Research Report

IDENTIFICATION OF INFLUENZA VIRUSES IN HUMAN AND POULTRY IN THE AREA OF LARANGAN WET MARKET SIDOARJO-EAST JAVA, INDONESIA

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ABSTRACT

Background: Influenza is a viral infection that attacks the respiratory system (nose, throat, and lungs) that commonly known as "flu". There are 3 types of influenza viruses, such as type A, type B, and type C. Influenza virus type A is the type of virus that can infect both human and animals, virus type B are normally found only in human, and Influenza virus type C can cause mild illness in human and not causing any epidemics or pandemics. Among these 3 types of influenza viruses, only influenza A viruses infect birds, particularly wild bird that are the natural host for all subtypes of influenza A virus. Generally, those wild birds do not get sick when they are infected with influenza virus, unlike chickens or ducks which may die from avian influenza. Aim: In this study, we are identifying the influenza viruses among poultry in Larangan wet market. Method: Around 500 kinds of poultry were examined from cloacal swab. Result: Those samples were restrained with symptoms of suspected H5. The people who worked as the poultry-traders intact with the animal everyday were also examined, by taking nasopharyngeal swab and blood serum. Conclusion: Identification of influenza viruses was obtained to define the type and subtype of influenza virus by PCR.

Key words: *subtype of influenza viruses, human, poultry, symptoms, PCR result*

ABSTRAK

Latar belakang: Influenza merupakan infeksi virus yang menyerang sistem pernafasan (hidung, tenggorokan, dan paru-paru), biasa dikenal sebagai "flu". Terdapat 3 tipe virus influenza yaitu tipe A, tipe B, dan tipe C. Virus influenza tipe A adalah jenis virus yang dapat menginfeksi manusia dan hewan, virus influenza tipe B normalnya hanya ditemukan pada manusia, sedangkan virus influenza tipe C dapat menyebabkan sakit ringan pada manusia tanpa mengakibatkan kasus epidemi maupun pandemi. Dari ketiga tipe virus influenza tersebut, hanya tipe A yang dapat menginfeksi hewan jenis burung, terutama burung liar yang secara alami merupakan host dari semua subtipe virus influenza A. Secara umum, burung liar tersebut tidak akan menjadi sakit ketika terinfeksi virus influenza, tidak seperti pada ayam mapun bebek jika terinfeksi kemungkinan besar dapat mati. Tujuan: Dalam penelitian ini, kami mengidentiifikasi virus influenza pada unggas di pasar tradisional di Larangan, Sidoarjo. Metode: Sekitar 500 unggas diidentifikasi berdasarkan hapusan kloaka. Hasil: Pengambilan sampel tersebut disesuaikan dengan gejala yang muncul pada hewan itu terkait dengan gejala virus H5. Selain itu, kami juga mengumpulan sampel dari responden yang merupakan pekerja di pasar Larangan. Sampel responden berupa hapusan hidung dan serum darah. Kesimpulan: Identifikasi virus influenza dilakukan untuk melihat tipe dan subtipe virus berdasarkan PCR.

Kata kunci: *subtipe virus influenza, manusia, unggas, gejala, hasil PCR*

INTRODUCTION

Influenza virus type A can infect humans and animals. Various subtypes of this influenza A virus which usually attack human are H1N1, H1N2, and H3N2 (Rendell et al., 2006). Meanwhile several other influenza A types of attacking animals like H7N9, H5N1, or H3N2. Only this influenza A virus that attacks poultry which actually attacking domestic birds. Human infections with avian influenza (AI or "bird flu") are rare but occur most commonly after exposure to infected poultry (bird to human spread). HIN1 is a flu virus that was first detected in 2009 called as "swine flu", caused a world wide pandemic. Currently the HIN1 is a seasonal influenza virus found in humans and it is now also circulates among pigs. In 2010, even though World Health Organization announced that the pandemic was over, H1N1 flue virus is still circulating (Corzo et al., 2013).

Recently, there is a new type of influenza virus. H7N9 is a new subtype of avian influenza that has been reported to be detected in poultry in China. However no cases of H7N9 outside China have been reported yet and no sustained person-to-person spread of the H7N9 virus has been found at this time.

H5N1 is a highly pathogenic avian flu virus that caused serious outbreaks in domestic poultry in parts of Asia and the Middle East (WHO, 2012). Although H5N1 does not usually infect humans, nearly 600 cases of human cases of H5N1 have been reported from 15 countries since 2003 in Asia, Africa, Europe, and the Near East. About 60% of these people died from their illness. In 2011, 62 human with H5N1 cases and 34 deaths were reported from five countries— Bangladesh, Cambodia, China, Egypt, and Indonesia. Six countries— Bangladesh, China, Egypt, India, Indonesia, and Vietnam—have widespread and ongoing infections in their poultry. In 1997 an outbreak of H5N1 occurred in the farms and traditional markets in Hong Kong. For the first time reported that the H5N1 virus can infect human with the number of deaths of 6 to 18 cases. Poultry outbreaks also happen in other countries recently as well. Most human cases of "highly pathogenic" H5N1 virus infection have occurred in people who had recent contact with sick or dead poultry that were infected with H5N1 viruses. However, unlike other types of flu, H5N1 usually does not spread between people and no further evidence discovers that this virus can spread easily between people. Thus, the symptoms and possible complications of H5N1 in people can include fever, cough, shortness/difficulty breathing leads to respiratory failure, or pneumonia (Iskander et al., 2013).

Markets in Indonesia are the center of social and economic activities, but the market can also be a source of spread of diseases. A number of outbreaks of the disease at this time can even be transmitted through food products and living animals that are sold in the market. Traditional animal market needs special attention due to the occurrence of direct contact between wild birds carrying the virus of avian influenza (AI) in poultry and human (the poultry traders or buyers).

Weak bio-security and poor hygiene and sanitation lead to the spread and transmission of AI virus in poultry markets. Survey on wild birds around the Larangan wet market Sidoarjo has been conducted and showed that the wild birds infected with avian influenza virus H5N1 (Poetranto, 2011). There were highly possibilities of transmission of H5N1 virus from wild birds to poultry sold in the market and to the people works in that market. Therefore the Influenza study group, Institute of Tropical Disease Airlangga University was planning to hold Surveillance Influenza virus in traditional community animal market, the Larangan wet market Sidoarjo.

The aims of the study are to detect any potential transmission of AI virus in the traditional animal Larangan wet market, and also to detect the presence of AI virus in poultry trade and wild birds around the traditional market. This study is only an identification project to obtain early detection of transmission of AI virus among wild birds to poultry and the impact to those who works as poultry traders. Furthermore, the early detection could be useful for surveillance of influenza planning.

MATERIALS AND METHODS

There were 3 sample activities in this study. The first one is the sampling on human. Some general health assessment was carried out by checking the condition of 63 poultry traders. Physical examination and nasal swab sampling was taken during the study. The second sampling was on the poultry. The types of poultry sell in Larangan market was variety, such as chicken and duck. During the study, examination on the poultry in the market was carried out, especially those that showed the symptoms of influenza. Nasal swab and cloacae swab was taken from around 350–400 poultry. And the last sampling was on wild birds and poultry around the market. The same examination was held, and cloacae swab was also taken from approximately 50–100 wild birds and poultry.

The 63 nasal swabs were taken with cotton swab tube. Each of the tube were given 2 ml 5% BSA-BHI (*Bovine Serum Albumin – Brain Heart Infusion*), and mixed by vortex twice. After all samples ready, the next step is filtration which were done inside the Bio-Safety Cabinet (BSC), with 5 ml syringe and sterilized filter, then collect the filtrate in 1.5 ml tube. These filtrates samples were inoculated in monolayer of MDCK cells. These samples were incubated at 33–35° C during 3–7 days. Daily CPE (*Cytopathic Effect*) was observed. MDCK cells were chosen considering their better virus sensitivity (possible positive samples). After that, the fluids were harvested for HA test. The positive samples were submitted to the serologic test such as the Influenza Rapid Test, and also evaluated with Haemagglutination test (HA) as described in WHO Laboratory's method (2007).

Around 500 animal samples with nasal and cloacae swab were collected. The samples were treated similar as human samples with the filtration procedures. After that, those samples were inoculated using 9–10 days old

embryonated hen's eggs. The treatment of animal samples was performed in the BSL3 laboratory according to the CDC guidelines (WHO, 2011).

After the samples has been harvested, and show HA test positive, the samples are extracted into RNA and followed by cDNA synthesis. The final product will be cDNA, and ready to use or storage at -20° C until the Polymerase Chain Reaction (PCR) techniques was performed.

PCR was performed using primer forward (F) and reverse (R). Because this is an identification study, the primers that we applied were: H1(F) – H1(R), H3(F) – H3(R), and H5(F) – H5(R). PCR Reaction for each sample: 2.5 μl cDNA were amplified in a volume of 25 μl containing 12.5 μl premix, 8 μl dilute water (DW), and 1μl primer (10 pmol) for each primer forward and primer reverse. This reaction mixture was then heated in the PCR machine for 3 hours 19 minutes, with the thermal cycler of 40 cycles as follows: 94° C for 2 minutes, 94° C for 30 second, 50° C for 40 second, 72° C for 2 minutes, and 72° C for 10 minutes. The mixture then held at 4° C for indeterminate period until the heat cools down. The amplified PCR product was analyzed by electrophoresis (ELP) on 1.5% agarose gel at 100 V for about 40 minutes. The bands were stained with 2 μl/Ml ethidium bromide, documented by Gel Documentation System.
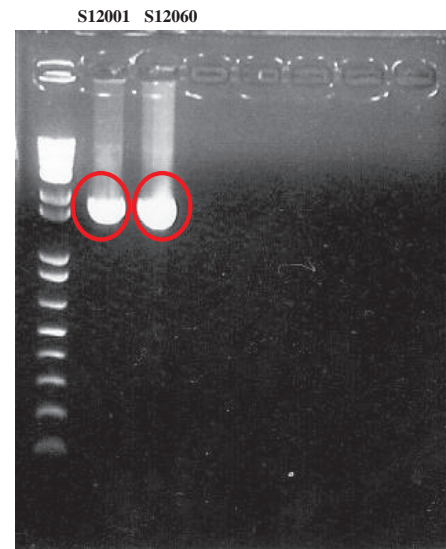
RESULT AND DISCUSSION

Human Samples

Among 63 people who worked as a poultry traders in Larangan market, several symptoms of influenza-like-illness (ILI) was identified such as cough, heavy breathing, arthritis, and diarrhoea. The samples were categorized by age and symptoms. Approximately around 36.5% samples were in the productive age between 31–40 years old, and only 6.3% were above 60 years old. Several symptoms also have been identified. Those symptoms are showed in the figure below.

The most common symptoms were coughing and heavy breathing, which appeared in all age categories. However, only among people age 51–60 years old that have other symptoms as arthritis and diarrhoea. And surprisingly, those who are above 60 years old did not have any symptoms of ILI even though they also working as poultry traders. This might relate on the length of working and how often they spent the time around the market, as well as how the antibody of the person who might resistant to the

Picture 1. The H3 result of Larangan human sample

influenza virus. Those are the limitation of this study, that the information on the length of working and the antibody serum were not collected.

Of these human samples, the serologic test has been taken using Influenza rapid test. The results were identified as influenza type B. As the PCR reaction was conducted, only 2 samples (3.17%) were identified of positive H3 (with 1000 base pair), as showed below:

ANIMAL SAMPLE

About 500 poultry samples were collected from the market and the area near the market. Several kinds of poultry and wild birds were identified with symptoms of influenza virus (particularly H5) are described below.

The HA test was conducted and the titter results using chicken RBC (HA ck+) and guinea pig RBC (HA gp+), and also using the diagnostic kit (dx +) that showing possible positive outcome. The diagnostic kit result was identified as influenza type A However, based on the assessment on the egg conditions after inoculation, it showed a relatively high number of possible positive results especially on 24 and 32 hpi (post infection).

Further assessments were attained with PCR technique using H5 primer. There were two steps of PCR to identify the types of protein on the surface. The first one is to

Table 1. Age category of human sample and the symptoms

Age	Cough	Arthritis	Diarrhoea	Heavy breathing
< 20–30 years old	21%	-	-	21%
31–40 years old	11%	-	-	11%
41–50 years old	9.7%	-	-	9.7%
51–60 years old	9%	4.5%	4.5%	9%
> 60 years old	-	-	-	-

Table 2. Poultry category with symptoms

Poultry category	With symptoms
Broiler chicken	45.5%
Backyard chicken	46.5%
Duck	5.9%
Migrant bird	1.0%
Owl	1.0%
Total	100.0%
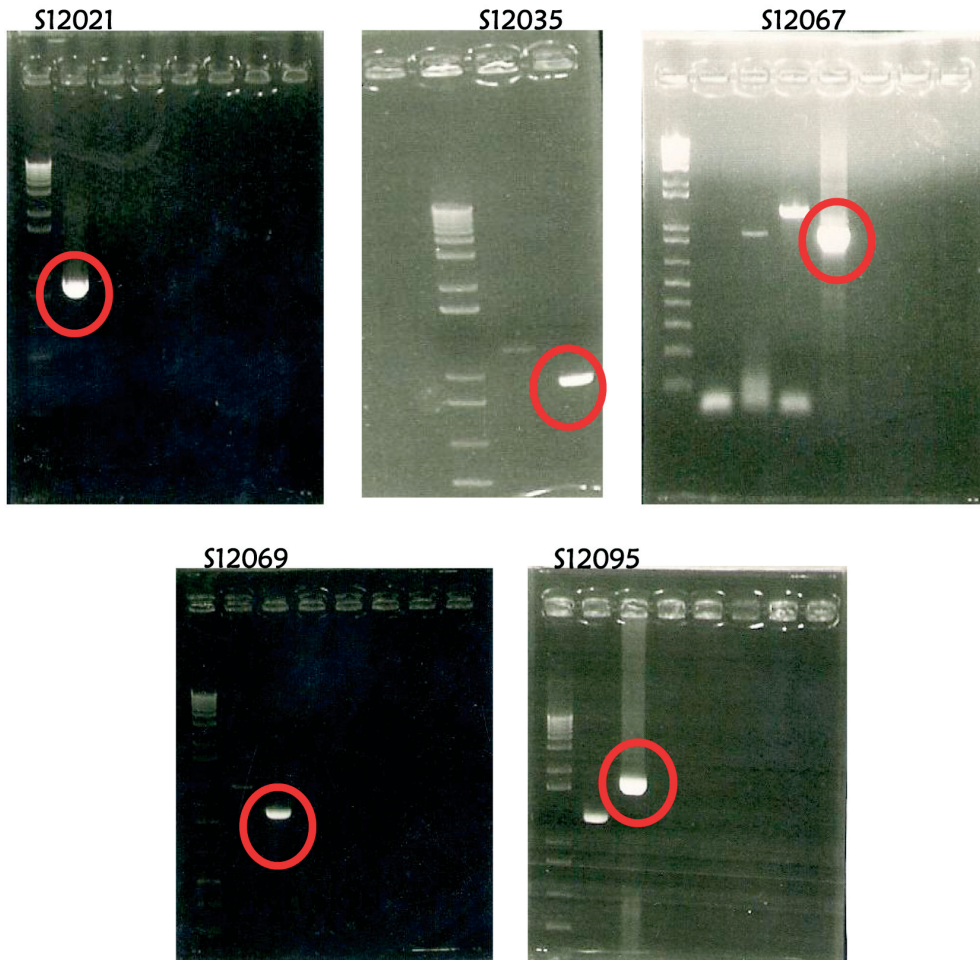
Picture 2. PCR result of HA

identify Hemagglutinin (HA) by using H5 primers. And the second one is to identify Neuraminidase (NA) by using H5N1 primer.

The H5 results are identified positive when the marks showed on 1.500 bp (base-pair). Only 5 samples were identified positive result H5, specifically the types of H5N1. The PCR outcome of HA types are illustrated as follow.

The PCR outcome of NA types are described below.

The NA results are identified positive when the marks showed on 1.500 bp (base-pair). Based on 5 positive sample of H5, we can identify the NA result as positive, specifically the types of H5N1.

Picture 3. PCR result of NA

CONCLUSION

There are some limitations on this study. For human sample, there are no information on the length of a person working in the market, the anthropometry measurement to check the nutrition aspect, the immune serum to identify the immune system and the antibody. As well as human sample, the animal sample also have are no specific assessments held, especially for the phylogenetic analysis to check any mutations occur.

For the next study, it will better if the anybody serum also taken to check the level of immunity. The anthropometry measurement and nutrition assessment also needed to be carried out to identify the nutrition condition related to the immune system. Furthermore, examinations such as sequencing need to be conducted, as well as the phylogenetic analysis. And last but not least, it is better to start developing a pandemic influenza planning as a result of the surveillance activity.

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