

DISSERTATION

**MOLECULAR EPIDEMIOLOGY AND ASSOCIATED RISK FACTORS OF
AVIAN INFLUENZA A VIRUS SUBTYPES H5 AND H9 IN POULTRY OF
LIVE BIRD MARKETS OF EAST JAVA, PROVINCE OF INDONESIA**



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**DOCTORAL PROGRAM
VETERINARY SCIENCE STUDY PROGRAM
FACULTY OF VETERINARY MEDICINE
AIRLANGGA UNIVERSITY
SURABAYA
2023**

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to obtain a Doctorate Degree
in Veterinary Science Study Program
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STATEMENT

I hereby declare that the dissertation entitled:

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Surabaya, 27 December 2022



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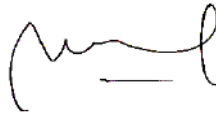
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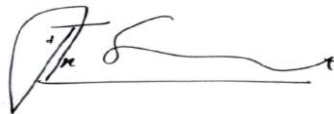


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SUMMARY

MOLECULAR EPIDEMIOLOGY AND ASSOCIATED RISK FACTORS OF AVIAN INFLUENZA A VIRUS SUBTYPES H5 AND H9 IN POULTRY OF LIVE BIRD MARKETS OF EAST JAVA, PROVINCE OF INDONESIA

Avian influenza (AI) is a highly infectious, contagious, zoonotic viral disease that belongs to the family “Orthomyxoviridae”. Initially, AI was named “fowl plague” and can be confused with Avian Pasteurellosis or Fowl Cholera. During the First International Symposium on Avian Influenza, which took place in 1981, the term “fowl plague” was eventually replaced with the more accurate term “avian influenza. The AIV subtype H9N2 outbreaks have been reported in domestic poultry including turkeys, domestic ducks, and chickens in many parts of the world.

Highly pathogenic avian influenza (HPAI) virus subtype H5N1 viruses have also had terrible in local communities of Southwest Asia. There had been reports of outbreaks in a number of Asian and Southeast Asian countries over the course of several years. To date, there have been 861 confirmed disease cases in people, and 455 have led to death. Indonesia reported 200 confirmed human cases and 168 fatalities. The 168 fatalities account for over one-third of all deaths among all affected countries. However, Indonesia had only two cases in 2014 and 2015, zero in 2016, and one in 2017, the last.

The first outbreak of the HPAI virus subtype H5N1 was identified in Indonesia in December 2003 affecting all provinces of Indonesia except one province. There are still endemic cases of the disease in various parts of the country. The avian influenza subtype H5N1 is highly pathogenic for humans and poultry as it causes huge economic losses in Indonesia and the rest of the world while the low pathogenic avian influenza A virus subtype H9N2 causes low fatality when compared to H5N1. Although AIV subtype H9N2 was initially detected in Indonesia in late 2016 and early 2017, there is still little evidence to support this.

The current study was carried out to estimate the prevalence, investigate molecular characterization, phylogenetic analysis and determination of associated risk factors of avian influenza A virus subtypes H5N1 and H9N2 among poultry in the live bird markets of four cities in East Java province, Indonesia. A cross-sectional study was designed from March 2021 to April 2022 to estimate the prevalence, determine the molecular characterization, phylogenetic analysis and potential risk factors associated with the positivity of AIV among the poultry (backyard, layer, and broiler) of LBMs of four cities of East Java, Indonesia.

A total of 600 cloacal and tracheal swab samples were collected from healthy live birds and inoculated into the allantoic cavity of 9–11-day-old embryonated specific-pathogen-free (SPF) chicken eggs. For the initial identification of viruses, we have used HA and HI tests, and then further identification was done using qRT-PCR.

The viral RNA copies were determined using the THUNDERBIRDTM probe one-step qRT-PCR kit (TOYOBO) with the specifically designed primers and probes by Indonesia-Japan Collaborative Research Center for Emerging and Re-emerging Infectious Diseases, Institute of Tropical Disease University of Airlangga.

City (Sidoarjo, Surabaya, Pasuruan and Malang) and poultry (backyard, broiler, and layer) level prevalence was calculated by dividing the number of positive cases by the total number of samples screened. The WGS of AIV subtype H5N1(EPI_ISL_13690275) and H9N2 (not yet uploaded on GISAID) from East Java were selected. Eight whole-genome sequences of AIV subtype H5N1 and five whole-genome sequences of AIV subtype H9N2 reference viruses that showed closeness were selected and downloaded from the GISAID EpiFlu database.

The Bio edit v7.2 was used to edit the WGSs of the highly pathogenic avian influenza virus subtype H5N1 and LPAIV H9N2. Mega 11 software CLUSTAL W program was used to obtain whole-genome sequence (WGS) alignment before analysis. The genetic information processing software Genetyx v14 (Genetyx Co., Tokyo, Japan) generated the phylogenetic trees using the RAxML with 100 bootstrap replicates used for H5N1, while Mega 11 software was used to do the phylogenetic analysis using the Neighbor-Joining method with a bootstrap of 1000 for H9N2. In addition, the NCBI protein BLAST web server was used to confirm the mutations of all segments with reference isolates of both viruses.

In the second aim of the research, we used a structured questionnaire contained 30 questions related to the demographics of LBMs and risk factors of AIVs prevalence. The information was gathered via hard proformas and personal interviews conducted by the first author in the native language. All of the information was meticulously entered into Microsoft Excel sheets and statistically examined. Statistical analyses were conducted using IBM SPSS version 25.

A multivariable logistic regression analysis and Chi-square tests were performed to determine the effect of various potential variables on the prevalence of avian influenza H5N1 and H9N2 viruses. To test the significant effect of associated risk factors, two distinct multivariable models were created, one for the H9N2 and the other for the H5N1. A total of 23 (3.8%) samples out of 600 samples tested positive utilizing the HA, HI, and qRT-PCR techniques for avian influenza A virus subtypes H5N1 and H9N2. The H5N1 subtype [14 (2.3%)] was detected more frequently than the H9N2 subtype [9 (1.5%)].

A total of 8 (34.78%) out of 23 samples were positive for both subtypes. The backyard had the highest prevalence of 16/267 (5.99%), followed by broiler 4/179 (2.23%), and layer 3/154 (1.68%). LBMs of Sidoarjo showed a maximum prevalence of 7/150 (4.67%), followed by Surabaya 6/150 (4%), Pasuruan 6/150 (4%), and Malang 4/150 (2.67%).

Phylogenetic analysis clearly showed that Av1955 was a virus of the H5 HA subtype (clade 2.3.2.1c) and the N1 NA subtype; the clade 2.3.2.1c had originally evolved in Eurasia before 2012, while Av1953 isolate belonged to the G-1 branch, was monophyletic, and genetically extremely similar to Indonesian strains found in 2017 and 2018, respectively.

The HA amino acid sequence of Av1955 contained multiple basic amino acids (PQRE-RRRKR↓G) at the cleavage site which shows the characteristics of the highly pathogenic avian influenza virus (HPAIV) while sequence motif 335RSSR/GLF341 at the hemagglutinin (HA) cleavage site provided evidence that Av1953 isolate had a low level of pathogenicity.

Av1955 showed inter-subtype and intra-subtype reassortment between Indonesian lineage and Eurasian lineage and maximum mammalian adaptation markers from circulating H5N1 viruses in Indonesia. The Av1953 revealed inter-subtype reassortment and found that the viral internal proteins (PB2, PB1, PA, NP, and NS) of the Av1953 isolate contain a variety of molecular markers related to pathogenicity and infections in mammalian hosts.

Rodents seen in the stall ($p=0.03$), stray dogs accessing the stall ($p=0.04$), presence of ducks ($p=0.061$), turkeys ($p=0.03$), chukars/partridges ($p=0.024$), and presence of peafowl in the stalls ($p=0.003$), rainy season ($p=0.001$) and birds from the household sources ($p=0.002$) showed significant relationship ($p<0.05$) with avian influenza A virus subtype H5N1 prevalence in LBMs.

The risk of H9N2 was higher among the poultry of live bird markets in the presence of the following potential risk factors: the presence of ducks ($p=0.003$), turkeys ($p=0.025$) and pheasants ($p=0.024$) in the stalls, dry ($p=0.006$) and the rainy season ($p<0.001$), and birds from the household sources ($p=0.04$) showed a significant association with the prevalence of avian influenza A virus subtype H9N2 in LBMs. Nineteen factors were found to be protective (i.e., having a p -value >0.05) of exposure in the LBMs with the prevalence of avian influenza A virus subtype H9N2 infection.

The findings of the current study illustrate the recurring infection and presence of both avian influenza viruses H5 and H9. The H5 subtype [(14 (2.3%)] was detected more frequently than the H9 subtype [(9 (1.5%)). 8/23 (34.78%) of samples were positive for both subtypes.

Phylogenetic analysis and molecular characterization of AIV in the current study showed that Av1955 belonged to the H5N1 clade 2.3.2.1c (Eurasian lineage) and Av1953 belong to the H9N2 clade h9.4.1 (G1 like) had mutations in their gene segments. The AIV subtype H5N1 showed maximum mammalian adaptation marker mutations among the currently circulating viruses in avian hosts by inter-subtype and intra-subtype reassortment. Av1953 H9N2 isolate revealed mutations related to a higher zoonotic potential through inter-subtype reassortment among viruses circulating in Indonesia.

Five risk factors for H9N2 infections, including ducks, turkeys, and pheasants in the stalls, dry and rainy seasons, and household birds, and seven factors for H5N1 infections, including rodents, stray dogs' access, turkeys, chukars/partridges, and peafowls in the stalls, rainy season, and household birds, revealed a significant association with prevalence of AIV subtypes H9 and H5 in live.

To better understand the pathogenic potentials of both viruses, more research in animal models is needed. To stop the zoonotic transmission of AIV to humans, it is essential to track their evolution in both migratory and domestic bird populations.

Appropriate biosecurity precautions must be put in place to prevent the introduction and dissemination of avian influenza A virus subtypes H5N1 and H9N2 in Indonesia.

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Saifur Rehman

ABSTRACT

Avian influenza A virus subtypes H5N1 and H9N2 are contagious zoonotic pathogens that are circulating in Indonesia and have raised concern about their potential impacts on poultry and public health. A cross-sectional study was carried out to estimate the prevalence, determine the molecular characterization, phylogenetic analysis and associated risk factors of avian influenza A virus subtypes H5 and H9 among poultry in the live bird markets of four cities in East Java province, Indonesia. A total of 600 tracheal and cloacal swab samples (267 from backyards, 179 from broilers, and 154 from layers) from healthy birds were collected. The samples were inoculated into specific 9-day-old embryonated eggs via the allantoic cavity. Initial screening was done through HA and HI. Positive samples were further identified through qRT-PCR. The complete genome has been sequenced, and phylogenetic and molecular characterizations have been performed. Risk factors were statically analysed through logistic regression. The overall prevalence of AIV subtypes H5 and H9 was 3.8% (23/600). Prevalence was higher in backyards at 5.99% (16/267) followed by broilers 2.23% (4/179) and layers 1.68% (3/154). Phylogenetic analysis revealed that Av1955 belonged to the H5N1 clade 2.3.2.1c (Eurasian lineage) and HA contains an HPAIV-type cleavage site sequence (PQRERRRKR/GLF). The six gene segments (PB1, PB2, HA, NP, NA, and NS) derived from viruses of the H5N1 Eurasian lineage, one (PB2) from the H3N6 subtype and the remaining one (M) from H5N1 clade 2.1.3.2b (Indonesian lineage). The donor of the PB2 segment was a reassortant among three viruses of H5N1 Eurasian and Indonesian lineages and the H3N6 subtype. Phylogenetic results for H9N2 isolate revealed that it was genetically similar to the Indonesian strains found in 2017 and 2018. The presence of the sequence motif 335RSSR/GLF341 at the HA cleavage site provided evidence that the Av1953 had a low level of pathogenicity. Av1953 revealed mutations that indicated a higher zoonotic potential. The segment NS1 of Av1953 had a PDZ domain C-terminal motif of ²²⁷KPEV₂₃₀ and no 80–84 deletion. The final multivariable model revealed seven factors for H5N1 infections including observing rodents (p=0.036, OR=0.005), stray dogs access (p= 0.004 OR= <0.001), presence of turkeys (p= 0.03 OR= 0.007), chukars/partridges (p= 0.024 OR= 2500), and peafowls in the stalls (p= 0.0043 OR= <0.001), rainy season (p=0.001) and birds from the household sources (p=0.002) and five risk factors for H9N2 infections “presence of ducks (p=0.003, OR= 38.2), turkeys (p=0.017 OR= 0.032), and pheasants in the stall (p=0.04, OR=18.422), dry (p=0.006) and the rainy season (p=<0.001), and household birds

($p=0.002$) in the live bird markets. The findings of the current study illustrate the presence of both avian influenza viruses and associated risk factors in the surveyed marketplaces. The mutational analysis depicted that nucleotides and amino acid sequences of study isolates might cause newly emerging subtypes in the study area. Effective protective measures and mitigation strategies for risks outlined in this study could help to reduce the burden of both subtypes in Indonesia. Appropriate biosecurity precautions must be put in place to prevent the introduction and dissemination of H9 and H5 in Indonesia.

Keywords: Avian influenza, Low pathogenic avian influenza H3N6, Whole-genome sequence, Inter-subtype and Intra-subtype reassortment, public health, East Java, Indonesia

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ABBREVIATIONS MEANS AND SYMBOLS

°C	= Degree celsius
AI	= Avian Influenza
bp	= Base pair
cDNA	= Complementary deoxyribonucleic acid
CEF	= Chicken embryo fibroblasts
DNA	= Deoxyribonucleic acid
dNTPs	= Deoxynucleotide phosphates
DPBS	= Dulbecco's phosphate buffer saline
EDTA	= Ethylenediaminetetraacetic acid
IRCVS	= Indonesian research center for veterinary science
ITD	= Institute of Tropical Diseases
GISAID	= Global science initiative and primary source
GFP	= Green fluorescent protein
HA	= Haemagglutinin
HI	= Hemagglutination Inhibition
HEF	= Haemagglutinin–Esterase–Fusion
HPAI	= High pathogenic avian influenza
hr	= Hour(s)
IFN	= Interferon
IL	= Interleukin
L	= Liter
LBM	= Live bird markets
LPAI	= Low Pathogenic Avian Influenza
M	= Matrix
min	= Minute(s)

mL	= Mililiter
mm	= Milimeter
mRNA	= Messenger Ribonucleic acid
NA	= Neuraminidase
NES	= Nuclear Export Signal
ng	= Nanogram
nm	= Nanometer
NP	= Nucleoprotein
NS	= Non structural
PA	= Polymerase acidic
OIE	= Office International des Epizooties
PB	= Binding buffer
PB1	= Polymerase basic 1
PB2	= Polymerase basic 2
PBS	= Phosphate Buffer Saline
PCR	= Polymerase Chain Reaction
PDRS	= Participatory disease surveillance response
PDS	= Participatory disease surveillance
PGS	= Potential glycosylation sites
RNP	= Ribonucleoprotein
qRT-PCR	= Real-Time quantitative reverse transcription PCR
RT-LAMP	= Reverse transcription loop-mediated isothermal amplification
S	= Second(s)
SA α 2,3-Gal	= Sialic acid linked to galactose by α 2,3 linkage
SA α 2,6-Gal	= Sialic acid linked to galactose by α 2,6 linkage
SPF	= Specific pathogenic-free
ssRNA	= Single stranded RNA

U	= Unit(s)
UV	= Ultra-violet
μ	= Micro
μg	= Microgram
μL	= Microliter
μm	= Micrometer
μM	= Micromolar
VLA	= Veterinary laboratories agency
WGS	= Whole genome sequence
WHO	= World health organization