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Genetic diversity of the H5N1 viruses in live bird markets, Indonesia

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ABSTRACT

Background: The live bird market (LBM) plays an important role in the dynamic evolution of the avian influenza H5N1 virus.
Objectives: The main objective of this study was to monitor the genetic diversity of the H5N1 viruses in LBMs in Indonesia.
Methods: Therefore, the disease surveillance was conducted in the area of Banten, West Java, Central Java, East Java, and Jakarta Province, Indonesia from 2014 to 2019. Subsequently, the genetic characterization of the H5N1 viruses was performed by sequencing all 8 segments of the viral genome.
Results: As a result, the H5N1 viruses were detected in most of LBMs in both bird' cloacal and environmental samples, in which about 35% of all samples were positive for influenza A and, subsequently, about 52% of these samples were positive for H5 subtyping. Based on the genetic analyses of 14 viruses isolated from LBMs, genetic diversities of the H5N1 viruses were identified including clades 2.1.3 and 2.3.2 as typical predominant groups as well as reassortant viruses between these 2 clades.
Conclusions: As a consequence, zoonotic transmission to humans in the market could be occurred from the exposure of infected birds and/or contaminated environments. Moreover, new virus variants could emerge from the LBM environment. Therefore, improving pandemic preparedness raised great concerns related to the zoonotic aspect of new influenza variants because of its high adaptivity and efficiency for human infection.

Keywords: H5N1 virus; live bird market; Indonesia; genetic diversity; reassortant viruses

INTRODUCTION

The live bird market (LBM) in developing countries such as Indonesia is a meeting point for sellers and buyers of poultry products and other necessities of life. Hence, LBMs are the most popular place for the community. LBM provides various goods of human needs such as basic food, eggs, meat, and poultry. LBM is generally located close to settlement where it is easily accessible to the public. It also offers price that tends to be cheaper compared with modern markets. However, LBM is one factor that could potentially be a source of the spread of diseases, including avian influenza (AI). This zoonotic disease can be transmitted from birds to other birds or even to humans.

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The live poultry in LBM is intermixed from different geographic locations resulting in a continuous virus circulation [1,2]. Several LBMs provide slaughter facilities on-site so the buyer can choose the desired poultry that can be slaughtered and cleaned in this facility. The vendor usually also keeps the birds in cages at the LBM until they are sold. Activities undertaken between sellers and buyers in the market have often caused the environment itself to become dirty. This condition becomes one of the factors for the contamination by the H5N1 virus which allows the transmission to humans. The spread of the AI viruses in the LBM environment is exacerbated by poor biosecurity implementation such as absence regular cleaning and disinfection as well as inappropriate waste disposal management [3,4].

The studies about LBM contamination by AI virus have been conducted in several countries such as China, Hong Kong, Vietnam, and Egypt [5-8]. The genetic materials and infectious viruses were detected predominantly at the LBM in Guangzhou and Bangladesh [9,10]. Meanwhile in Indonesia, environmental contamination and poultry infection in LBM by the H5N1 virus were also reported [11,12]. In addition, the LBMs have been suspected as a major source of human infection with AI viruses [3,13,14]. Numerous patients with illness due to H5N1 and H7N9 infection were caused by indirect exposure to infected birds or as a result of aerosol generated by poultry slaughtering process at markets [14-16]. Based on the report by the World Health Organization, Indonesia is the second number of the H5N1 human infection with up to 200 cases (168 mortality). This evidence should give more awareness of the virus's dynamic evolution.

The LBMs are a major source of AI virus transmission, mixing, and reassortment. Reassortment could occur for both high and low pathogenic viruses [17]. For instance, reassortant viruses of 3 genotypes (A, B, and C) were detected in both wild and duck in LBMs in China [18]. The novel H5N6 reassortant virus that contains a set of H9N2-like internal genes with high and low pathogenic AIV subtypes was identified circulating in domestic and wild birds [19]. Moreover, LBM can also become an important source of novel AI viruses.

Since 2012, 2 clade AI viruses have circulated in Indonesia, namely clade 2.1.3 and clade 2.3.2. Later, LBMs in Indonesia are suspected to have a major role in maintaining the H5N1 circulation, including both clades. Therefore, the objective of this study was to identify the circulation of the AI subtype H5N1 in the LBM area in Indonesia. In addition, this study also aimed to characterize the genetic diversity of the H5N1 virus circulating in LBMs by sequencing all 8 segments of the virus genome, particularly to identify the presence of reassortant viruses between clades 2.1.3. and 2.3.2.

MATERIALS AND METHODS

Sampling collection

Collection of swab environment and poultry performed on LBM selling and accommodating live poultry as well as poultry meat from 2014 to 2019 in large cities or towns/districts in the Province of Banten (Serang), Jakarta (East Jakarta), West Java (Cirebon and Cianjur), Central Java (Solo, Sukoharjo, Karanganyar, Semarang, Brebes, and Ambarawa) and East Java (Surabaya, Malang, and Lamongan). The environmental samples were taken from the floor, knife, chopping board, scales, de-feathering machine, table for poultry display, waste bin, and wet clothes for cleaning [12]. For the live bird, swabs were taken from birds' cloaca and pooled for the same species maximal 3 samples. The cloacal and environmental samples

were kept in the transport medium (Dulbecco's Modified Eagle Medium, GIBCO; Thermo Fisher Scientific, USA) and maintained in the cool condition during transportation to the Indonesian Research Center for Veterinary Science.

Virus identification

Cloacal swabs and environmental samples in the transport media were processed for viral RNA isolation using QIAmp Viral RNA Mini Kit (Qiagen, Germany) according to the manufacturers' instruction. The reverse-transcriptase polymerase chain reaction (RT-PCR) was performed to identify the influenza type A for the matrix gene [20]. Then, the positive samples were confirmed using the RT-PCR for H5 subtyping [11]. The positive samples for H5 subtyping were cultured in specific pathogenic free embryonated eggs 9–11 old days. The H5 positive allantoic fluid was processed for DNA sequencing. The sample preparation, RNA isolation, and virus isolation were conducted in the BSL-3 facility belonged to the Indonesian Research Center for Veterinary Sciences.

DNA sequencing and analysis of influenza virus genes

The amplification of 8 segments of H5N1 viruses (PB2, PB1, PA, HA, NP, NA, MP, and NS) was conducted by following the previous methodology [21]. The sequencing was performed by direct sequencing using Cycle sequencing kit (BigDye Terminator version 3.1; Applied Biosystems, USA) on Genetyx Analyzer 3130 (Applied Biosystems). Multiple alignments analysis was carried out by using BioEdit version 7. Later, phylogenetic trees were generated using maximum likelihood (1,000 replicates) in MEGA version 5.2.

RESULTS

The LBM profiles

In 2014–2019, the surveillance of the H5N1 virus at the LBMs was conducted in 21 LBMs in Indonesia, including Banten (2 LBMs), Jakarta (3 LBMs), West Java (2 LBMs), Central Java (10 LBMs) and East Java (4 LBMs). The largest LBM was selected that sells live poultry, provides slaughter services and sells poultry products. For example, LBM in Solo is very large because it sends poultry to other big cities of Indonesia such as Jakarta. LBMs in Surabaya, East Jakarta, Ambarawa, Brebes, and Malang are also large because they have cage facilities to keep unsold poultry or wait for the slaughtering process. The LBMs in Serang, Karanganyar, and Sukoharjo only sell poultry meat with no poultry slaughter services. For Lamongan and Semarang, LBM is close to the slaughterhouse that not only provides services within the market but also accepts live bird processing from particularly the neighboring areas. In general, the merchants usually transport the live poultry with modified motorcycles with baskets from wood or bamboo. The LBM profile in Indonesia is quite similar to other developing countries (**Fig. 1**).

Identification of H5N1 virus in the LBMs area

The results of market surveillance in **Table 1** showed that most of LBMs were contaminated with influenza virus for both birds' cloacal and environmental samples. On the one hand, the RT-PCR results showed that 22% of all bird pool samples were positive for the influenza A. From these positive influenza A samples, about 53% of samples were positive for H5 subtyping. On the other hand, 48% of all environmental pool samples were positive for influenza A. Thus, from these positive results, about 51% of samples were positive for H5 subtyping. Subsequently, 35% of overall pool samples were positive for influenza A, whereas 52% of these samples were positive for subtype H5.

Fig. 1. The general profiles of live bird markets in Indonesia. (A) The slaughter facility for poultry on site in the markets. (B) The modified motor cycle for poultry transportation. (C) The cages to keep poultry for temporary time. (D) The merchants who display chicken carcass or meat in the traditional market.

Our data results indicated that the environment of LBM was the highest place contaminated by the influenza virus. Several environmental sampling points namely knives, chopping boards, scales, display tables, wet cloths, de-feathering apparatus, waste bin, floor in slaughter area, and water run-off had nearly the same risk of contamination with influenza virus A. The most contaminated LBM occurred in Solo where about 80% of the pool samples were identified as influenza A and 60% of the positive samples were detected as subtype H5. The waste bin, display table for poultry, knives, and de-feathering apparatus are next points after the floor that gets massive virus contamination. Moreover, other cities in the region of East Java Province such as Malang, Surabaya, and Lamongan also had high-level virus contamination, either influenza A or subtype H5. Meanwhile, the LBMs in the other cities and provinces had a varied level of influenza A virus contamination from about 15% up to 72%.

The genetic characterization of H5N1 viruses from the LBMs

The results of the phylogenetic analysis of 14 viruses isolated in this study showed that there were 2 clades circulating in LBM in Indonesia, including clade 2.1.3 and 2.3.2 (**Fig. 2A**). Besides in the LBMs, these 2 clades have also circulated in the poultry population in Indonesia. The clade 2.3.2 viruses newly identified in 2012 seem to compete with the earlier predominant clade 2.1.3 [21]. On the one hand, the SLO.70, SLO.76, ML.19, and CIA.77 viruses are grouped with clade 2.1.3.2.a with the genetic characters similar to AI viruses isolated around 2013–2014. On the other hand, the other ten viruses namely SLO.33, SB.48, SRG.C1, LA.101, SM.32Muks, JTM.45, JTM.164, CI.37, BBS96, and Abr1, are included in the clade 2.3.2.1.c. These clade 2.3.2 viruses showed little genetic difference with clade 2.3.2 viruses isolated in 2012.

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Table 1. Identification of the H5N1 virus in the live bird markets, Indonesia, 2014–2019

Year	Province	City/district	Sample origin*	Total sample	Number of pool	Positive RT-PCR	
						Flu A	H5
2014	Banten	Serang	Birds	109	65	7	3
			Environment	40	40	18	8
2014	Central Java	Surakarta	Birds	63	12	5	1
			Environment	55	50	40	24
2014	Central Java	Karanganyar	Birds	30	6	2	0
			Environment	50	10	5	0
2014	Central Java	Sukoharjo	Birds	6	1	1	0
			Environment	60	12	6	0
2014	East Java	Surabaya	Birds	60	8	0	0
			Environment	74	74	45	13
2014	East Java	Malang	Birds	108	15	4	1
			Environment	56	56	22	16
2014	East Java	Lamongan	Birds	192	34	21	7
			Environment	34	32	23	10
2015	Central Java	Semarang	Birds	118	42	2	0
			Environment	50	50	13	9
2016	Jakarta	East Jakarta	Birds	68	23	2	2
			Environment	114	46	7	7
2017	West Java	Cirebon	Birds	30	30	11	11
			Environment	8	8	3	3
2018	West Java	Cianjur	Birds	82	82	8	8
			Environment	0	0	0	0
2019	Central Java	Brebes	Birds	118	39	13	7
			Environment	29	13	2	2
2019	Central Java	Ambarawa	Birds	109	32	13	8
			Environment	0	0	0	0
			Birds (%)†		389	87 (22)	46 (53)
			Environment (%)†		391	186 (48)	94 (51)
			Total (%)†		780	273 (35)	140 (52)

RT-PCR, reverse-transcriptase polymerase chain reaction.
*Birds (backyard, chicken, layer, broiler, duck, Muscovy duck, etc). Enviroment (floor, knife, chooping board, scales, de-feathering apparatus, table for poultry display, waste bin, and wet clothes); †Percentage for Flu A is calculated by dividing number of Flu A positive RT-PCR with number of pool, whereas percentage for H5 is calculated by dividing number of H5 positive RT-PCR with number of Flu A positive RT-PCR.

Moreover, 5 reassortant H5N1 viruses were identified, including SLO.70, SLO.76, CIA.77, BBS.96, and Abr1 (**Figs. 2**-**5**). The SLO.76 virus showed that HA and PB2 genes belong to H5N1 clade 2.1.3 with the 6 remaining genes that are included in the clade 2.3.2 group. The HA gene of SL0.70 and CIA.77 virus belong to clade 2.1.3, whereas the other 7 genes belong to clade 2.3.2. Although these 3 viruses were in same clade 2.1.3, they showed considerable variation evident among the clades. On the other hand, the matrix gene of BBS.96 virus belong to clade 2.1.3, whereas other 7 genes belong to clade 2.3.2. Meanwhile, the Abr1 virus showed that the matrix gene belongs to H5N1 clade 2.1.3 with the 6 remaining genes that are included in the clade 2.3.2; however, its PB2 gene does not belong to neither the clade 2.1.3 nor clade 2.3.2.

Analysis of cleavage site indicates that viral HA protein has multiple basic amino acids which are characteristic of highly pathogenic AI (**Table 2**). All the influenza viruses have the SA α-2,3-Gal receptor. The viruses also have a PDZ binding motif on NS protein indicated for avian origin. Moreover, the viruses also showed resistance to amantadine based M2 sequence. Most of them have no mutation on 626 amino acids of the PB2 gene and have truncated of PB1-F2 gene (**Table 2**).

The gene sequences that were obtained were submitted to the GISAID database with accession numbers as follows: (A/Environment/Central_Java/SLO.33/2014) EPI_ISL_409004:

Fig. 2. The phylogenetic trees of the H5N1 viruses were generated in MEGA 5.2 (maximum likelihood analysis and 1,000 bootstrap replicates) for HA (A) and NA gene (B). The characterized viruses in the study were indicated with triangle marking.

(PB2) EPI1683102, (PB1) EPI1683079, (PA) EPI1683069, (HA) EPI1683011, (NP) EPI1683051, (NA) EPI1683036, (MP) EPI1683027, (NS) EPI1683064; (A/Environment/Central_Java/SLO.70/2014) EPI_ISL_409003: (PB2) EPI1683093, (PB1) EPI1683081, (PA) EPI1683072, (HA) EPI1683010, (NP) EPI1683052, (NA) EPI1683034, (MP) EPI1683025, (NS) EPI1683061; (A/Environment/Central_Java/SLO.76/2014) EPI_ISL_409005: (PB2) EPI1683094, (PB1) EPI1683105, (PA) EPI1683073, (HA) EPI1683012, (NP) EPI1683053, (NA) EPI1683038, (MP) EPI1683028, (NS) EPI1683058; (A/Muscovy_duck/Banten.SRG.C1/2014) EPI_ISL_409006: (PB2) EPI1683088, (PB1) EPI1683083, (PA) EPI1683068, (HA) EPI1683013, (NP) EPI1683054,

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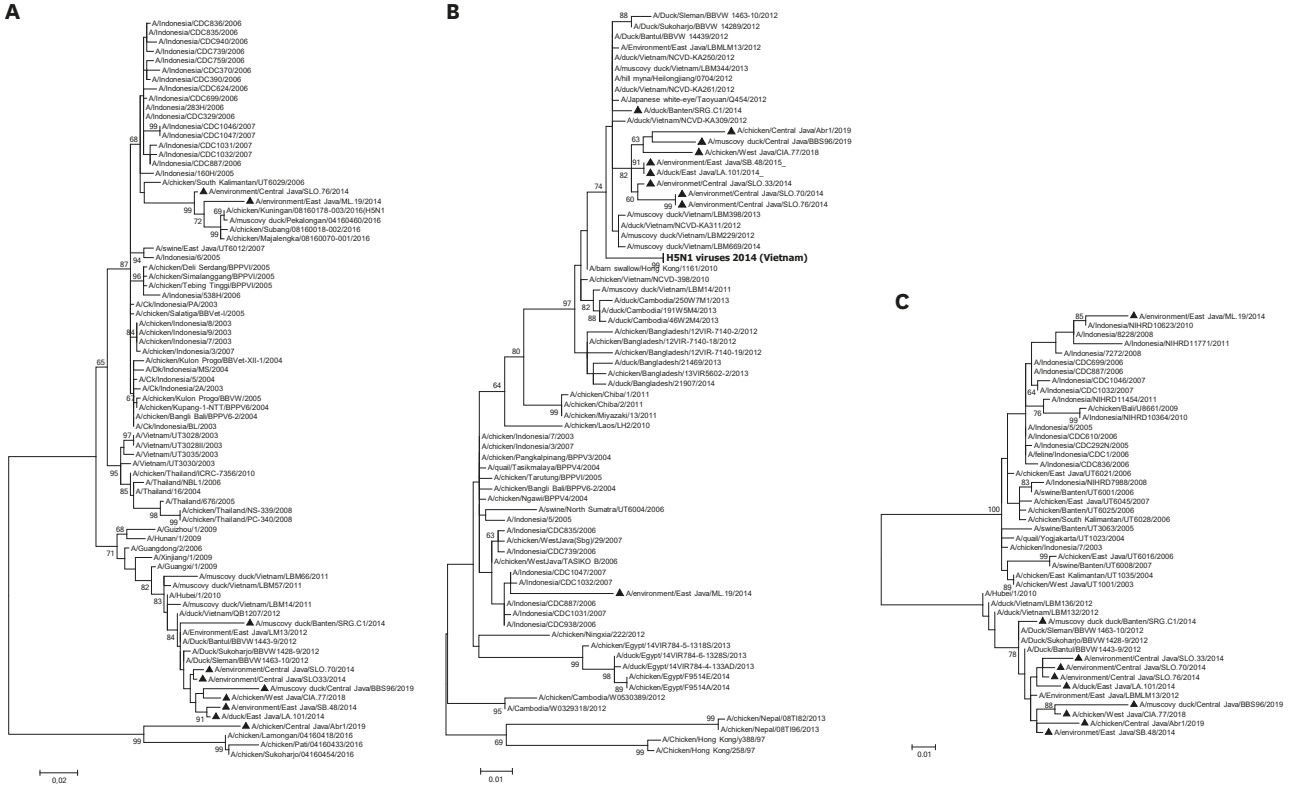

Fig. 3. The phylogenetic trees of the H5N1 viruses were generated in MEGA 5.2 (maximum likelihood analysis and 1,000 bootstrap replicates) for PB2 (A), PB1 (B), and PA gene (C). The characterized viruses in the study were indicated with triangle marking.

Fig. 4. The phylogenetic trees of the H5N1 viruses were generated in MEGA 5.2 (maximum likelihood analysis and 1,000 bootstrap replicates) for NP (A), MP (B), and NS gene (C). The characterized viruses in the study were indicated with triangle marking.

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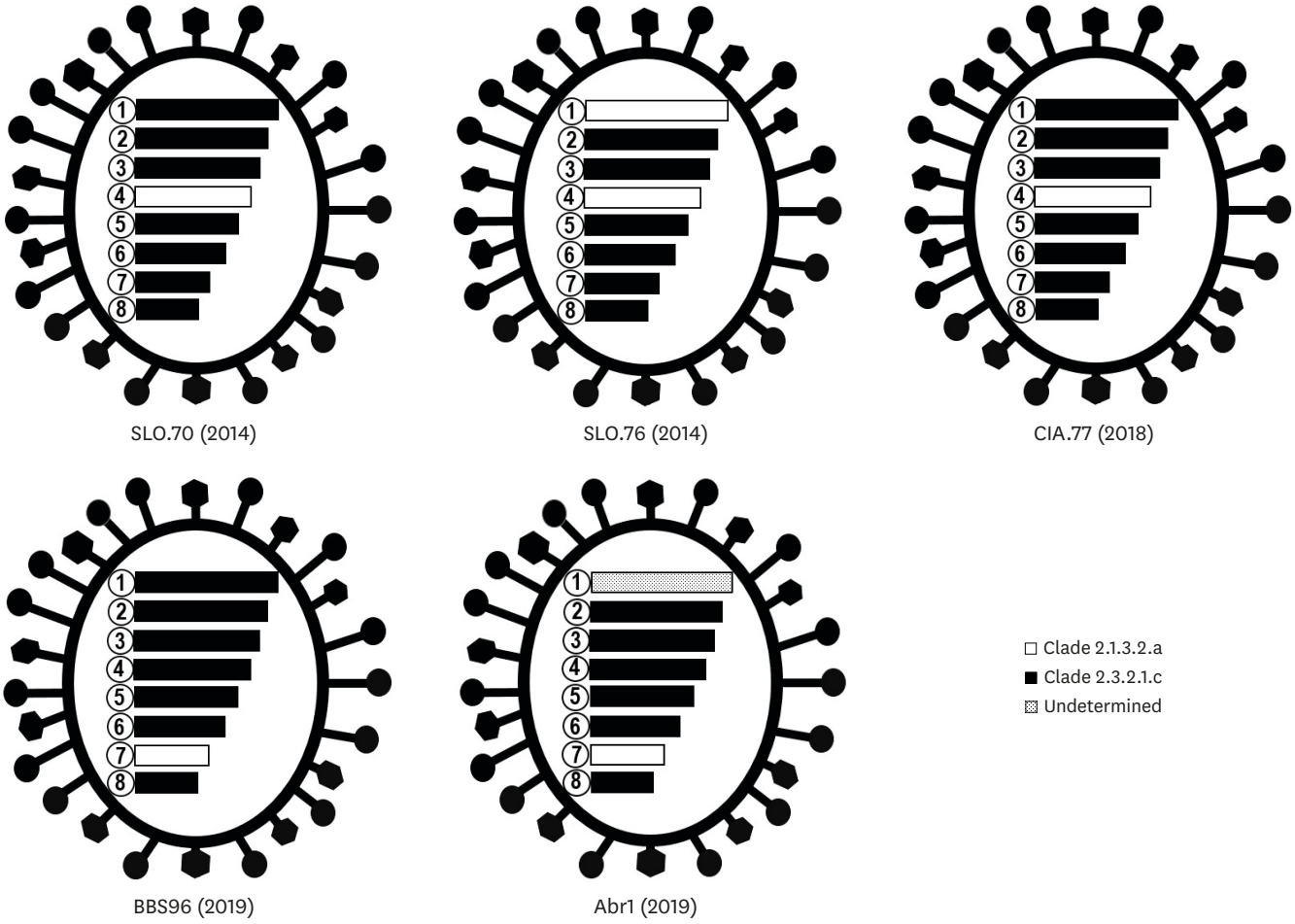

Fig. 5. The gene constellation of the Indonesian reassortant H5N1 viruses from live bird markets from 2014–2019 between clade 2.1.3 and 2.3.2. (1) PB2 gene, (2) PB1 gene, (3) PA gene, (4) HA gene, (5) NP gene, (6) NA gene, (7) MP gene, and (8) NS gene.

Table 2. The molecular characteristics of the H5N1 viruses from the live bird markets, Indonesia, 2014–2019

Isolate	City/district origin	Sample origin	Year	HA				NS		M2		PB2	PB1-F2
				Clade	222	224	Cleavage site	Deletion of amino acid 80 to 84	PDZ-binding ligand	27	31	627	
SLO33	Solo	Environment	2014	2.3.2.1.c	Q	G	PQRESRRKKR	YES	ESEV	V	S	E	57
SLO70	Solo	Environment	2014	2.1.3.2.a	Q	G	PQRERRRKR	YES	ESEV	I	S	E	57
SLO76	Solo	Environment	2014	2.1.3.2.a	Q	G	PQRERRRKR	YES	ESEV	I	S	E	57
SRGC1	Serang	Muscovy duck	2014	2.3.2.1.c	Q	G	PQRERRKR	YES	ESEV	I	S	E	57
SB48	Surabaya	Environment	2014	2.3.2.1.c	Q	G	PQRERRKR	YES	ESEV	I	S	E	57
LA101	Lamongan	Duck	2014	2.3.2.1.c	Q	G	PQRERRKR	YES	ESEV	I	S	E	57
ML19	Malang	Environment	2014	2.1.3.2.a	Q	G	PQRESRRKR	YES	ESEV	A	N	E	90
SM32Muks*	Semarang	Environment	2015	2.3.2.1.c	Q	G	PQRERRKR	-	-	-	-	-	-
JTM45*	Jakarta	Environment	2016	2.3.2.1.c	Q	G	PQRERRKR	-	-	-	-	-	-
JTM164*	Jakarta	Environment	2016	2.3.2.1.c	Q	G	PQRERRKR	-	-	-	-	-	-
Ci37*	Cirebon	Duck	2017	2.3.2.1.c	Q	G	PQRERRKR	-	-	-	-	-	-
CIA77	Cianjur	Chicken	2018	2.1.3.2.a	Q	G	PQRERRKR	YES	ESEV	V	S	E	57
BBS96	Brebes	Muscovy duck	2019	2.3.2.1.c	Q	G	PQRERRKR	YES	ESEV	A	N	E	57
Abr1	Ambarawa	Chicken	2019	2.3.2.1.c	Q	G	PQRERRKR	YES	GESEV	A	N	E	57

*The gene sequencings were performed to only HA and NA genes.

(NA) EPI1683035, (MP) EPI1683024, (NS) EPI1683059; (A/Environment/East_Java/SB.48/2014) EPI_ISL_409009: (PB2) EPI16830089, (PB1) EPI1683078, (PA) EPI1683109, (HA) EPI1683016, (NP) EPI1683048, (NA) EPI1683039, (MP) EPI1683026, (NS) EPI1683062; (A/Duck/East_Java/

LA.101/2014) EPI_ISL_409008: (PB2) EPI1683091, (PB1) EPI1683080, (PA) EPI1683070, (HA) EPI1683015, (NP) EPI1683049, (NA) EPI1683040, (MP) EPI1683030, (NS) EPI1683060; (A/Environment/East_Java/ML.19/2014) EPI_ISL_409007: (PB2) EPI1683090, (PB1) EPI1683084, (PA) EPI1683071, (HA) EPI1683014, (NP) EPI1683050, (NA) EPI16837, (MP) EPI1683029, (NS) EPI1683063; (A/Environment/Central_Java/SM.32Muks/2015) EPI_ISL_409016: (HA) EPI1683023, (NA) EPI1683047; (A/Environment/Jakarta/JTM.45/2016) EPI_ISL_409014: (HA) EPI1681683021, (NA) EPI1683045; (A/Environment/Jakarta/JTM.164/2016) EPI_ISL_409015: (HA) EPI1683022, (NA) EPI1683046; (A/Duck/West_Java/CI.37/2017) EPI_ISL_409013: (HA) EPI1683020, (NA) EPI1683044; (A/Chicken/West_Java/CIA.77/2018) EPI_ISL_409012: (PB2) EPI1683097, (PB1) EPI1683087, (PA) EPI1683077, (HA) EPI1683019, (NP) EPI1683057, (NA) EPI1683043, (MP) EPI1683033, (NS) EPI1683067; (A/Muscovy_duck/Central_Java/BBS96/2019) EPI_ISL_409011: (PB2) EPI1683096, (PB1) EPI1683086, (PA) EPI1683108, (HA) EPI1683018, (NP) EPI1683056, (NA) EPI1683042, (MP) EPI1683031, (NS) EPI1683066; (A/Environment/East_Java/Abr1/2019) EPI_ISL_409010: (PB2) EPI1683095, (PB1) EPI1683085, (PA) EPI1683075, (HA) EPI1683017, (NP) EPI1683130, (NA) EPI1683041, (MP) EPI1683032, (NS) EPI1683065.

DISCUSSION

The LBM usually sells live birds that are kept in the cages until sold. Several vendors in the LBM provide slaughter services, de-feathering and carcass cleaning for costumers. These activities can cause contamination of AI viruses in the LBM environment that becomes a source of virus transmission to human, particularly people working in or visiting the market [12]. Since LBM become a source of AI viruses, zoonotic transmission in the market could be happened directly from infected birds or indirectly from contaminated personnel, poultry products, and fomites [8,22].

The AI viruses could survive for such a long period of time in both porous and non-porous materials depending on the environmental conditions; however, the virus survival is longer in the non-porous material [23,24]. Moreover, the AI virus can remain viable on non-porous materials after contamination [24]. The virus still can be recovered in about 10^2–10^3 infectious particles up to 72 h after artificial contamination with AI virus subtype H13N7 with a dose of 6.3×10^6 tissue culture infection dose ($TCID_{50}$/mL) from several materials such as latex gloves, gumboots, cotton fiber, feathers, and plastics. The other study demonstrated that the infectivity of the H1N1pdm virus remained on the smooth nonporous surface for at least 7 days at 35°C and up to 66 days at 4°C and persisted in water and on the glass surface for extended periods of time, even at 35°C [25]. The infected materials either porous or nonporous become a source of viral transmission; thus, the virus remains circulating in larger areas of LBM. Furthermore, the AIV transmission mode via aerosol exposure was the most prominent route transmission for the H6N2 virus at the LBM, even though the virus in fecal exposure from infected birds was detected earlier than in birds with aerosol exposure only [26].

Like other RNA viruses, influenza viruses lack a proofreading activity in the replication of viral RNA that results in error-prone in transcription which leads to a high mutation rate. However, the virus mutation can also be occurred by reassortment between different influenza viruses because of the characteristic of the segmented genome. This study found 5 reassortant H5N1 viruses between clade 2.1.3.2a and clade 2.3.2.1.c confirmed by the full genome sequencing, namely SLO.70, SLO.76, CIA.77, BBS96, and Abr1. Several previous studies also confirmed the occurrence of the H5N1 reassortant virus either among or with other subtypes. For example, reassortant viruses between subtype H5N1 occurred in 24

of 25 human Indonesian H5N1 viruses [27]. The next study also found a reassortant H5N1 virus in Indonesia with the donor NS1 gene originated from the human H3N2 virus [28]. For comparison, the reassortant viruses from clade 1 were also identified from the H5N1 human cases in Vietnam [29]. In addition, the novel H5N6 reassortant virus containing H9N2-like internal genes was identified in both wild and domesticated birds in LBM [19].

In this study, reassortant H5N1 viruses occurred between clade 2.1.3 circulating in Indonesia since 2003 and new clade 2.3.2 circulating since late 2012. The discovery of 5 reassortant H5N1 viruses provides evidence for the LBM role as a hot spot for the emergence of new virus variants because a wide range of influenza viruses may circulate simultaneously. The emergence of the new variant influenza A viruses needs great concern because it can affect existing AI disease control policies such as surveillance programs, vaccination, and identification/detection disease methods. However, the bigger concern is the zoonotic aspect, when a new AI virus variant becomes more adaptive and efficient for infecting humans. For example, past human influenza pandemics were caused by reassortant viruses. The ample evidence of is the emergence of a novel avian-origin influenza A(H7N9) virus reassorting from 3 parental viruses, namely H7N3 (ZJ12-like), H7N9 (KO14-like), and H9N2 (BJ16-like) [30]. Despite the H7N9 virus has low pathogenicity characteristics, this reassortant virus can infect humans with severe causalities.

Birds may secrete the virus for 7–10 days and the virus can be maintained for a longer time in a large population such as in chicken farms, in the backyard, or in LBM [31]. Under appropriate environmental conditions, AI virus can survive for more than 6 months causing continuous disease transmission [22]. The environmental aspect of LBMs is essential for an AI control program. Firstly, a contaminated environment becomes a source of disease transmission for the bird population as well as for the community in the market. Secondly, environmental sampling in LBM is more likely to be favorable to traders/vendors and field officers rather than invasive bird sampling that may be infected with the influenza viruses [12].

These study findings provided ample evidence that the contamination of the H5N1 viruses is common in the LBM environment. Table for poultry display, chopping board, knife, de-feathering apparatus, the floor in slaughter area, waste bin, scale, and wet clothes were all contaminated. The virus contamination may be originated from droplets generated from the slaughtering process, de-feathering, and carcass cleaning of infected birds, especially internal organs with potentially high viral loads. Furthermore, the opportunity of human exposure for acquiring AIV infection at LBM is quite high as proposed in the previous study [22].

In this study, we rarely detected positive samples from birds. On the other hand, more positive samples came from the LBM environment. Moreover, the rate of virus isolation is lower than the RT-PCR positive result. The rationale for this evidence because virus isolation only detects the viable virus, whereas RT-PCR can detect the genetic material of the virus, even from the inactive virus. A load of viruses in samples, duration, and temperature of exposure, and humidity in the LBM environment are several factors that can influence the virus isolation rate.

The LBMs are considered to the main pathways for disease transmission because they provide a condition for virus amplification, interspecies transmission, and virus reassortment, especially for H5N1 viruses. The evolution of H5N1 viruses in a certain region is influenced by geological properties such as migratory bird staging areas, river networks, LBMs, literacy rate, poultry density, road networks and household density [32]. In this study, the H5N1

surveillance in the LBMs was conducted in 13 cities/districts with divergent topography features with a long time of observation. Thus, the genetic diversity of the Indonesian H5N1 virus can be observed in virus mutation and evolution.

The H5N1 virus has been detected from both the animal and environment in the LBM where the viruses circulated silently becoming risk factors for human infection. There was an H5N1 human infection when the victim visited LBM with his relative [33]. A high prevalence of virus infection in LBMs could occur if the virus is amplified within the market following the introduction of a single infectious bird [1]. The infected birds may be sold and slaughtered before showing any clinical signs or dying from the infection. These conditions cause virus circulations to remain silent, so the LBM environment is continuously contaminated. As a consequence of virus amplification in LBMs, the risk of poultry-to-human infection is also increasing [34].

The risk factors related to the zoonotic aspect of the H5N1 virus identified in this study provide important consideration in developing interventions to reduce the burden of virus contamination in LBMs. The influenza virus can be inactivated by various commercial disinfectants, even with detergents. Therefore, the combination of regular cleaning and periodic market rest day is effective to eliminate the AI virus contamination from LBMs [35,36]. When keeping live birds overnight in LBMs is banned, the rate of virus isolation is declined.

The LBM is known as an important place for maintaining and changing the AI virus, including the H5N1 virus [37]. Circulation of the H5N1 virus on the market is usually associated with the epidemic or increased mortality in poultry in the market [38]. However, this study showed no such correlation. The H5N1 virus silently circulated and replicated in LBM representing variants of the virus circulating in Indonesia. The diversity of types and species of birds such as chicken, duck, and Muscovy duck, which are sold in LBM facilitates virus reassortment. Furthermore, LBMs have a potential role for occupational exposure and interspecies transmission to create the novel reassortant of AI viruses [16]. Since the reassortant or novel strains are potential for pandemic influenza, the genetic measures to monitor the endemicity of AI viruses remain as key elements of pandemic preparedness.

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