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A real-time PCR assay for the monitoring of influenza a virus in wild birds

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Abstract

A screening system including a new real-time PCR assay for the monitoring of influenza A virus in wild birds was developed. The real-time PCR assay uses SYBR green chemistry and the primers are targeting the matrix gene of influenza A virus. The performance of the assay was compared with two other assays, one assay also using SYBR green chemistry and one assay using TaqMan chemistry, i.e. a specific probe. A total of 45 fecal bird samples were analysed for influenza A virus in three different PCR reactions. Overall, 26 samples were positive in at least one of the three real-time PCR assays. Of the 26 samples, 18 were positive by all three reactions. Eight samples were found positive exclusively by the two SYBR green reactions, six of which were detected by both SYBR green reactions. Of the 26 positive samples, 15 samples were verified as positive either by virus isolation or influenza A M2-gene PCR. The results showed that the two SYBR green systems had a higher performance regarding the detection of influenza A as compared to the PCR reaction using a specific probe.
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1. Introduction

Influenza viruses are segmented negative-sense single-stranded RNA viruses that belong to the family *Orthomyxoviridae*. The influenza viruses can be divided into types A, B or C and influenza A viruses can further be divided into different subtypes according to its surface glycoproteins, i.e. hemagglutinin (HA) and neuraminidase (NA) (Hindiyeh et al., 2005). In humans, influenza A viruses mainly infect the respiratory tract and are the cause of yearly epidemics affecting 5–15% of the world population resulting in 250,000–500,000 deaths annually. Three times during the last century, influenza virus caused major pandemics, i.e. "Spanish flu" 1918–1919, "Asian influenza" 1957 and "Hong Kong influenza" 1968, with a high mortality rate all over the world (http://www.who.int/mediacentre/factsheets/fs211/en/ (2005-10-19)).

Sixteen HA and nine NA subtypes have been identified to date. All known HA and NA subtypes have been identified in the wild bird population, predominantly ducks, geese and shorebirds, which form a reservoir for the influenza A virus (Fouchier et al., 2005; Olsen et al., 2006). While the influenza virus does not usually cause clinical signs of disease in wild aquatic birds, severe illness may occur when the virus crosses the species border to domestic poultry. Swine has traditionally been considered to serve as an intermediate host in the transmission of avian influenza viruses to humans, but in recent years, cases of directly transmitted avian influenza A from domestic poultry to

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humans have occurred (Kaye and Pringle, 2005). Therefore, a monitoring system for influenza A viruses in wild aquatic birds might prepare us for what may emerge in poultry and the human population.

The real-time PCR technique has improved the rapid detection of viral nucleic acid sequences, also for influenza A viruses. Several real-time PCR assays for influenza A virus using SYBR green chemistry, hybridisation probes or hydrolysis probes (TaqMan chemistry) have been described (Stone et al., 2004; Smith et al., 2003; Ward et al., 2004; Templeton et al., 2004; Spackman et al., 2002, 2003a,b).

In the present study, a real-time PCR assay was developed using SYBR green chemistry with primers targeting the influenza A matrix gene, thereby establishing an optimal influenza A screening tool for large numbers of bird samples. The performance of this assay was compared to two other assays with published sets of primers (Ward et al., 2004): one assay using SYBR green chemistry and the other one using TaqMan chemistry, i.e. a specific probe.

2. Materials and methods

2.1. Samples

The samples used in the study originated from an influenza A screening study among wild ducks and were collected at Ottenby bird observatory, Öland, Sweden in 2003.

Cloacal swabs from a total of 45 birds were placed in transport media consisting of Hank's balanced salt solution, supplemented with 10% glycerol, 0.5% lactalbumin, 200 U/ml penicillin, 200 μg/ml streptomycin, 100 U/ml polymyxin B sulphate, 250 μg/ml gentamicin and 50 U/ml nystatin (all reagents from ICN, Zoetermeer, The Netherlands). The samples were stored at −70 °C until analysed.

2.2. RNA extraction

A sample volume of 150 μl, including transport medium, was mixed with 450 μl Trizol® LS Reagent (Invitrogen, Paisley, Scotland). A volume of 160 μl chloroform was then added and the tube was shaken for 15 s and incubated at room temperature for 3 min, followed by centrifugation for 15 min at 4 °C, 12,800 × *g*. The aqueous phase containing the RNA was removed and 300 μl were extracted in the Qiagen M48 robot using the Magattract viral RNA M48 kit (Qiagen Sciences, Maryland, USA). For each extraction a total of 48 samples could be processed. The RNA was eluted to a final volume of 65 μl. A positive control and a water control were included for each extraction. As positive control, a human influenza virus isolate, A/Malmö/2/03 (H3N2), with a concentration of 100,000 genome copies/ml, corresponding to 140 genome copies/PCR well was used. The concentration of the positive control was determined using a subtype specific TaqMan real-time PCR, an EBV standard and a count of influenza virus particles by electron microscopy (data not shown) (Enbom et al., 2001).

Table 1
Sequences for primers and probe used in the real-time PCR assays

Primer sets/probe	Sequence
Primer set I	5′ AAGACCAATYCTGTCACCTCTGA 3′ (forward) 5′ TCCTCGCTCACTGGGCA 3′ (reverse)
Primer set II	5′ AAGACCAATCCTGTCACCTCTGA 3′ (forward) 5′ CAAAGCGTCTACGCTGCAGTCC 3′ (reverse)
Specific probe	6-FAM 5′ TTTGTGTTCACGCTCACCGT 3′TAMRA

2.3. cDNA synthesis

The cDNA synthesis reaction mixture contained 1× first strand buffer, 0.01 M of DTT, 0.5 mM of each dNTP, 0.27 μM of random primer (hexamer), 100 U of M-MLV reverse transcriptase, 10 μl of sample RNA and RNase free water up to a final reaction volume of 40 μl, (all reagents from Invitrogen). The reaction mixture was then incubated with the following thermal conditions: 22 °C for 10 min, 37 °C for 60 min, 95 °C for 5 min and then cooled down to 4 °C (GeneAmp PCR System 2700, Applied biosystems, Foster City, USA).

2.4. Primers and probe

In order to develop a real-time PCR system, a conserved region in the influenza A matrix gene was identified using the influenza sequence database (ISD) (Macken et al., 2001) and a set of primers (primer set I) was designed for this region. The second primer set (primer set II) and probe used in the study, were also targeting the influenza A matrix gene (Ward et al., 2004). The same forward primer was used in both sets. The probe was labelled with FAM5′-3′TAMRA. All primers (Invitrogen) and probe (DNA technology A/S, Aarhus, Denmark) sequences are shown in Table 1.

2.5. Real-time PCR

Three different PCR assays were performed for each sample. Two SYBR green reactions with primer set I or II and one TaqMan reaction with primer set II, including the specific probe. All samples were run in triplicate in all three PCR reactions and were regarded as positive when at least two out of the three wells showed positive signals.

For the PCR reactions a 96 well plate was used (Bio-Rad Laboratories Inc., Hercules, USA) and the PCR thermal cycling was performed in an iCycler (Bio-Rad Laboratories Inc.) with the following cycling conditions: 50 °C for 2 min and then 95 °C for 10 min (SYBR green reactions)/5 min (TaqMan reaction) followed by 43 cycles at 95 °C for 15 s and 60 °C for 1 min. For the SYBR green reactions a melting curve analysis was performed after the PCR cycling, where the temperature was increased by 0.5 °C every 10 s, starting with a temperature of 55 °C and with an ending point at 95 °C. For all three PCR systems the positive control was run in four 10-fold dilutions.

2.5.1. PCR SYBR green reaction with primer set I

The real-time PCR reaction consisted of 1× SYBR green supermix (Bio-Rad Laboratories Inc.), 300 nM of forward primer, 900 nM of reverse primer, 5 mM of $MgCl_2$, 5 μl of sample cDNA and RNAse free water up to a final volume of 25 μl.

2.5.2. PCR SYBR green reaction with primer set II

The real-time PCR reaction consisted of 1× SYBR green supermix (Bio-Rad Laboratories Inc.), 900 nM of each forward and reverse primer (Ward et al., 2004), 5 μl of sample cDNA and RNAse free water up to a final volume of 25 μl.

2.5.3. PCR TaqMan reaction with primer set II including specific probe

The real-time PCR reaction consisted of 1× supermix (Bio-Rad Laboratories Inc.), 900 nM of each forward and reverse primer, 225 nM of the specific probe, 2 μl of sample cDNA (Ward et al., 2004) and RNAse free water up to a final volume of 25 μl.

2.5.4. Virus isolation

For 20 of the 26 samples showing positive results in any of the three real-time PCR assays, virus isolation in embryonated chicken eggs were performed as described earlier (Fouchier et al., 2005) (Table 2). Virus isolates were characterized by hemagglutination and neuraminidase inhibition assays and by nucleotide sequence analysis as described earlier (Fouchier et al., 2005).

2.5.5. Influenza A M2-gene PCR

For some of the samples showing discordants results in the three real-time PCR systems an additional PCR analysis was performed targeting part of the influenza A M2 gene. The cDNA synthesis was performed using primer 5′-TTG CAG GCC TAY CAG AAA CG-3′. The PCR was performed with the same forward primer used in the cDNA synthesis and reverse primer 5′-CAA GTA GTA GAA ACA AGG TAG-3′. The product of 325 bp was visualized on a 1.5% agarose gel.

3. Results

A total of 45 fecal wild bird samples were analysed for influenza A in three different real-time PCR reactions; two SYBR green reactions with two different primer sets and one TaqMan reaction using one of the primer sets and a specific probe. Overall, 26 samples out of the 45 samples were analysed as positive in at least one of the three real-time PCR assays. Of these 26 positive samples, 18 samples were positive in all three PCR assays. Eight additional samples were found positive exclusively by the SYBR green assays, six of which were positive by both SYBR green assays. One sample was detected exclusively by each SYBR green assay respectively (Fig. 1, Table 2).

For 20 of the 26 samples with positive results in any of the three PCR assays, virus isolation in embryonated chicken eggs was performed. Of these 20 samples, 13 were successfully isolated and characterized as influenza A virus (Table 2).

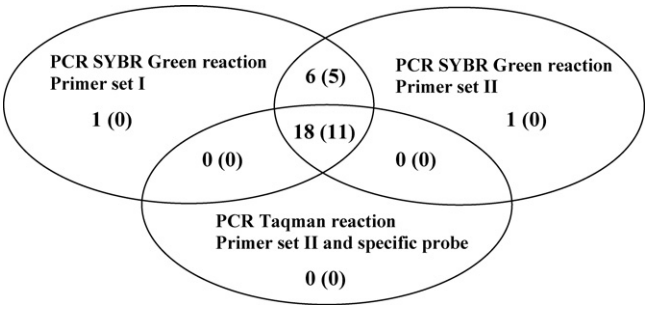

Fig. 1. The number of positive samples for influenza A matrix gene in the three different real-time PCR reactions. The numbers in parenthesis show how many of the positive samples that were verified by either virus isolation or influenza A M2-gene PCR.

For 13 samples, positive by any of the three real-time PCR assays, but negative for virus isolation, an additional PCR targeting the influenza A M2 gene was performed. Of these 13 samples, three samples showed positive results. Of these three samples, two samples could be further verified as influenza A by sequencing (data not shown) (Table 2).

As a total, from the 26 samples positive by any of the three real-time PCR assays, 16 samples were verified as influenza A by additional assays (isolation and/or M2-PCR). From the 18 samples positive by all three real-time PCR reactions, 11 samples were verified as influenza A by additional assays. From the six samples found positive exclusively by both SYBR green reactions, five samples were verified as influenza A by additional assays (Fig. 1, Table 2).

All three real-time PCR assays had the ability to detect the positive control at a dilution of 1:10, corresponding to 14 genome copies/PCR well but not in further dilutions.

4. Discussion

A real-time PCR was developed using SYBR green chemistry with primers targeting the influenza A matrix gene. The PCR assay was compared to two other real-time PCR assays, one SYBR green assay and one assay using TaqMan chemistry, including different sets of primers and a specific probe. The results showed that the two SYBR green systems had a higher performance regarding the number of detected influenza A samples as compared to the PCR reaction using a specific probe.

No differences could be observed between the two SYBR green assays regarding the number of positive samples. The total number of positive samples for the two SYBR green reactions together was 26, and 24 of those were detected by both reactions. One positive sample, respectively, was detected exclusively by each SYBR green reaction. This was the case, although the forward primers were identical and the two different reverse primers were positioned close to each other in the influenza A nucleotide sequence, with three nucleotides overlapping (the influenza sequence database, ISD) (Macken et al., 2001).

As a total, 18 samples were positive by the PCR assay including the specific probe. All of these were also positive by both SYBR green assays.

Table 2
Results for all samples tested in the different assays

Sample name	PCR SYBR green reaction. Primer set I	PCR SYBR green reaction. Primer set II	PCR TaqMan reaction. Primer set II and specific probe	Virus isolation of influenza A	Influenza A M2-gene PCR
S6:1	+++	+++	− − −	Negative	Positive
S6:2	+++	+++	+++	Positive	ND
S6:3	− − −	− − −	− − −	ND	ND
S6:4	− − −	− − −	− − −	ND	ND
S6:5	+++	+++	+++	Positive	ND
S6:6	+++	+++	+++	Positive	ND
S6:7	− − −	− − −	− − −	ND	ND
S6:8	− − −	− − −	− − −	ND	ND
S6:9	++−	+++	+++	Negative	Negative
S6:10	− − −	− − −	− − −	ND	ND
S6:11	+++	+++	+++	Positive	ND
S6:12	− − −	− − −	− − −	ND	ND
S6:13	− − −	− − −	− − −	ND	ND
S6:14	− − −	− − −	− − −	ND	ND
S6:15	+++	+++	+++	Positive	ND
S6:16	− − −	− − −	− − −	ND	ND
S6:17	− − −	− − −	− − −	ND	ND
S6:18	++−	+++	++−	ND	Negative
S6:19	+++	+++	+++	Negative	Positive
S6:20	− − −	− − −	− − −	ND	ND
S6:22	+++	+++	+++	Negative	Negative
S7:33	+++	+++	+++	Positive	ND
S8:12	+++	+++	+++	Positive	ND
S8:18	++−	++−	− − −	ND	Positive
S90:419	+++	+++	+++	Negative	Negative
S90:520	+++	++−	+++	ND	Negative
S90:594	− − −	− − −	− − −	ND	ND
S90:629	++−	+++	++−	Positive	ND
S90:668	+++	+++	+++	Negative	Negative
S90:673	− − −	++−	+− −	Negative	Negative
S90:674	+++	+++	− − −	Positive	ND
S90:683	− − −	− − −	− − −	ND	ND
S90:687	+++	+++	+++	ND	Negative
S90:689	++−	+++	+− −	ND	Negative
S90:692	+++	+++	+++	Positive	ND
S90:699	+++	+− −	+− −	ND	Negative
S90:711	− − −	− − −	− − −	ND	ND
S90:727	− − −	− − −	− − −	ND	ND
S90:728	− − −	− − −	− − −	ND	ND
S90:729	− − −	− − −	− − −	ND	ND
S90:782	+++	+++	− − −	Positive	ND
S90:817	− − −	− − −	− − −	ND	ND
S90:948	− − −	− − −	− − −	ND	ND
S90:962	+++	+++	+++	Positive	ND
S90:970	+++	+++	+− −	Positive	ND

The plus and minus signs correspond to each of the three PCR wells run for each sample in the different real-time PCR assays. Two or more plus signs correspond to a positive result. ND, not done.

All samples positive in any of the three real-time PCR assays were tested in additional assays for influenza A, i.e. virus isolation and influenza A M2-gene PCR. The results showed that five samples out of the eight, positive exclusively by the two SYBR green reactions, but not detected using the specific probe, were positive for influenza A virus by additional assays. This revealed that the SYBR green assays have higher sensitivities as compared to the real-time PCR assay using a specific probe.

Of the positive samples not verified as influenza A by any of the additional assays, it is not possible to confirm whether they are true or false positives. One reason for discordant results could be a low viral content in the fecal samples. For the positive control, the detection level for all three real-time PCR assays corresponded to 14 genome copies/PCR well. A sample with a lower genome copy content could therefore result in a non-detectable result. Also, for detection to occur when using TaqMan chemistry, both the primers and the specific probe all have to match the nucleotide sequence. Thus, the risk for a mismatch is greater for the probe assay as compared to using primers alone, as in the SYBR green assays.

In conclusion, when choosing an assay for detection of unknown influenza A virus strains in wild bird specimens, any

one of the two studied real-time PCR assays using SYBR green chemistry seem to be a better option than using the assay with specific probe. All of the tested real-time PCR assays are suitable for large-scale analysis but the SYBR green assays have the advantage of better detection ability as compared to the studied real-time PCR assay using specific probe.

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