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Detection and subtyping (H5 and H7) of avian type A influenza virus by reverse transcription-PCR and PCR-ELISA

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Summary. Avian influenza virus infections are a major cause of morbidity and rapid identification of the virus has important clinical, economical and epidemiological implications. We have developed a one-tube Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) for the rapid diagnosis of avian influenza A. A panel of reference influenza strains from various hosts including avian species, human, swine and horse were evaluated in a one tube RT-PCR using primers designed for the amplification of a 218 bp fragment of the NP gene. The PCR products were detected by PCR-ELISA by use of an internal catching probe confirming the NP influenza A origin. The PCR-ELISA was about 100 times more sensitive than detection of PCR products by agarose gel electrophoresis. RT-PCR and detection by PCR-ELISA is comparable in sensitivity to virus propagation in eggs. We also designed primers for the detection of the influenza. A subtypes H5 and H7 shown to have pathogenic potential in poultry. The H5 primers cover the cleavage site of the HA gene and specifically amplify influenza A subtype H5. The H7 primers also cover the HA cleavage site and detected all H7 reference strains investigated. In addition, the H7 primers also amplified very weak and/or additional bands on an agarose gel from other subtypes. However, the H7 origin and the pathogenic potential defined by the presence or absence of basic amino acids at the cleavage site can be determined by sequencing of the PCR product. As far as we know this is the first demonstration of RT-PCR detection on a panel of H7 strains using only one primer set.

Introduction

Influenza A viruses have a segmented genome of single stranded negative sense RNA, which together with the virally encoded nucleoprotein (NP) form the ribonucleoprotein (RNP). The NP protein is encoded by viral genome segment

number 5 which is 1565 nucleotides long. There is frequent antigenic variation in the envelope protein of influenza A whereas the sequence of NP is relatively conserved [12].

The influenza haemagglutinin (HA) and neuraminidase (NA) viral surface antigens are classified serologically into subtypes. Currently, 15 HA and 9 NA subtypes are known [15, 22]. Host enzyme proteolytic cleavage of the HA protein is a prerequisite for the infectivity of avian influenza virus (AIV). The outcome of an AIV infection depends on the virus strain and on host. The virulence of a virus strain can be determined by inoculation in chickens and by determination of the amino acid sequence of the HA cleavage site [21]. The pathogenicity of AIV in chickens is influenced by the presence or absence of multiple basic amino acids at the cleavage site of the HA protein. Until now only H5 and H7 subtypes have been characterised as highly pathogenic in chickens, although many strains of these subtypes have been shown to be low pathogenic [17, 23, 24]. Therefore, attention should be paid to avirulent viruses with multiple basic residues at the HA cleavage site, as they may have the potential to become highly pathogenic by a single mutation [11].

Since the circulating influenza virus has the potential to change by genetic drift and shift, methods used for surveillance should have specificity allowing detection of antigenically and genetically diverse influenza strains. Due to the potential of AIV to spread to avian and non-avian hosts the identification of an isolate is important. Early detection of field isolates will improve the control of influenza in domestic and feral birds. It is expected that the use of RT-PCR technique for influenza diagnostics in birds could be a valuable tool in the control of influenza outbreaks and in surveillance of birds in quarantine.

Reverse Transcription Polymerase Chain Reaction (RT-PCR) has previously been used to detect influenza A virus in throat and nasal specimens collected from humans, pigs and horses [7, 14, 16, 25]. These methods are at least as sensitive and specific and much faster compared to conventional cultivation procedures.

In this paper we describe the development of a one tube NP RT-PCR assay and PCR-ELISA for the rapid diagnosis of influenza A virus. Additionally, we have developed primers for the amplification of the HA cleavage site primarily for the subtypes H5 and H7. To our knowledge this is the first demonstration that the identification of the cleavage site of various H7 strains can be done with only one primer set. Primers designed for use in this assay are specific for three templates: 1) the NP gene, 2) the HA gene including the cleavage site for H5 and 3) the HA gene including the cleavage site for all H7 strains tested. However, the H7 primers do also amplify gene sequences from other HA subtypes.

The RT-PCR assay offers a fast, sensitive and specific alternative to propagation in eggs/culture for avian influenza detection. In addition, the presence of infectious virus is not necessary for influenza detection by PCR.

Materials and methods

Viruses

Virus strains used: A/Turkey/England/N28/73 (H5N2); A/Starling/Africa/983/79 (H7N1); A/FPV/Dutch/27 (H7); A/Tern/S.Africa/61 (H5); A/Chicken/Scotland/59 (H5); A/Chicken/Brescia/1902 (H7); A/Turkey/England/50-92/91 (H5); A/Chicken/Germany/34 (H7); A/Turkey/England/63 (H7); A/Turkey/England/69 (H3N2); A/DK/ALB/35/76 (H1N1); A/DK/Germany/1215/73 (H2N3); A/Duck/Czech/56 (H4); A/Rhea/NC/41014/93 (H7N1); A/S.Africa/P/10/9/91 (H7N1); A/parrot/N. Ireland/P3987/73 (H7); A/Turkey/Canada/63 (H6); A/Turkey/Ontario/6118/67 (H8N4); A/Turkey/England/384/79 (H10N4); A/Duck/England/56 (H11N6); A/DK/ALB/60/76 (H12N5); A/Mallard/Gurjev/263/82 (H14N6); A/Turkey/Wisconsin/66 (H9N2); A/Ostrich/Denmark-Q/72420/96 (H5N2). The AIV strains were propagated in SPF chicken eggs (Lohmann Tierzucht, Cuxhaven, Germany) and harvested from the allantoic fluid. Haemagglutination (HA) titres of the viruses ranged from 1: 32 to 1: 512 when tested according to the guidelines given by the European Union (EU) Council Directive 92/40/EEC [6].

RNA extraction from virus stocks

RNA purification was performed using the RNeasy Kit (Qiagen, Germany) according to the instructions by the manufacturer. Briefly, 400 µl of allantoic fluid harvested from inoculated embryonated eggs were mixed with 300 µl of RTL buffer and 7 µl mercaptoethanol and left for at least 10 min. at room temperature. If the suspension was cloudy, the supernatant was transferred to a new tube after a short spin. After the addition of 700 µl 70% ethanol and mixing, the liquid was applied to a spin-column in two steps followed by washing. Finally, RNA was eluted in 50 µl H_2O (free of RNase).

RT-PCR

The oligonucleotide primers were commercially synthesised (DNA Technology, Aarhus, Denmark). The oligonucleotides are listed in Table 1. The primers MMU 42 and MMU 43 were synthesised with a 5′ biotin group. The one-tube RT-PCR was carried out according to the manufacturer instructions (Titan One Tube RT-PCR System, Boehringer Mannheim). The 50 µl reaction contained 5 µl of extracted RNA, 0.4 mM each dATP, dGTP, dCTP, dTTP, 20 pmol of each primer, 5 mM DTT, 1×RT-PCR buffer and Titan enzyme mix. Samples were amplified by the following conditions: Reverse transcription at 50 °C for 30 min. After an initial denaturation at 94 °C for 2 min, 35 cycles of heat denaturation at 94 °C for 30 sec, primer annealing at 58 °C for 30 sec and primer extension at 68 °C for 1 min (from cycle 10 to 35 five seconds were added to the elongation time per cycle) were followed by a final primer extension step at 68 °C for 7 min. The annealing temperature was 55 °C for the amplification of the HA gene using the primers MMU 2+9 for subtype H5 and MMU 21+31 for H7. Precautions were taken to minimise contamination risks. RNA purification, the one-tube RT-PCR set-up, run and agarose gel electrophoresis were performed in separate rooms. Negative reagent controls were included in each assay. No contamination was detected at any time.

Sequence analysis

Some of the PCR products were analysed by sequencing. As a first step, the products were cleaned using a “QIAquick PCR purification kit” (Qiagen, Germany). Sequencing was performed in both the sense and anti-sense direction with primers MMU 19 and MMU 39 for the NP gene and the RT-PCR primers MMU 2,9 (H5) and 21,31 (H7), respectively

Table 1. The oligonucleotides used

Name	Sense[a]/Target	Sequence of oligonucleotide[b]	Location[c]	Size[d]
MMU42	− / NP	B-C_6-AG-AGC-TCT-TGT-TCT-CTG-ATA-GGT-G	506-483[a]	
MMU43	+ / NP	B-C_6-CA-TCC-CAG-TGC-TGG-GAA-RGA-YCC-TAA-GAA	288-317[a]	218
MMU41	Probe	Phosphate-TG-GCG-CCA-AGC-GAA-CAA-TGG-AGA-AGA	403-428[a]	
MMU19	− / NP	AG-AGC-TCT-TGT-TCT-CTG-ATA-GGT-G	506-483[a]	
MMU39	+ / NP	CA-TCC-CAG-TGC-TGG-GAA-RGA-YCC-TAA-GAA	288-317[a]	218
MMU2	− / H5	ATA-CCA-TCC-ATC-TAC-CAT-TCC	1120-1099[b]	
MMU9	+ / H5	TAT-GCC-TAT-AAA-ATT-GTC-AAG	830-850[b]	290
MMU21	− / H7	TCT-CCT-TGT-GCA-TTT-TGA-TGC	1133-1113[c]	
MMU31	+ / H7	GGG-GCT-TTC-ATA-GCT-CCA-GAT-CGT-GC	793-818[c]	340

[a] + = sense; − = anti-sense

[b] B = Biotin; R and Y represent mixtures of nucleotides of A/G and C/T, respectively

[c] The nucleotide location of the oligonucleotides at the GenBank sequence according to accession numbers: [a] M22576 (NP), [b] X07826 (H5), [c] AF028020 (H7). The gene/subtype is indicated in parenthesis

[d] The size (base pairs) of the PCR product predicted from the sequence

for the HA gene. Samples were subjected to electrophoresis using an automated sequencer ABI 377 (PE Biosystems, Foster City, CA). Sequence data were aligned (using the program Clustal X) with NP and HA influenza A sequences from NCBI database to assess homology.

Detection of PCR product by gel electrophoresis

Ten μl of the PCR products were analysed by agarose gel electrophoresis, using a 1.5% SeaKem GTG (FMC) agarose in 1×TAE buffer. Amplified products were visualised by ultraviolet light transillumination followed by staining with 0.1 μg/ml ethidium bromide. A 100 base pair ladder was used as a molecular weight marker (DNA Technology, Aarhus, Denmark).

Dilution series of virus to assess the sensitivity of the one tube NP RT-PCR

The following avian influenza strains were used: A/Starling/Africa/983/79; A/Turkey/England/N28/73; A/Ostrich/Denmark-Q/72420/96. Titration of virus stock was performed by inoculation of 10-fold virus stock dilutions into 10-day embryonated hen's eggs. After incubation allantoic fluid was harvested and the 50% end-point dilution determined by the presence/absence of dead eggs and by HA. The same virus dilutions were, in parallel, amplified by the NP RT-PCR and PCR product detected by PCR-ELISA.

Detection of PCR product by PCR-ELISA

PCR-ELISA is based on the formation of a heat stable covalent bond between a solid plastic surface of a 96-well plate and an oligonucleotide used as a catching probe. After hybridisation to the probe the denatured PCR-product can be detected spectrophotometrically as the PCR primers are labelled by biotin. The principle of the PCR-ELISA is

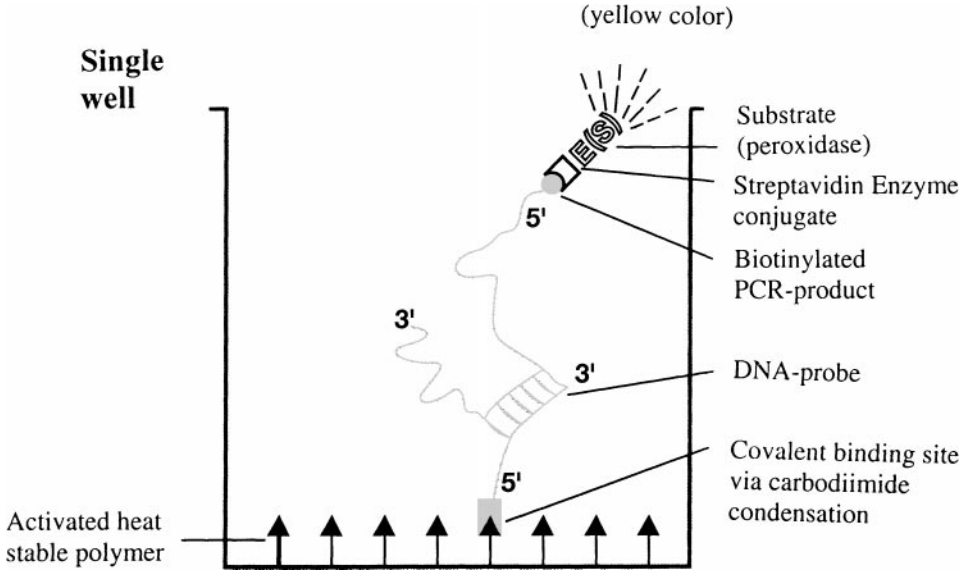

Fig. 1. Illustration of the PCR-ELISA principle

illustrated in Fig. 1. The detection probe, MMU41, was phosphate labelled at the 5′ end. MMU41, diluted in 10 mM 1-methyl-imidazole, was bound, by the addition of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide to NucleoLink microtiter plates (Nunc, Roskilde, Denmark) according to the manufacturer's instructions for hybridisation. Following the NP (primer MMU 42+43) RT-PCR, 25 µl of PCR product (denatured 5 min at 95 °C) was added to the wells containing 75 µl of 5× Saline-sodium citrate (SSC); 0.1% Tween 20. Incubation at 45 °C for 2 h. Three times wash in 0.5×SSC; 0.1% Tween 20. 100 µl of Horse-Radish-Peroxidase conjugated Streptavidin (Dako, Glostrup, Denmark) diluted 1:4000 in PBS; 0.5% Tween 20. Incubation 1 h at RT. The plate was emptied and washed 3 times in PBS; 0.05% Tween 20. Detection by adding 100 µl OPD/H_2O_2 solution in H_2O. The reaction was stopped after 10 min by adding 100 µl 0.5 M H_2SO_4. The plate was read spectrophotometrically at 492 nm.

Results

The NP RT-PCR (primer MMU 42+43) was evaluated using several influenza A reference strains. We showed that the RT-PCR is able to amplify the NP gene from a series of different avian influenza strains (HA 1 to 12+14) as demonstrated in Fig. 2. Furthermore, the ability of the procedure to amplify the NP gene of H5 and H7 serotypes was evaluated by testing five and eight of them, respectively (not shown).

Compared to detection by agarose gel electrophoresis the PCR-ELISA is at least 10 to 100 times as sensitive. This is illustrated by NP RT-PCR of 10-fold dilutions of the influenza. A strain: A/Turkey/England/N28/73 (mean embryo infective dose (EID_{50}) = $10^{8.4}$/ml) and detection of PCR products by either agarose gel electrophoresis or PCR-ELISA is shown in Fig. 3. In this case, comparison of the two methods demonstrate that the PCR-ELISA is about 100 times as sensitive as detection on the gel and able to detect PCR product also in the

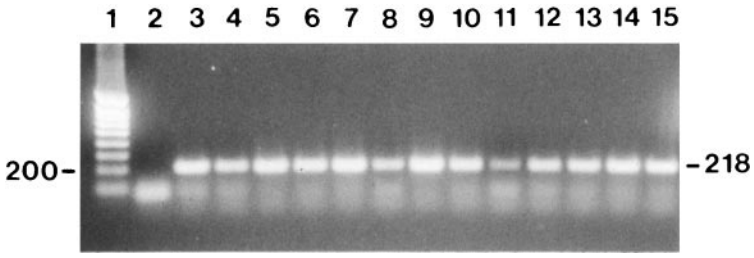

Fig. 2. The NP RT-PCR was applied to a panel of avian influenza strains representing various subtypes (HA 1 to 14). Ten μl of PCR product was applied on a 1.5% gel in *1–16* as follows: *1* Mr, *2* H_2O control, *3* H1, *4* H2, *5* H3, *6* H4, *7* H5, *8* H6, *9* H7, *10* H8, *11* H9, *12* H10, *13* H11, *14* H12, *15* H14

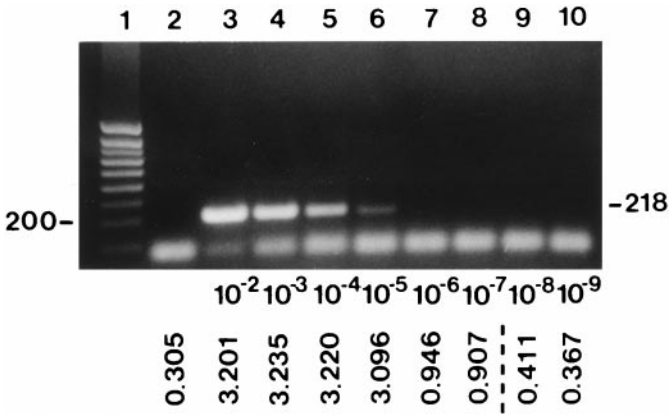

Fig. 3. Comparison between detection of PCR-products by agarose gel electrophoresis and PCR-ELISA. The avian influenza A strain A/Turkey/England/N28/73 was diluted 10-fold and the dilutions amplified by the NP RT-PCR. PCR products (15 μl each) were applied on a 1.5% gel and in parallel detected by PCR-ELISA. *1–10* represent: *1* Mr, *2* H_2O control, *3* virus diluted 10^{-2}, *4* 10^{-3}, *5* 10^{-4}, *6* 10^{-5}, *7* 10^{-6}, *8* 10^{-7}, *9* 10^{-8}, *10* 10^{-9}. The PCR-ELISA value/reading is indicated beneath the respective lane on the gel. The cut-off value is set to 0.5

10^{-7} virus dilution. To test the sensitivity of the NP RT-PCR serial ten-fold dilutions of virus pools of known titre were propagated in eggs and in parallel the dilutions were RNA purified and PCR amplified. Three different avian strains were tested namely A/Ostrich/Denmark-Q/72420/96, A/Turkey/England/N28/73 and A/Starling/Africa/983/79 with 50% end-point dilutions of 10^{-6}, $10^{-7.3}$, and $10^{-7.7}$, respectively. The ability of influenza A virus detection by the NP RT-PCR assay, including PCR-ELISA, was estimated to be as sensitive as virus isolation by inoculation in eggs. We also tested some human influenza A strains (H1N1 and H3N2) having HA titers between 1:80 and 1:1280, by the parallel sensitivity test, but these strains were not able to grow in eggs when diluted 10^{-2} (1:100).

The NP RT-PCR did not only detect influenza A virus from avian strains but when applied on virus strains from other species influenza A virus were detected too. Thus, influenza A was detected by the NP RT-PCR and PCR-ELISA from the human strains: A/Bayern/7/95 (H1N1), A/Sydney/5/97 (H3N2), A/TW/1/86 (H1N1), A/Wuhan/359/95 (H3N2), A/JHB/82/95 (H1N1), A/JHB/33/94 (H3N2), A/SA/1197/96 (H3N2), A/Beijing/262/95 (H3N2), the porcine strains: A/swine/Denmark/6019/81 (H1N1), A/swine/Denmark/4744/81 (H1N1), A/swine/Denmark/19126/93 (H1N1), A/swine/Denmark/15027A/90 (H3N2) and the equine strains: A/E2/Miami/63 (H3N8) and A/E1/Prague/56 (H7N7).

The specificity of the primers for the detection of influenza A was examined by including RNA from other viruses infecting avian species. Thus, RNA isolated from paramyxovirus (PMV)1 (La Sota), PMV1 (Ulster), PMV3, Infectious Bronchitis Virus (IBV) (Beaudette) and IBV (793B) did not yield amplified products.

The HA RT-PCR (primer MMU 2+9) for the detection of H5 influenza A subtypes amplified a band of 290 bp. A sharp band of the expected size was obtained from all five H5 strains tested and no PCR product was amplified from non-H5 influenza subtypes (not shown). The H5 origin was verified by sequencing. The HA RT-PCR (primer MMU 21+31) for the detection of H7 influenza A subtypes amplifies a band of 340 bp. A single band of the expected size was detected from all eight avian H7 strains tested, see Fig. 4, and HA H7 subtype origin verified by sequencing. When applying the HA H7 RT-PCR on the 24 different avian influenza A strains and the human strains PCR products were amplified from different HA subtypes except H4 (based on gel verification). However, for the non-H7 isolates the bands were either very weak or additional bands appeared on the gel (not shown). The sensitivity of the H5 and H7 subtype RT-PCRs varies between the different strains. For two strains with the H5 subtype and one with the H7 subtype having 50% end-point dilutions of 10^{-6}, $10^{-7.3}$ and $10^{-7.7}$ when

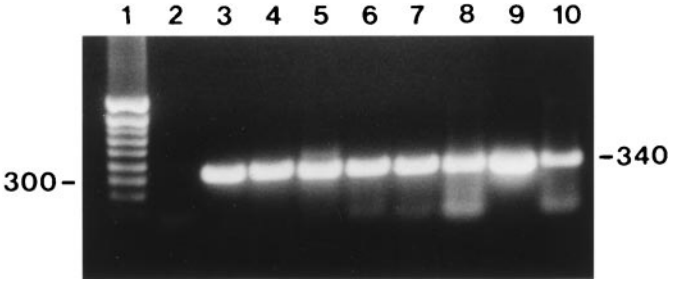

Fig. 4. RT-PCR amplification of a 340 bp fragment of the HA gene including the cleavage site of eight influenza A strains of the H7 subtype. Ten μl of PCR product was applied on a 1.5% agarose gel in *1–10* as follows: *1* Mr, *2* H_2O control, *3* A/African Starling/983/79, *4* A/FPV/Dutch/27, *5* A/Chicken/Brescia/1902/67, *6* A/Chicken/Germany/34, *7* A/Turkey/England/63, *8* A/Rhea/NC/41014/93, *9* A/S. Africa P/10/9/91, *10* A/Parrot/N. Ireland/73

grown in eggs, subtype specific bands on the gel suitable for sequencing were detected in the 10^{-3}, 10^{-4} and 10^{-6} dilution, respectively.

Discussion

The NP RT-PCR is a fast, sensitive and specific way, within one day, to determine if influenza A is present. The specificity of the NP RT-PCR reactions were confirmed by PCR-ELISA and an internal probe for hybridisation of the PCR product. The PCR-ELISA is a very convenient detection method as it is easy and fast to perform compared to traditional hybridisation methods. Our general diagnostic NP RT-PCR detects at least 38 influenza A strains from different species (primarily avian).

Wild ducks and other aquatic birds normally show no clinical symptoms of influenza infection [18] and outbreaks caused by highly pathogenic viruses have shown that the clinical appearance of the virus in different species is difficult to predict [5]. Experimental inoculation of ducks, turkeys, quail and ostriches with viruses known to be pathogenic in fowl generally results in no disease symptoms [1, 2, 13]. A general influenza A diagnostic RT-PCR is important both for the detection of virus from an actual outbreak but also for the screening of potential carriers of influenza A. Therefore, further studies concerning the detection of influenza A virus in clinical samples, by the NP RT-PCR and PCR-ELISA method, developed in the present study, are in progress.

Over the years especially avian influenza A viruses have been propagated routinely in embryonated chicken eggs, where the HAs of all strains are cleaved to yield infectious virus [18]. This is a very time consuming process, which may lead to changes of the viral genome compared to the strain of origin due to the adaptation. Nevertheless, propagation in eggs/culture is the only diagnostic technique that allows the collection of viral strains.

The pathogenicity of influenza A in birds has previously been determined by RT-PCR [10] in a study based on two strains, namely one avirulent (H5N2) and one virulent (H5N1) strain not including any avian influenza strains of the H7 subtype. So far, no data are available of H7 HA cleavage site RT-PCR amplification of several H7 strains using only one primer pair. We have developed a set of primers for the amplification of the H7 cleavage site. Sequencing and alignment/Blast search of the product that included the HA cleavage site confirmed the HA H7 origin of those isolates. The primer set also amplifies some non-H7 HA subtypes although either the gel band(s) were relatively weak or additional bands were present. However, the identification of the H7 specific product was evident. Since the PCR-ELISA is very sensitive the potential of that method for specific diagnosis of H5 and H7 subtypes should be investigated. A further aim could be to design probes specific for the different HA subtypes and/or the pathogenic potential of an isolate (the cleavage site). Nevertheless, the extensive sequence variation in the HA gene makes that difficult.

Even though field isolates have been shown to be avirulent by field and by experimental infection in chickens, additional determination of the HA cleavage

site sequence is recommended to assess their potential of becoming virulent [21]. In some cases only one nucleotide mutation has been required for the change from a non-virulent to a virulent avian influenza virus strain. Recently, it has been indicated that low pathogenic viruses may mutate and become high pathogenic [20].

Although avian viruses generally do not grow well in humans and vice versa [4, 9], influenza A viruses can be transmitted from one species to another [3, 8, 26]. Comparisons of H5N1 influenza A virus isolated at the same time, in 1997, from humans and chickens from Hong Kong showed that the isolates were close to identity. These isolates possessed multiple basic amino acids at the hemagglutinin cleavage site, which is associated with a highly pathogenic phenotype in poultry [19]. It is speculated if such isolates have the potential to cause a human pandemic under certain conditions.

In conclusion, the NP RT-PCR is rapid, it detects various influenza A strains from different species and is at least as sensitive as traditional methods of virus isolation using embryonating chicken eggs followed by serological identification. The pathogenicity of the avian influenza A subtypes H5 and H7 can be determined by RT-PCR and subsequent sequencing.

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