural VP2 protein have been shown to play a decisive role in pathogenicity of IBDV.

Choosing target sequences based on virus groups and lineages relevant to Australia is important for designing molecular diagnostic tests that can be used for index case diagnosis. We have developed sets of real-time PCR assays for each of the targeted viruses, to provide a robust platform and to minimise the risk of not detecting a pathogen due to failure of a single specific test. The tests developed here include a generic TaqMan real-time PCR assay to detect all of either serotype 1 IBDV strains, Australian NDV strains as identified in the National NDV survey (Kite, 2000), or all Type A AIV strains. The generic tests ensure that all viruses will be detected for index case diagnosis and in conjunction with the pathotype or subtype-specific assays, a rapid diagnosis can be made. In a situation where a new virus that contains genetic changes has emerged, and which results in the failure of the specific tests, one would expect that the generic tests would still be able to detect the virus. Such an unexpected reaction pattern in the diagnostic test results would signal the need for follow up with other diagnostic assays to further characterise the new pathogen. The assays designed here were all developed for the same uniform reaction conditions, so any combination of tests for one or different viruses could be performed simultaneously on the same reaction plate. The 18S rRNA internal control served as an extraction control for presence of intact RNA and absence of PCR inhibitors and provided a convenient marker for relative quantification, adding confidence for the validity of the assays. The assays were developed for a one-step format combining the RT step with the subsequent PCR in a single tube or well. This eliminates the need for transfer of material from RT into PCR reaction mix, thus reducing the possibility of contaminations and facilitating the adaptation to robotics and high throughput testing.

The detection and quantification of IBDV in blood (Moody et al., 2000) by TaqMan RT-PCR has been described, as has a real-time RT-PCR that employed strain specific primers and probes different from TaqMan technology for detection of a major neutralising epitope in VP2 of IBDV (Jackwood et al., 2003). Other real-time RT-PCR have been published that differentiated vvIBDV from classical strains by dual-labelled probes binding specifically to VP4 sequence (Peters et al., 2005) or in the VP2 region (Jackwood and Sommer, 2005). The IBDV TaqMan tests described here were able to detect all serotype 1 IBDV strains and to specifically differentiate vvIBDV from classical IBDV, including US variants and Australian strains. A single assay containing highly conserved primers and probe was used for the detection of all serotype 1 IBDV strains. The differentiation between vvIBDV and classical IBDV strains was achieved in separate reactions using vvIBDV or classical strain-specific MGB detection probes and distinctive primer combinations. The three characteristic nucleotide substitutions in the type-specific MGB probes were sufficient for the unambiguous discrimination between vv and classical strains without the need for DNA sequencing. The rapid detection of all IBDV strains and differentiation of vvIBDV from classical strains will support rapid index case diagnosis and ongoing surveillance in vaccination or eradication programs.

The TaqMan assays described here for NDV detection and typing of Australian strains were based on sequences of the fusion gene from recent Australian isolates (Gould, 2001, 2004) and from those

obtained during the National NDV survey (Kite, 2000). Other TaqMan assays, predominantly for North American isolates, have been based on the matrix gene or the fusion gene (Wise et al., 2004). Australian NDV V4, an avirulent field strain and a virulent strain were all detected by a generic NDV-all TaqMan assay and could be differentiated when the type-specific tests were performed in parallel. Results for strain typing should be interpreted carefully and confirmed by sequencing of the fusion protein cleavage region as some cross reactivity of the type-specific tests could confuse diagnosis. The correct type-specific test always had the lowest CT value of the type-specific tests and a CT value closest to that of the generic NDV-all test.

The TaqMan RT-PCR assays for detection of all Type A influenza strains and the H5 haemagglutinin gene (HA) of the Eurasian lineage of influenza both utilise the highly conserved gene-specific fluorescent probes developed for American isolates (Spackman et al., 2002). Newly designed forward and reverse primers incorporating multiple nucleotides to include sequences of Eurasian H5N1 isolates increased the analytical sensitivities of both assays for H5N1 strains. A new TaqMan assay specific for Australian H7 strains was developed as these strains form a distinct subgroup within the Eurasian lineage (Banks et al. 2000) which was also confirmed by the analysis of the newly sequenced (GenBank AY943924) HPAI isolate from the 1997 outbreak in NSW . The tests developed here had a wide linear range of detection over 5 to 6 logs of template concentration and were suitable for detection and differentiation of H7 and H5 strains in various clinical samples. Such a high analytical sensitivity makes the tests valuable for detection of virus in surveillance studies where only low viral loads are expected in infected birds. The assays have been used to identify AI in diagnostic submissions from Vietnam, Cambodia, Indonesia and other Asian countries sent to AAHL as the SE Asian OIE regional reference laboratory. These tests allow definitive confirmation of an AI virus as H5 within hours, which is crucial for rapid implementation of control measures in the event of an outbreak.

Implications

- The TaqMan RT-PCR assays are suitable for the rapid and sensitive detection of very virulent IBDV, virulent NDV and highly pathogenic AIV including H5N1 strains.
- The tests can reduce the time for index case diagnosis facilitating the rapid implementation of control measures in an outbreak.
- Knowledge of circulating virus strains and associated sequences is critical to the successful application of highly specific molecular assays as Australian viruses that differ significantly from those in other parts of the world and may not be detected by tests developed elsewhere.
- TaqMan RT-PCR assays are highly specific and are affected by genetic changes in the target virus population. Subtype- or strain-specific tests should be performed in conjunction with a generic test for the virus family.
- TaqMan RT-PCR assays are highly sensitive allowing the detection of traces of genetic material in samples from which virus cannot be isolated. The diagnostic implications of TaqMan results, especially near the limit of detection, should be considered carefully together with other clinical observations and laboratory results.

Recommendations

The TaqMan RT-PCR assays developed here should be used in qualified accredited diagnostic laboratories for early and sensitive detection of virus and to recognize the potential emergence or

outbreak of virulent or highly pathogenic strains. Strict quality controlled procedures through all stages from the field to the lab should be maintained to ensure sample integrity and minimise contamination events.

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Plain English Compendium Summary

Project Title:	Rapid identification and pathotyping of virulent IBDV, NDV and AIV isolates
RIRDC Project No.:	CSA-24J
Researcher:	Hans Heine
Organisation:	CSIRO Livestock Industries – Australian Animal Health Laboratory Private Bag 24 Geelong VIC 3220
Phone:	03 5227 5278
Fax:	03 5227 5555
Email:	hans.heine@csiro.au

Objectives

- To develop real-time PCR assays for detection of all serotype 1 IBDV strains and differentiation of very virulent IBDV from classical strains
- To develop real-time PCR assays for detection of NDV and differentiation of endemic low/non-pathogenic and vaccine strains to distinguish from highly pathogenic strains
- To develop real-time PCR assays for detection of all AIV strains and identification of subtypes H5 (including H5N1) and Australian H7.

Background

Outbreaks of very virulent IBDV, virulent NDV or highly pathogenic AIV are major threats to the Australian poultry Industry. Rapid index case diagnosis is essential for the swift implementation of disease control measures. New real-time RT PCR methods based on gene-specific amplification and detection by TaqMan fluorescent probes are fast and highly sensitive, and able to differentiate between closely related isolates within a few hours.

Research

TaqMan real-time RT-PCR assays were designed to target nucleotide sequences specific for each of the virus families (IBDV, NDV, AIV) and for virulent and non-virulent isolates in each family. The assays were evaluated in the microbiologically secure laboratories at AAHL using virulent and non-virulent virus strains.

Outcomes

Rapid and highly sensitive molecular diagnostic tests based on TaqMan real-time RT-PCR have been developed and implemented to detect infectious bursal disease virus (IBDV), Newcastle disease virus (NDV) and avian influenza virus (AIV) and to differentiate harmless non-pathogenic isolates that may circulate in Australia from emerging or exotic virulent and pathogenic strains.

Implications

The new TaqMan RT-PCR assays for diagnostic laboratories have been implemented at AAHL and will reduce the time for the identification of important poultry viruses. The early recognition of a potential emergence or outbreak of virulent or highly pathogenic strains will assist the affected industries in the rapid implementation of control measures to limit the spread of disease.

Publications

- Heine, H.G, L. Trinidad, P. Selleck. Influenza virus type A and subtype H5-specific real-time reverse transcription (RRT)-PCR for detection of Asian H5N1 isolates. Technical Report for Australian Biosecurity Cooperative Research Centre for Emerging Infectious Disease. 2005. Download from http://www1.abcrc.org.au/
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