RESEARCH COMMUNICATION

A rapid and sensitive real-time reverse transcription PCR for the pathotyping of South African H5N2 avian influenza viruses

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ABSTRACT

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A Fluorescence resonance energy transfer (FRET) real-time reverse-transcription (rRT-PCR) assay was developed that distinguishes stains of South African and European highly pathogenic (HPAI) from low pathogenicity (LPAI) H5 avian influenza viruses in the absence of virus isolation, irrespective of the length of insertion at the hemagglutinin cleavage site (H_0). The assay was used to pathotype H5-type viruses detected by rRT-PCR in ostrich tracheal swabs collected during the 2006 HPAI H5N2 outbreak in the Western Cape Province.

Keywords: Avian influenza virus, H5N2, pathotyping, real-time reverse transcription PCR, FRET

INTRODUCTION

Highly pathogenic avian influenza (HPAI) is a devastating disease of poultry that has zoonotic potential for humans and is caused by strains of the H5 and H7 subtypes of influenza A viruses (family *Orthomyxoviridae*). The low pathogenicity avian influenza (LPAI) virus precursors to HPAI viruses are ubiquitous in wild waterfowl and shorebirds and usually only cause asymptomatic infections in these reservoir hosts. Once transferred to terrestrial poultry, LPAI viruses can convert into HPAI (Webster, Bean, Gorman, Chambers & Kawaoka 1992).

Although the virulence determinants of avian influenza viruses are multigenic in nature, an insertion of multiple basic amino acids at the cleavage site of the haemagglutinin precursor protein, H_0, targets this protein for cleavage by ubiquitous subtilisin-like endonucleases, resulting in rapid systemic spread of the virus. In contrast, LPAI viruses contain a monobasic composition at H_0 which is targeted by trypsin-like proteases that are limited to cells of the intestinal and respiratory tracts (Rott 1979; Stieneke-Gröber, Vey, Angliker, Shaw, Thomas, Roberts, Klenk & Garten 1992; Vey, Adler, Klenk, Rott & Garten 1992; Wood, McCauley, Bashiruddin & Alexander 1993). The amino acid sequence at H_0 is thus recognized as an important and reliable molecular virulence marker (OIE Terrestrial Manual 2004).

South Africa was affected by two separate outbreaks of HPAI H5N2 in intensively-farmed ostriches between 2004 and 2006, and intensive serological and virological surveillance was conducted during both outbreaks. Of thousands of tracheal swabs or organ samples tested, 46 were positive or suspect positive for the presence of AIV by real-time reverse transcription-PCR (rRT-PCR) or nucleic acid-based sequence assay (NASBA) (M. Romito, unpublished laboratory data). H5-type viruses were isolated in only 3 (6.5%) of these cases, viz. the HPAI H5N2 virus isolated in the Eastern Cape outbreak in 2004 (OSZA04N227), the HPAI H5N2 virus isolated in

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the Western Cape outbreak of 2006 (OSZA06AI1091) and the LPAI H5N2 virus isolated in the same outbreak (OSZA06AI1160). The isolate OSZA06AI1091 contained fewer multiple basic amino acids at H_0 than did isolate OSZA04N227, but was clearly HPAI according to the OIE definition (Abolnik 2007b). Unfortunately however, the nucleic acids detected by rRT-PCR/ NASBA are present in insufficient quantities to allow further molecular characterization (Hoffmann, Starick, Depner, Werner & Beer 2007; unpublished laboratory data 2007), yet for control purposes there is an urgent need to be able to differentiate between LPAI and HPAI viruses in ostriches especially where virus isolation has not been successful. At least two other groups have described sensitive rRT-PCR assays for HPAI H5 pathotyping, hoewever the SYBR Green method described by Payungporn, Chutinimitkul, Chaisingh, Damrongwantanapokin, Nuansrichay, Pinyochon, Amonsin, Donis, Theamboonlers & Poovorawan (2006) may give false positive results (Fernández, Gutierrez, Sorlozano, Romero, Soto & Ruiz-Cabello 2006). In the method of Hoffmann and co-workers (2007), a set of hydrolysis probes was described that detects the Asian group of HPAI H5N1, and the H_0 cleavage site sequence of the Qinghai strain in particular. However, insertions/substitutions in the H_0 cleavage site abolished signal generation. As demonstrated for isolates from in South Africa (Fig. 1), potential variations in the H_0 cleavage site sequence need to be taken into account. Therefore a rapid and sensitive rRT-PCR assay was developed to distinguish between South African HPAI and LPAI H5N2 viruses in the absence of virus isolation that does not rely on the sequence at H_0.

MATERIALS AND METHODS

Primer and probe design

A set of primers (InfA_H5_F and InfA_H5_R) and three probes (InfA_H5_640, InfA_H5_FL and InfA_H5_705) were designed for the amplification and detection of a fragment spanning the cleavage site sequence of the H5N2 HA gene (Table 1). The LCRed640 fluorophore-labelled InfA_H5_640 probe was designed to hybridize over the consensus LPAI sequence that is conserved within the H5 lineage. The InfA_H5_FL binds a sequence adjacent to this, and is labelled at both the 5' and 3' ends with fluorescein. The LCRed705 fluorophore-labelled InfA_H5_705 probe binds at the opposite end of InfA_H5_FL, in a conserved region within the H5 sub-lineage. In the presence of an LPAI sequence, the three probes will bind in tandem and both acceptor probes will be excited by the flourescein donor probe, resulting in

TABLE 1 Primers and probes used to differentiate between HPAI and LPAI in this study[a]

Primer/probe	Sequence (5'-3'), -fluorophore	Nucleotide position (Fig. 1)
InfA_H5_F	GTGCCCCAAATACGTGAARTCA	951–972
InfA_H5_R	CCATCTATTGCTTKKTGAGTGGACTC	1169–1143
InfA_H5_640	640-TCCTCTTGTTTCTYTTTGAGGGACATT-p	1029–1067
InfA_H5_FL	F-CCTCCTTCTATAAARCCTGCTATRGCCCCAAATA-F	1069–1102
InfA_H5_705	-pCCATACCAACCRTCTACCATKCCTTGCC-705	1105–1133

[a] Primers and probes were designed in collaboration with and manufactured by TIB MOLBIOL, Eresburgstr. 22-23, D-12103 Berlin, Germany

TABLE 2 Results of the H5 rRT-PCR pathotyping assay

Sample		$F2_{(640)}$ Cp	$F3_{(705)}$ Cp	Pathotype
dH_20	Negative control	–	–	–
OSZA06AI1091	HPAI positive control	–	22.34	HPAI
OSZA06AI1160	LPAI positive control	20.46	20.59	LPAI
AI1133.1	Ostrich tracheal swab	–	–	–
AI1133.11	Ostrich tracheal swab	–	29.90	HPAI
AI1133.12	Ostrich tracheal swab	26.34	26.21	LPAI
AI1133.13	Ostrich tracheal swab	–	28.83	HPAI
AI1133.15	Ostrich tracheal swab	–	31.12	HPAI
AI1133.16	Ostrich tracheal swab	–	–	–
AI1133.17	Ostrich tracheal swab	–	–	–
AI1133.23	Ostrich tracheal swab	–	–	–
AI1120.37	Ostrich tracheal swab	–	29.80	HPAI
AI1149.6	Ostrich tracheal swab	–	–	–

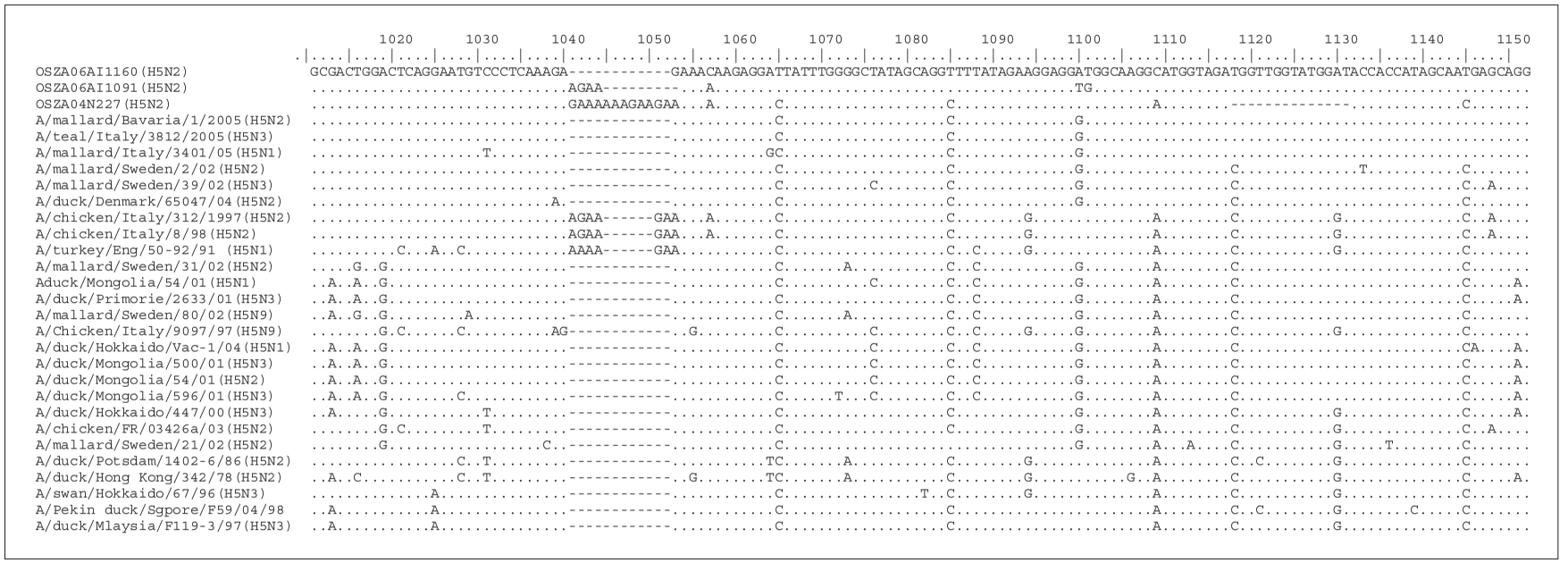

FIG. 1 Multiple nucleotide sequence alignment of H5 strains for the region spanning the H0 cleavage site (numbering upstream of the H0 cleavage site is based on Genbank sequence DQ851561 [not shown])

two signals that are detectable at different wavelengths (LightCycler® channels F2 and F3). The presence of insertions or substitutions in the H_0 cleavage site of HPAI virus will however abolish the binding of InfA_H5_640 and only one signal in channel F3 would thus be visible.

RNA extraction and first-strand cDNA synthesis

Viral RNA was extracted from tracheal swabs or infective allantoic fluid with a Total Nucleic Acid Isolation kit (Roche) using a MagNALyser and TRIzol® LS Reagent (Gibco, Invitrogen), respectively. First-strand cDNA synthesis was accomplished by incubating 5 µℓ RNA, 2 µℓ random hexamer and 6 µℓ dH_20 at 65 °C for 10 min. After snap-cooling in an ice bath for 5 min, 4 µℓ of 5 X RT buffer, 0.5 µℓ of RNAse inhibitor, 2 µℓ 10 mM dNTPs and 0.5 µℓ Transcriptor Reverse Transcriptase® (Roche) were added. The reactions were incubated at 55 °C for 30 min.

rRT-PCR

A LightCycler® FastStart DNA Master^{PLUS} HybProbe kit (Roche) and LightCycler® 2.0 device were utilized for real-time PCR using 5 µℓ of the cDNA in a total volume of 20 µℓ. The temperature profile was used as follows: 10 min at 94 °C, and 45 cycles of 20 s at 95 °C, 15 s at 55 °C, and 20 s at 72 °C. Fluorophore-specific emission data were collected during the annealing step. Crossing point (Cp) values were calculated with LightCycler 3.5 software using the second derivative maximum method. The assay was applied to ten samples from the recent outbreaks in 2006 that were positive or suspect positive for the presence of H5 virus by rRT-PCR during surveillance but where virus isolation was unsuccessful (Table 2).

RESULTS AND DISCUSSION

The positive controls, OSZA061160 (LPAI) and OSZA06AI1091 (HPAI), produced fluorescent signals in both and in one channel, respectively (Table 2). Unknown samples AI1133.11, AI1133.13, AI1133.15 and AI1120.37 were HPAI positive, whereas AI1133.12 was LPAI positive. Five of the samples gave negative results, which may have been due to RNA degradation during prolonged storage. Interestingly, LPAI and HPAI were detected within the same flock (AI1133 samples). OSZA04N227 (HPAI, 2004) did not amplify, due to an unusual sequence deletion detected between nucleotides 1121 and 1134 (Fig. 1) (Abolnik 2007a). (The assay has been adapted for high throughput on the LightCycler480® system by replacing the LCRed710 fluorophore with an LCRed610 fluorophore, and use of a one-step RT-PCR kit).

In South Africa the reemergence of H5N2 in poultry is a constant threat because the LPAI strain exists in the wild bird reservoir (Abolnik 2007b) but this particular LPAI H5N2 lineage is also circulating in the European wild waterfowl population (Abolnik 2007a) and therefore after further validation the primer and probe set described here could be used in Europe since poultry in that region is also at risk of infection and consequent mutation. I have described here a rapid and sensitive method to pathotype H5-positive swabs where virus isolation has been unsuccessful. This approach of detecting the consensus LPAI H_0 sequence with abolishment of binding as an indicator of the presence of an HPAI strain (provided that adjacent or upstream controls are included) could be adapted for the pathotyping of other lineages of AIV H5 and extended to hydrolysis probe chemistries.

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