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Molecular detection of avian influenza virus from birds sold in a multi-species animal market at Jakarta-Indonesia



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ABSTRACT

Avian Influenza (AI) is a zoonotic disease caused by influenza virus A of the family *Orthomyxoviridae*. The Avian Influenza virus infects various birds and mammals, including humans. Multi species live animal markets, as a meeting places for human, birds, mammals, and reptiles, can potentially transmit the AI virus from animals to humans. The purpose of this study was to detect the presence of Avian Influenza virus by the detection of Matrix and H5 genes from various bird species sold in a multi species live animal market in Jakarta using real-time Reverse Transcriptase-Polymerase Chain Reaction (rRT-PCR). 862 field samples from 28 families and 96 bird species were collected using a cross-sectional sampling technique. These field samples consisted of

649 fecal samples, 114 cloacal swab samples, and 99 tracheal swab samples. The samples were pooled based on the type of sample and kiosk where the samples were collected. The presence of AI virus was screened using rRT-PCR targeting the matrix gene, followed by further test targeting the H5 gene for AI virus subtyping. Positive AI samples were only detected in 7 out of 649 fecal samples (1.08%); however, all of them were not H5 AI virus. Positive AI samples were detected in four bird species, which were three magpie-robins (*Copsychus saularis*, family *Turdidae*), two white-eyes (*Zosterops palpebrosus*, family *Zosteropidae*), a Yellow-vented bulbul (*Pycnonotus goiavier*, family *Pycnonotidae*), and a robin (*Leiothrix lutea*, family *Sylviidae*).

Key words: Avian Influenza, birds, H5 gene, matrix gene, real-time RT-PCR

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INTRODUCTION

Infections by influenza virus A of the family *Orthomyxoviridae* have a significant impact on health, social and economic sectors.^{1,2} As a zoonotic disease, Avian Influenza viruses (AIV) can infect various birds and mammals including humans,^{1,2,3} therefore live bird markets (LBM) as a meeting place for humans and poultry can potentially transmit the AI virus among birds or from birds to humans.⁴

It has been shown that LBM plays a significant role in facilitating emergence or reemergence of Avian Influenza.⁴ In Indonesia, LBMs are present in many places, including in the major cities like Jakarta where they sell not only live birds but also multi species animals, such as mammalians, reptiles, and amphibians. Studies show that the market as a meeting place for humans and avian has the potential to become a means of spreading AIV in avians or the possibility of transmission from avians to humans. It was recorded in Guangzhou, China that a patient was infected with Highly Pathogenic AI (HPAI) from a food market that simultaneously sold live poultry.⁴

This study examined the presence of AIV in various bird species sold in the biggest multi species live animal market in Jakarta via the

detection of Matrix and H5 genes in the tracheal swab, cloacal swab, and fecal samples using real time Reverse Transcriptase-Polymerase Chain Reaction (rRT-PCR). The relationship between the geographical origin of the birds and the prevalence of AIV was discussed.

RESEARCH DESIGN AND METHODS

This study collected cloacal swab, tracheal swab and fecal samples from various bird species using a cross-sectional sampling technique for six months in a multi species live animal market, Jakarta. The bird origin was based on the information from the seller, and its identification was made according to bird identification methods.^{5,6}

The samples were pooled based on the type of sample and kiosk where the sample was collected. A pool contains a maximum of 5 individual-samples. For the pooled-sample, the individual-samples were vortexed, and then 100 µl of each individual-samples that would be added to the pool were combined and homogenized. The RNA isolation was performed using the AI/ND Viral RNA Isolation kit from Ambion®.

To detect the presence of AI virus, a molecular technique by using One-step real-time Reverse

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Transcriptase-Polymerase Chain Reaction (rRT-PCR) was accomplished with an ABI 7500 (Applied Biosystems). Initial screening was done by rRT-PCR targeting matrix gene (M gene), which then further assessed to detect H5 gene (H5 rRT-PCR) for AI virus subtyping. The primer and probe used in this study are shown in Table 1.

A total of 8 µl of RNA extract was amplified in a volume of 20 µl by employing the following temperature profile for MA rRT-PCR: 5 minutes at 50°C, 20 seconds at 95°C, and 45 cycles of 3 seconds at 94°C, and 42 seconds at 60°C. The amplification condition for H5 rRT-PCR was: 5 minutes at 50°C, 20 seconds at 95°C, and 40 cycles of 3 seconds at 95°C, 32 seconds at 56°C, and 10 seconds at 72°C.

RESULTS

Sample Composition

The study was done in the biggest multi species live animal market in Jakarta. The market was established in 1976. It has 289 kiosks with 152 sellers. Various live animals were sold in this market, i.e. avians, pet animals, wild animals, amphibians,

reptiles and exotic animals. The avian species sold in this market were diverse and can be grouped into ornamental chickens, waterfowls, wetland birds, bridge species, ornamental or singing birds, and exotic birds.

Based on groups and sample type, the sample composition taken from the multi species live animal market in Jakarta are shown in Table 2. The samples consisted of 649 fecal samples, 114 cloacal swab samples, and 99 tracheal swab samples. Examination of Matrix rRT-PCR was conducted on 131 pools of fecal samples, 30 pools of cloacal swab samples, and 26 pools of tracheal swab samples.

Matrix Gene Determination and H5 Subtyping

Based on the rRT-PCR examination, six pools of fecal samples (containing 30 individual samples) were positive for the AI Matrix (M) gene while all cloacal and tracheal samples were negative. TheM rRT-PCR detection was further performed on each individual sample in the positive pools. Individual MrRT-PCR testing determined that 7 out of 649 fecal samples (1.08%) were positive. Overall, AIV was detected in 0.81% (7 out of 862 samples) of samples from a multi species live animal market in Jakarta (Table 2). However, based on H5 rRT-PCR, all of them were not subtype H5 AIV.

Positive fecal samples were detected in four bird species, which are three magpie-robins (*Copsychus saularis*, family *Turdidae*), two white-eyes (*Zosterops palpebrosus*, family *Zosteropidae*), a Yellow-vented bulbul (*Pycnonotus goiavier*, family *Pycnotidae*), and a robin (*Leiothrix lutea*, family *Sylviidae*). The rRT-PCR Ct value for positive Matrix gene samples was 30.64 – 38.55. All positive samples were ornamental or singing birds. The birds' were from Indonesia (Sumatera Island, Java Island and South East Nusa Island) and one from China.

Table 1 Primer and probe used in this study

Primer/Probe	Sequence of primer/probe	Reference
Primer Matrix forward M+25* 5'	5'-AgATgAgTCTTCTAACCgAggTCg-3'	7,8
Primer Matrix reverse M-124*3'	5'-TgCAAAAACATCTTCAA g TCTCTG-3'	
Probe AIV Matrix M+64*	5'DFAM-TCAggCCCCCTCAA g CCgA-BHQ1-3	
Primer H5 forward IVA D148 H5	5'-AAACAgAgAggAAATAAgTggAgTAAAATT-3'	9
Primer H5 Reverse IVA D149 H5	5'-AAA gATAgACCgCTACCAT gATTgC-3'	
Probe AIV H5 H5+1637*	5'd FAM-TCAACAgTggCgAg TTCCCTAgCA-BHQ1-3'	7

Table 2 Samples composition and Avian Influenza Positive samples by avian group and sample type

Nr.	Avian Group	Species Amount	Type of Sample (Positive/subtotal)			Positive/Total sample
			Fecal	Cloacal swab	Oropharyngeal swab	
1	Ornamental chicken	17	0/146	0/68	0/59	0/273
2	Waterfowl	6	0/12	0/28	0/33	0/73
3	Wetland birds	4	0/4	0/4	0/4	0/12
4	Bridge Species	4	0/57	0/14	0/3	0/74
5	Ornamental or singing birds	63	7/425	0/0	0/0	7/425
6	Exotic Birds	2	0/5	0/0	0/0	0/5
	Total	96	7/649 (1.08%)	0/114 (0%)	0/99 (0%)	7/862 (0.81%)

DISCUSSION

Molecular Detection of Avian Influenza Virus

Influenza A viruses have a high genetic diversity and are spread in wild birds all over the world.² The influenza viruses can infect a wide variety of birds and mammals, but the natural hosts of the virus are wild waterfowl, shorebirds, and gulls.¹⁰ In this study, AIV was detected in four bird species. Based on the rRT-PCR test, the AIV prevalence in fecal samples was 1.08%, and all of them were ornamental or singing birds. The AIV prevalence in this study was lower than in previous publications.^{11,12}

A study in wild birds in Central-South Spain showed that the prevalence of the AIV was 2.6%,¹¹ whereas a similar study revealed that isolation of AIV had been reported from 12 orders and 88 species of free-living birds with an isolation level of 10.9%.¹² Most isolations are reported from species in the orders Anseriformes and Charadriiformes, and it is recognized that species in Anseriformes represent important reservoirs of AIV.¹² In the study of Stallknecht and Shane (1988),¹² most of the samples were collected from the Order Anseriformes with an isolation level of 15.2%. However, in our study, more than 50% of samples were collected from ornamental or singing birds.

All of the samples detected in this study were non-H5 AIV. This finding was supported by studies which show that Highly Pathogenic Avian Influenza (HPAI) subtype H5 is rarely detected in wild birds,^{1,11} but the isolation rate of Low Pathogenic Avian Influenza (LPAI) is quite high, i.e., 11% for ducks and geese and about 2% of other species.¹³ Since the number of waterfowl samples (ducks and geese) in this study was lower than samples from ornamental or singing birds, it is assumed that this may cause the absence of AIV from waterfowl samples. In addition, some factor could lead to the failure of H5 detection using rRT-PCR, including inhibitor factor in the sample,¹⁴ the amount of viral genetic material in the sample that did not reach the limit of detection of rRT-PCR,¹⁵ and the diversity of HA gene sequences in isolates from wild birds.^{14,16}

Over 75% of field samples in this study were fecal samples. In fecal samples, various inhibitor factors can decrease rRT-PCR detection sensitivity. A false negative could be obtained if the amount of AIV genetic material in the sample was below the detection limit. The rRT-PCR detection limit for the matrix gene is ten fg or about 1,000 copies of the target RNA and can detect 0.1 50% Egg infective dose (EID₅₀).¹⁵ Meanwhile, the rRT-PCR detection limit for H5 and H7 is 100 fg or about 1,000-10,000 copies of the gene and can detect 10 EID₅₀¹⁵ or it can be 5×10^{-2} EID₅₀.¹⁷

HA genes in AIV have high variability. Therefore the appropriate pair of primers and probes is an essential consideration to increase the sensitivity of rRT-PCR. The primer and probe pairs used in this study were known to be able to detect AIV in waterfowl in Indonesia,¹⁸ but its sensitivity to samples from wild bird species still needs further investigation. But until now, there were no report that the primer and probe used in this study failed to detect AIV in Indonesia. Differences between poultry detection results have been reported by Das *et al.* (2007)¹⁹ in Xing *et al.* (2008),¹⁶ where the sensitivity of rRT-PCR for samples from wild birds was lower than domestic poultry. The proper use of primers and probes for isolates from Indonesia is particularly noteworthy due to HPAI infection from the new clade in ducks in Indonesia in 2012. The new AIV clade in Indonesia was clade 2.3 which is very different from the previous clade, i.e. Clade 2.1.3.²⁰ But, further study using the same primer and probe as in this study revealed that the primer and probe used were able to detect AIV from clade 2.3 (*unpublished data*). Despite high variability, RNA viruses such as AIV have high mutation rates.

The mutation rate of AIV Matrix gene is 1.64×10^{-4} substitution/site/year for AIV of North American lineage and 5.76×10^{-4} substitution/site/year for AIV of Asian lineage.²¹ The non-synonymous substitution rate of HA AIV gene is higher than the Matrix gene, i.e., 5.7×10^{-3} /site/year.²² It is, therefore, advisable to conduct further research and confirm the diagnosis using viral isolation in embryonated chicken eggs and sequencing.

LPAI usually infects wild birds.^{1,2} The LPAI virus remains a risk for poultry, humans and the environment because LPAI virus can mutate into HPAI virus.²³ HPAI virus mutations may result from nucleotide substitution or insertion¹³ and transcriptional failure by polymerase enzyme complexes resulting in amino acid insertion at the HA0 cleavage site.²⁴

HPAI H5N1 outbreaks in poultry farms (ducks) since September 2012 in Indonesia showed that mutation of AIV circulating in Indonesia continues to occur. The death of ducks in various provinces in Indonesia at the end of 2012 was caused by HPAI H5N1 clade 2.3.2 which is different from the previous clade circulating in Indonesia, namely clade 2.1.3.²⁰ The cause of the emergence of the new clade in Indonesia was still not understood. It is thought to be due to genetic drift mutations or genetic drift of previous viruses or the introduction of new viruses from abroad.²⁰

Various LPAI cases that eventually turned into HPAI showed that wild birds infected with LPAI have an important role in the spread of the virus.

Most avian influenza viruses replicate preferentially in the gastrointestinal tract of wild ducks, are excreted at high levels in feces, and are transmitted through the fecal-oral route.²⁵ Cages, vehicles, contact with feral birds, even human activities could be a source of AIV transmission.¹³ In contrast to HPAI virus infection that causes rapid deaths and lower viral shedding,¹³ the LPAI infected-birds do not die quickly, allowing the virus to replicate and shed into the environment. If the LPAI virus mutates into HPAI, then it can cause serious outbreaks and losses.

Geographical Origin of the Sample

The birds sold in the multi species live animal market in Jakarta were obtained from native capture, local bird breeders, and import. Based on its geographical origin, AI positive bird samples were from different islands in Indonesia and one from China. According to the bird-seller, it is known that the magpie-robins, white-eyes, and Yellow-vented bulbul were captured from nature, while Robin birds were obtained from bird breeding farms and imported from China. Influenza viruses may infect both domestic and captive birds, the frequency with which primary infections occur in any bird usually depending on the degree of contact there is with wild birds.¹³ Secondary spread of AIV is usually associated with human involvement, either by bird or bird product movement or by transferring infective feces from infected to susceptible birds, but potentially wild or captive birds could be involved.¹³

This study was a cross-sectional study. Therefore, it can not be determined exactly when the birds were infected by AIV. Birds could be infected in the area of origin, in the process of transportation, or in the market. The route of influenza virus introduction into the market remains to be ascertained. We suspect that contamination occurred as a consequence of virus-contaminated cages, trucks, and equipment, and the contact with wild birds.

CONCLUSION

Avian Influenza virus non-H5 subtyped was detected in 1.08% of fecal samples sold in a multi species live animal market in Jakarta. All of the AIV positive samples came from feces of ornamental or singing birds both domestic and imported.

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