Short communication

Identifying the species-origin of faecal droppings used for avian influenza virus surveillance in wild-birds

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ARTICLE INFO

ABSTRACT

Background: Avian influenza virus (AIV) surveillance in birds is important for public health. Faecal droppings from wild-birds are more readily available for such studies, but the inability to identify the species-origin of faecal samples limits their value.

Objectives: To develop, optimise, and field-test a method to simultaneously detect AIV and identify the species-origin from faecal samples.

Study design: Analytical sensitivity of the species-identification RT-PCR was assessed on serial dilutions of faecal droppings. Overall sensitivity of the methods for species-identification and AIV detection was assessed on 92 faecal and cloacal samples collected from wildlife, poultry markets, and experimentally H5N1-infected birds.

Results: All 92 samples were correctly identified to 24 different species, with a detection limit of 2.8 μg of faecal material. All 20 specimens previously shown by virus culture to be positive for influenza virus were correctly identified by RT-PCR for influenza A using the same nucleic-acid extracts used for species-identification.

Conclusion: We have optimised and evaluated a method for identifying the species of origin and detecting AIV from bird faecal droppings that can be applied to routine surveillance of influenza viruses in wild-birds.

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1. Background

Aquatic wild-bird-species are the natural reservoir for all 16 haemagglutinin (HA) and 9 neuraminidase subtypes of influenza virus.[1] Highly pathogenic avian influenza (HPAI) outbreaks in poultry arise from low pathogenic avian influenza (LPAI) viruses in wild-birds[2]. Some AIV also pose zoonotic and pandemic threats and are the causes of concern for human health.[3–5] Past pandemics arose from LPAI viruses; thus, surveillance for pandemic preparedness must focus on both LPAI and HPAI viruses. An understanding of the ecology, evolution, and antigenic characteristics of LPAI and HPAI in wild-bird reservoirs is important for animal and public health.[6,7]

Surveillance is best carried out through oropharyngeal and cloacal swabs from trapped and identified wild-birds, but without ornithological expertise, species-identification in wild-bird cloacal swabs and/or faeces may not be reliable. Many LPAI viruses are readily detected in cloacal swabs and from faeces.[1] While faeces are more accessible and often the only specimens available, it is more difficult to determine species-origin. A method to accurately identify species-origin will allow these specimens to provide valuable information. Therefore, a reliable method to accurately identify species-origin of faecal samples without compromising the sensitivity to detect virus is beneficial for routine surveillance work.

2. Objectives

To develop, optimise, and field-test a technique to recover adequate DNA from low-level/quality host genomic DNA found in wild-bird faeces in order to identify the species-origin of faeces using DNA barcoding.[8] To detect AIV by RT-PCR using the same nucleic-acid extract.

Table 1
Experimental conditions and methodological details of the single PCR and nested PCR methods.

	Single PCR		Nested PCR			
			First round		Second round[a]	
Primers[b]	BirdF1(8)	BirdR1(8)	ExternalF1 (10)	ExternalR1 (10)	InternalF1 (10)	InternalR1 (10)
Sequence	5′-TTCTCCAACCAC-AAAGACATTGGCAC-3′	5′-ACGTGGGAG-ATAATTCCAAATCCTG-3′	5′-TGTAAAAAG-GWCTACAGCCTAACGC-3′	5′-GTRGCNGAY-GTRAARTATGCTCG-3′	5′-AACAAACCA-CAAAGATATCGG3′	5′-TGGGARATA-ATTCCRAAGCCTGG-3′
Pre-incubation	95 °C, 10 min		95 °C, 10 min		95 °C, 10 min	
Amplification						
Cycles	45		45		35	
Denaturation	95 °C, 10 s		95 °C, 10 s		95 °C, 10 s	
Annealing	58 °C, 10 s		58 °C, 10 s		55 °C, 10 s	
Elongation	72 °C, 30 s		72 °C, 30 s		72 °C, 30 s	
Final extension	72 °C, 5 min		72 °C, 5 min		72 °C, 5 min	
Size of Product	~700 bp[c]		~700 bp		~670 bp[d]	

[a] A 1000-fold dilution was performed after the first (external) round of PCR before adding 5uL of the PCR reaction to the second (internal) round RT-PCR reaction mix.

[b] For both methods, the master mix was prepared as follows: each tube had 9.8uL water, 2.4 μL 25 μM MgCl$_2$, 0.4 μL 25 μM forward and reverse primers and 2 μL enzyme-buffer mix. 5 μL of DNA sample was used for 20 μL PCR reaction.

[c] Final PCR product from the single PCR method was run in a 2% gel and the 700-bp product was subsequently extracted using Qiagen Gel Extraction Kit (Qiagen, Hilden, Germany).

[d] The final PCR product from the nested PCR method was purified with the Qiagen PCR Purification Kit.

3. Study design

3.1. Specimens

92 cloacal and faecal samples collected from wild-bird habitats and poultry markets, as well as experimentally H5N1-infected chicken (*Gallus gallus*) were used to evaluate our new methods. The swabs were collected into viral transport medium (VTM) prepared as previously mentioned[9] and stored at −80 °C for up to 4.5 years. They had been tested for AIV by embryonated-egg inoculation and subtyped using standard methods.[9]

3.2. Host genomic DNA and viral RNA extraction

The faecal suspension in VTM was centrifuged to recover the faecal residue/pellet, which was then suspended in lysis buffer. Viral RNA and genomic DNA were extracted together using the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) with modifications to manufacturer's protocol. Samples were vortexed in a 5 ml vial with lysis buffer and 1 tablet of InhibitorEX (Qiagen) to facilitate mixing. 2 μg Carrier RNA was added to the lysis buffer-proteinase K-sample mixture to increase nucleic-acid yield. Purified DNA eluted from the column was concentrated with Microcon YM-100 (Millipore, Billerica, MA) at 4 °C to remove PCR inhibitors and concentrate the nucleic-acid.

3.3. PCR amplification of mitochondrial cytochrome oxidase I (COI) gene for host-species-identification and AIV M gene and H5 HA detection

PCR for the *COI* gene was performed using single and nested PCR methods (Table 1).[8,10] Standard precautions were taken to minimize PCR cross-contamination. The nested PCR amplicons were purified directly using QIAgen PCR Purification Kit and used for sequencing. The amplified ~700 bp PCR fragment of the *COI* gene was sequenced using 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA), and analyzed by the barcoding software BOLD, which provides a taxonomic assignment to the query sequence by employing a linear search to collect nearest neighbours (lowest % divergence) from a global alignment of all reference sequences.[11] The BOLD database has 18,206 specimens with barcodes collected from 117 countries. 5779 bird-species have been deposited into the database for species-identification.[11]

AIV *M* gene and H5 *HA* were detected by RT-PCR using previously described methods.[12,13] The forward and reverse primers for detection of *M* gene were 170801F-GGCATTTTGGACAAAKCGTCT and 170801R-CTTCTAACCGAGGTCGAAACG and for H5 *HA* were H5F-GCCATTCCACAACATACACCC and H5R-CTCCCCTGCTCATTGCTATG.

3.4. Determination of PCR sensitivity

Dilutions of the faecal swab pellet using lysis buffer were done before DNA extraction. Extraction and PCR was then performed as described above. To analyse the detection limit of the PCR methods in terms of copy number, serial dilution was performed on the DNA extract, and real-time PCR was performed using LightCycler480 according to manufacturer's protocol (Roche, Basal, Switzerland). Copy numbers determined from two different standards (746 bp *G. gallus* COI PCR fragment cloned in a plasmid and 990 bp PCR fragment of the cloned plasmid spanning the 746 bp *COI* insert) were averaged.

4. Results

The single *COI* barcoding PCR method successfully amplified 57% of 47 faecal swab and 76% of 45 cloacal swab samples while the nested PCR successfully amplified all the specimens (Table 2). All PCR amplicons were successfully sequenced and led to identification of the correct species. In contrast, all 47 faecal and 45 cloacal samples were successfully PCR amplified and identified to the correct species-level by barcoding. Successfully identified specimens include 24 bird-species from 8 different Orders. Furthermore, all 20 of the 92 faecal and cloacal samples known to be AIV positive in egg culture were successfully detected by RT-PCR for AIV *M* gene or H5 *HA*. There was no evidence of PCR-cross-contamination in the negative controls, which in some runs were added flanking each faecal specimen.

Typically, a swab collected from faeces picks up 45–75 mg of faecal material and contains on average 1.52×10^4 copies of *COI* ($n = 11$ samples). Fig. 1 shows the sensitivity of the single (panel A) and nested PCR (panel B) methods when applied to serial dilutions of a faecal specimen. The single PCR method (gel extraction required) allows for successful amplification and DNA barcoding of a 3125-fold dilution of the initial faecal material (i.e. 14 μg of the initial faecal sample). The nested PCR method can detect the equivalent of 2.8 μg (15,625-fold dilution) of the initial faecal sam-

Table 2
Comparison of the two PCR methods in identifying the host species through DNA barcoding using faecal and cloacal samples.

Species	Order	No. tested	Single PCR		Nested PCR	
			Successful PCR and correct species-identification	Mean specimen similarity ± standard deviation (%)[a]	Successful PCR and correct species-identification	Mean specimen similarity ± standard deviation (%)
Faecal samples						
Anas clypeata	*Anseriformes*	3	3	100 ± 0	3	100.0 ± 0.1
Anas platyrhynchos	*Anseriformes*	1	1	95.9 ± 0	1	100 ± 0
Ardea cinerea	*Ciconiiformes*	1	1	100 ± 0	1	100 ± 0
Columba livia	*Columbiformes*	2	1	100 ± 0	2	100 ± 0
Gallus gallus	*Galliformes*	29	18	97.9 ± 4.4	29	99.9 ± 0.3
Numenius phaeopus	*Charadriiformes*	1	1	100 ± 0	1	100 ± 0
Phalacrocorax carbo	*Pelecaniformes*	2	1	100 ± 0	2	100 ± 0
Phasianus colchicus	*Galliformes*	3	1	97.7 ± 0	3	100 ± 0
Charadrius leschenaultii	*Charadriiformes*	1	0		1	99.9 ± 0
Platalea minor	*Pelecaniformes*	3	0		3	99.7 ± 0.1
Rostratula benghalensis	*Charadriiformes*	1	0		1	99.9 ± 0
Total		47	27		47	
Cloacal samples						
Anas acuta	*Anseriformes*	4	4	100 ± 0	4	100 ± 0.1
Anas penelope	*Anseriformes*	1	1	100 ± 0	1	97.8 ± 0.0
Anas platyrhynchos	*Anseriformes*	1	1	99.7 ± 0	1	100 ± 0
G. gallus	*Galliformes*	13	13	100 ± 0	13	98.8 ± 2.9
Muscicapa dauurica	*Passeriformes*	1	1	100 ± 0	1	95.9 ± 0
Prinia gracilis	*Passeriformes*	1	1	100 ± 0	1	100 ± 0
Sturnus sericeus	*Passeriformes*	8	8	99.9 ± 0.3	8	99.9 ± 0.1
Tringa nebularia	*Charadriiformes*	1	1	100 ± 0	1	100 ± 0
Tringa totanus	*Charadriiformes*	3	3	100 ± 0	3	100 ± 0
Zosterops japonicus	*Passeriformes*	1	1	100 ± 0	1	100 ± 0
Acrocephalus orientalis	*Passeriformes*	1	0		1	100 ± 0
Halcyon smyrnensis	*Coraciiformes*	1	0		1	94.9 ± 0
Hirundo rustica	*Passeriformes*	1	0		1	100 ± 0
Luscinia cyane	*Passeriformes*	1	0		1	99.9 ± 0.0
Phalacrocorax carbo	*Pelecaniformes*	1	0		1	100 ± 0
Pycnonotus jocosus	*Passeriformes*	1	0		1	99.9 ± 0
Rostratula benghalensis	*Charadriiformes*	5	0		5	99.9 ± 0.0
Total		45[b]	34		45	

[a] Mean and standard deviation of specimen similarity in one species group: similarity scores (specimen similarity) are calculated from the alignment of query sequence to that of known species in the barcoding database.

[b] Four of the 45 cloacal samples did not yield a specific PCR product by the above methods. The DNA extracts from these 4 specimens, were re-extracted using the Qiagen Investigator Kit. Nested PCR was done for all 4 samples.

ple. Serial dilution of the DNA extract shows that the nested PCR method can successfully detect an average of as low as 3–4 genome copies of *COI* gene ($n = 4$ samples). In all cases, the amount of faecal material required was well within what routine sampling provides.

5. Discussion

We demonstrate that DNA barcoding can be reliably applied to faecal and cloacal swabs to accurately identify species-origin, even in specimens stored for up to 4.5 years. The quantity of
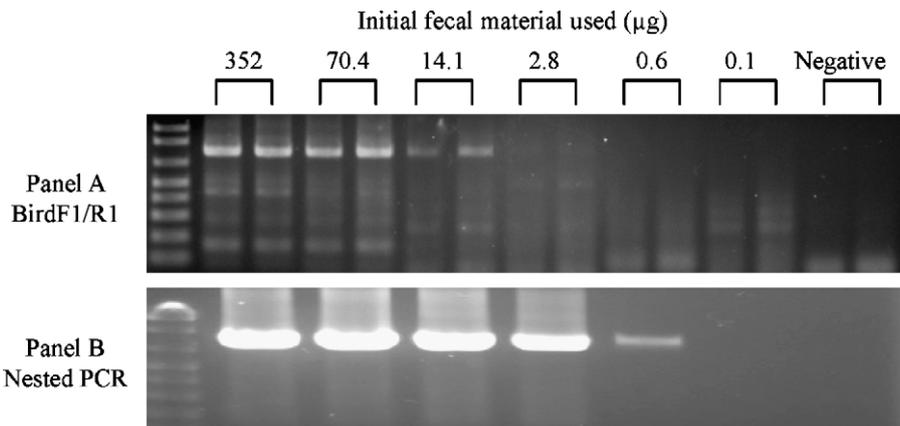

Fig. 1. Comparison of the sensitivities of the single (panel A) and nested PCR (panel B) methods: agarose gel electrophoresis products from PCR amplification of partial fragments of mitochondrial *COI* from faecal samples. Serial dilutions were done to determine the minimal amount of initial faecal sample needed for successful species-identification. Faecal sample in each reaction was respectively 352 μg (representing a dilution of 1/625 of faecal swab material), 70.4 μg, 14.1 μg, 2.8 μg, 0.6 μg and 0.1 μg. Negative control was distilled water.

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faecal material obtained on a swab is ample for this purpose. Furthermore, AIV detection by RT-PCR and DNA barcoding can be performed on the same nucleic-acid extract that contains both host DNA and viral RNA without loss of sensitivity. This method can be applied to field surveillance of AIV in wild-birds in two ways. Identification of species-origin of faeces by DNA barcoding can be done on the minority (usually <1%) of specimens found to be AIV positive by virus-culture or RT-PCR. Alternatively, all faecal surveillance samples can be processed with one nucleic-acid extraction using PCR to detect species-origin of faeces and RT-PCR to detect the presence of AIV RNA. Other genes, mitochondrial (*cytochrome b*), ribosomal (*16S*), and nuclear (*c-mos* and *glyceraldehyde-3-phosphodehydrogenase*) can be amplified from these samples for a more detailed phylogenetic analysis of the host species (data not shown).

The use of faeces is less intrusive to sensitive ecological locations, and can provide information to generate environmental risk-maps for AIV transmission. In addition to AIV surveillance, this method is useful when investigating the role of wild-birds in AIV poultry outbreaks since lack of wildlife expertise often leads to the misidentification of wild-birds in the vicinity of outbreaks.[14] Identifying the species-origin of wild-bird faeces by our method in the vicinity of poultry outbreaks is therefore beneficial to AIV surveillance.

Available experimental studies suggest that HPAI H5N1 viruses are shed preferentially in the respiratory tract of birds[7,15] and if so faeces may not be the optimal specimen for HPAI H5N1 detection. However, many LPAI viruses are more readily detected in cloacal and faecal swabs,[1] and such viruses are equally, if not more relevant, to pandemic preparedness.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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