Veterinary Microbiology 186 (2016) 52–58

Contents lists available at ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

Molecular analysis of hemagglutinin-1 fragment of avian influenza H5N1 viruses isolated from chicken farms in Indonesia from 2008 to 2010

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ARTICLE INFO

Article history:
Received 10 October 2015
Received in revised form 15 February 2016
Accepted 23 February 2016

Keywords:
Avian influenza virus
H5N1
Chicken industry
Indonesia
Vaccine

ABSTRACT

Highly pathogenic avian influenza virus of subtype H5N1 (AIV-H5N1) has been circulating in Indonesia since 2003. To understand the genetic diversity of these viruses, and to predict vaccine efficacy, the hemaglutinin-1 (HA-1) fragment of viruses isolated from chicken farms in Indonesia from 2008 to 2010 was sequenced and analyzed. The effects of these molecular changes were investigated in challenge experiments and HI assays of homologous and heterologous strains. Molecular analysis showed that these AIV-H5N1 isolates had evolved into three distinct sub-lineages from an ancestor circulating since 2003. Although no significant positive selection of residues was detected, 12 negatively selected sites were identified ($p < 0.05$). Moreover, four sites showed evidence of significant episodic diversifying selection. The findings indicated complete protectivity and high HI titers with homologous strains, compared with protectivity ranging from 40 to 100% and lower HI titers with heterologous strains resulting from polymorphisms at antigenic sites. Our findings provide valuable insight into the molecular evolution of AIV and have important implications for vaccine efficacy and future vaccination strategies.

1. Introduction

Highly pathogenic avian influenza virus of H5N1 subtype (AIV-H5N1) has been circulating in Indonesia since 2003. This has led to a countrywide epizootic and the death of millions of birds, either due to infection or culling to reduce the spread of the virus. AIV-H5N1 is also responsible for human fatalities, with Indonesia having the highest fatality rate in the world, until a recent increase in the number of human cases in Egypt (WHO, 2015). Human infection is believed to result from transmission from infected poultry, as around 80% of cases in Indonesia have been linked with direct or indirect contact with sick poultry (Kandun et al., 2008).

Investigations into molecular changes in AIV-H5N1 circulating at commercial poultry farms, so-called sector 1, 2, and 3 facilities according to the FAO definition (FAO, 2013), are lacking. Most reports on AIV-H5N1 have dealt with domestic poultry from live bird markets (which may include some poultry from intensive farms) or birds from backyard settings, and data on the commercial poultry industry are scarce. Intensive poultry production is a huge industry worldwide and will play a critical role in the spread and enhancement of the pathogenicity of AIVs (Olsen et al., 2006). An intensive poultry farm with a high degree of genetic uniformity between birds provides the optimum conditions for viral mutation and transmission. The cases in backyards and wild birds could represent propulsion from intensive farms, as has been found at Poyang Lake, southern China (Chen et al., 2006). Biosecurity is a major issue in controlling diseases such as that caused by AIV, and inevitably breaches in biosecurity occur across the world as a result of the mass movement of people, materials and vehicles internationally. However, despite the need for surveillance, access to large commercial poultry farms is often strictly limited in Indonesia (Daniels et al., 2013) and other countries. Therefore, information on AIV-H5N1 in the integrated poultry enterprise sector is not publicly available.

To control AIV-H5N1 in poultry, vaccination has been implemented in Indonesia. Various vaccine seeds have been registered, including a homologous seed based on H5N1 isolates, and heterologous seeds based on subtypes H5N2 and H5N9, prior to

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http://dx.doi.org/10.1016/j.vetmic.2016.02.023
0378-1135/

2012. In 2012, the Livestock and Animal Health Authority issued a directorate decree, which allowed only the homologous vaccine seeds to be used in Indonesia. Vaccination is claimed to have a masking effect on influenza evolution, as well as being a driving force for antigenic drift (Webster et al., 2006). To understand the molecular evolution of AIV, and to predict vaccine efficacy, analysis of the HA-1 fragment of the hemagglutinin of AIV-H5N1 provides valuable information. HA-1 is the primary target for neutralizing antibodies and responsible for host receptor specificity; it also harbors the pathogenic domains of the virus (Horimoto and Kawaoka, 2001; Webby and Webster, 2001). Therefore, HA-1 analysis may also provide important information on possible adaptations in circulating viruses that may facilitate human transmission. Here we report the results of ongoing efforts to analyze molecularly AIV-H5N1 isolated from chicken farms in Indonesia.

2. Methods

2.1. Safety procedures

All work with infectious material was carried out in a Bio-safety Class III facility (PT Medion, Bandung, Indonesia). RNA isolation and RT-PCR were performed in the Animal Biomedical and Molecular Biology Laboratory of Udayana University, Denpasar, Bali, and the Research and Development Laboratory of PT Medion, Bandung, Indonesia. Sequencing was performed at the Research and Development Laboratory of PT Medion, Bandung, Indonesia. Ethical clearance for the study involving animal samples and experiments was granted by the Ethics Commission for the Use of Animals in Research and Education of the Faculty of Veterinary Medicine Udayana University, Bali, Indonesia, in accordance with the Terrestrial Animal Health Code of the World Organization for Animal Health.

2.2. Virus transport and propagation

Collection, transport, and propagation of field specimens were carried out in accordance with the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2004 (FAO, 2014; WHO, 2002). The supernatant fluids of tissue specimens or fecal swabs were obtained by centrifugation at 1000g and ultrafiltration. The supernatants were then inoculated into the allantois sac of five fertile 10-day-old specific-pathogen-free (SPF) chicken eggs. The eggs were incubated at 37 °C for 5 days. Eggs containing dead or dying embryos and all eggs remaining at the end of the incubation period were first chilled to 4 °C and then the allantois fluids were harvested. The clinical and epidemological history of each isolate is presented in Table 1.

2.3. Hemagglutination (HA) and hemagglutination inhibition (HI) assay

The allantois fluids were tested for HA activity. In the HA assay, 25 μL of allantoic fluid were titrated in two-fold serial dilutions and reacted with 1% chicken red blood cells. In the HI assay, 0.25 μL positive control sera (Animal Biomedical and Molecular Biology Laboratory, Udayana University, Bali) were two-fold serially diluted in PBS, then an equal volume of 4HA units of allantois fluid or control virus were added and incubated at room

Table 1
Characteristics of the AIV subtype H5N1 isolates from Indonesia, isolated from 2008 to 2010, and the farms from which they originated.

No	Simplified isolate name	Estimated population size	Type of farm	Vaccination history	Age of outbreak (weeks)
1	West Java/M04/2008	50.000	Layer	Vaccinated	Unknown
2	West Java/M05/2008	100.000	Layer	Vaccinated	Unknown
3	West Java/M06/2008	100.000	Breeder	Vaccinated	Unknown
4	West Java/M07/2008	50.000	Layer	Vaccinated	Unknown
5	East Java/M08/2008	10.000	Layer	Vaccinated	70
6	East Java/M09/2008	10.000	Broiler	Unvaccinated	Unknown
7	North Sumatra/M10/2008	40.000	Layer	Vaccinated	Unknown
8	East Java/M11/2008	50.000	Layer	Unknown	87
9	East Java/M12/2009	10.000	Broiler	Unvaccinated	4.71
10	West Java/M13/2009	30.000	Layer	Unvaccinated	6.14
11	East Java/M14/2009	10.000	Broiler	Unvaccinated	4.86
12	Banten/M15/2009	50.000	Broiler	Vaccinated	3.71
14	West Java/M16/2009	100.000	Broiler	N/A	4.57
15	East Java/M17/2009	100.000	Broiler	N/A	4.14
16	Central Java/M18/2009	10.000	Broiler	Vaccinated	3.5
17	East Java/M19/2009	10.000	Broiler	N/A	5
18	Central Java/M20/2009	50.000	Broiler	N/A	4.86
19	South Sulawesi/M21/2009	10.000	Broiler	No	3.5
20	Central Java/M22/2009	10.000	Broiler	No	4.29
21	East Java/M23/2009	100.000	Layer	Vaccinated	58
22	West Java/M24/2009	50.000	Broiler	No	4.86
23	West Java/M25/2009	N/A	Broiler	Vaccinated	4.57
25	Bali/M26/2009	5.000	Broiler	No	4
24	North Sumatra/M27/2009	N/A	Layer	Vaccinated	3.86
26	South Kalimantan/M28/2010	10.000	Broiler	No	5
27	South Kalimantan/M29/2010	100.000	Broiler	No	4
28	West Java/M30/2010	20.000	Broiler	Vaccinated	3.71
29	Central Java/M31/2010	10.000	Broiler	Vaccinated	5.71
30	Central Sulawesi/M32/2010	10.000	Layer	Vaccinated	10
31	South Sumatra/M33/2010	10.000	Broiler	No	3.29
32	West Java/M34/2010	N/A	Breeder	Vaccinated	32
33	West Java/M35/2010	100.000	Layer	Vaccinated	20