Original Article

Avian Influenza Virus (H5N1) Inactivation by Binary Ethylenimine

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Abstract

Avian influenza virus (AIV) subtype H5N1 is known as a highly pathogenic agent for man and various kinds of animals, especially avian species. Herein, AIV H5N1 was inactivated by binary ethylenimine (BEI), an aziridine compound, produced from 2-bromoethylamine hydrobromide. BEI was used at concentrations of 0.001 and 0.01 M for AIV inactivation. At the lower concentration (0.001 M), AIV could be inactivated within 24 hr. At the higher concentration (0.01 M), AIV could be inactivated within 6 hr. After inactivation, no hemagglutination activity had been found meaning that AIV had been completely inactivated.

Keywords: avian influenza virus subtype H5N1, binary ethylenimine, hemagglutination activity, virus inactivation

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บทคัดย่อ

การทำลายฤทธิ์ของไวรัสไข้หวัดนก (เอช 5 เอน 1) ด้วยไบนารี เอธิลลินิมิน

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ไวรัสไข้หวัดใหญ่สัตว์ปีก ซับไทป์ เอช5เอน1 เป็นไวรัสก่อโรครุนแรงในมนุษย์และสัตว์หลายชนิด โดยเฉพาะสัตว์ปีก ในการทดลองนี้ ไวรัสไข้หวัดใหญ่สัตว์ปีก ซับไทป์ เอช5เอน1 ถูกทำให้หมดฤทธิ์ด้วยด้วยไบนารี เอธิลลินิมิน ซึ่งเป็นสารประกอบอะซิริดิน ผลิตจาก 2-bromoethylamine hydrobromide ไบนารี เอธิลลินิมิน ใช้ในความเข้มข้นที่ 0.001 และ 0.01 โมลาร์ ในการทำให้ไวรัสไข้หวัดใหญ่สัตว์ปีกหมดฤทธิ์ ความเข้มข้นต่ำ (0.001 โมลาร์) ไวรัสไข้หวัดใหญ่สัตว์ปีกจะถูกทำให้หมดฤทธิ์ ภายใน 24 ชั่วโมง ความเข้มข้นสูง (0.01 โมลาร์) ไวรัสไข้หวัดใหญ่สัตว์ปีกจะถูกทำให้หมดฤทธิ์ ภายใน 6 ชั่วโมง หลังจากทำให้ไวรัสหมดฤทธิ์ ไม่พบการตกตะกอนเม็ดเลือดแดง แสดงถึงไวรัสไข้หวัดใหญ่สัตว์ปีกถูกทำให้หมดฤทธิ์โดยสมบูรณ์

คำสำคัญ: ไวรัสไข้หวัดใหญ่สัตว์ปีก ซับไทป์ เอช5เอน1 ไบนารี เอธิลลินิมีน การตกตะกอนเม็ดเลือดแดง การทำให้ไวรัสหมดฤทธิ์

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Introduction

Avian influenza (AI) caused by influenza A virus is a disease of many kinds of poultry, wild and caged birds and is characterized by marked variations in morbidity, mortality, clinical signs and lesions. In addition, the infection causes periodical epidemics in humans, pigs, seals, and a variety of birds (Swayne and Halvorson, 2003). AI viruses (AIV) are members of the Orthomyxoviridae family. The viruses are segmented, negative sense, single stranded RNA viruses belonged to the genus influenza A (Portela and Digard, 2002). The virus is enveloped, sensitive to ether, chloroform, and different chemical disinfectants. Influenza A viruses are divided on the bases of the antigenic relationships in the surface glycoproteins, hemagglutinin and neuraminidase into subtypes. Up to now, 16 hemagglutinin subtypes and 9 neuraminidase subtypes have been revealed (Rezza, 2004). AIV can be categorized as having a high pathogenicity (HPAI) and low pathogenicity (LPAI). Not only are HPAI viruses primarily of concern to the poultry industry but they have also become a human health concern because of their ability to transmit directly to humans (Fouchier et al., 2005). Recently it has been suggested that the term HPAI bases on the surface antigen and pathogenicity can be used. Currently, only viruses of H5 and H7 subtype have been shown to cause HPAI in susceptible species, but not all H5 and H7 viruses are virulent. However, it has been proved that HPAI viruses emerge in domestic poultry from LPAI progenitors of the H5 and H7 subtypes (Garcia et al., 1996).

According to the highly virulent and human infectious properties of the viruses, inactivation of the viruses should be focused upon and studied. The appropriate treatments to inactivate the virus in egg products, serum samples, or immunodiagnostic antigens could reduce the risk associated with the shipment of those materials. Binary Ethylenimine (BEI), a member of the group of alkalating substances "aziridines", is more specific to the nucleic acid and reacts very little with protein. Therefore, BEI does not alter the antigenic component of viruses (Bahnemann, 1990). Moreover, BEI preserves the conformation and accessibility of epitopes to formalin and β-propiolactone (Blackburn and Besselaar, 1991, Kyvsgaard et al., 1997). The aim of this study was to evaluate the effect of BEI treatment on the infectivity of HPAI AIV H5N1.

Materials and Methods

Virus preparations: Influenza virus A/Chicken/Nakorn-Pathom/Thailand/CU-K2/04 (H5N1) was inoculated into the allantoic sac of 10-day-old specific antibody negative, embryonated chicken eggs (Kasetsart University, Bangkok, Thailand). The eggs were observed for 24-72 hr post inoculation. Allantoic fluid of the inoculated eggs was collected and centrifuged 1000 x g, 15 min. The supernatant was stored at -70°C until used.

BEI inactivations: BEI was prepared as described by King (King, 1991). Briefly, 0.1 M BEI was prepared by dissolving 0.041g. of 2-bromo-ethylamine HBr (BEA) (Sigma, USA) in 2 ml of 0.175 N NaOH (Merck, Germany). The solution was incubated at 37°C for 60 min. The BEI solution was used for virus inactivation when the pH was reduced from 12 to 8.5. The BEI was diluted 1:10 and 1:100 in tested materials (allantoic fluids) to yield a final concentration of 0.01 and 0.001M, respectively. Both concentrations of BEI-allantoic fluid solution were incubated at 37° C and were collected at 2, 4, 6, 8 and 24 hr to test for pH,

hemagglutination-inhibition (HI) titer and infectivity. The BEI-allantoic reactions were stopped by using sterilized 1M sodium thiosulfate (Merck, Germany) at the concentration 10 times of the BEI final concentration. The experiment was performed 3 times.

Virus infectivity assay: The BEI-allantoic fluid reactions were tested for virus infectivity by the inoculation of 10-day-old embryonated eggs. One hundred microliters of 0.01 or 0.001M BEI-allantoic fluid solution at each time point were inoculated into 10-day-old embryonated eggs. Five embryonated eggs were used for each time interval and concentration. The inoculated eggs were observed for 24-72 hr. Allantoic fluid from dead or surviving embryos was tested for hemagglutination (HA) activity. The HA-positive samples were determined by HI with antiserum specific for the test virus (Department of Livestock Development, Thailand).

HA and HI assays: The HA and HI assays were performed in V-bottom 96-well plates with 1% chicken red blood cells, as described in the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial animals (Alexander, 2000). For the HA test, phosphate buffered saline (PBS) was dispensed into each well of the first row of the plate. The virus suspension was placed in the first well. A twofold serial dilution was performed. Then, the PBS and 1% chicken red blood cells were dispensed to each well, respectively. The solution was mixed and allowed to settle for 40 min before observing the results. The titration was to be read to the highest dilution giving complete HA. For the HI test, twofold serial dilutions of the positive control serum were incubated with four hemagglutinating units (HAU) of AIV at room temperature for 30 min. Thereafter, chicken red blood cells (1% v/v in PBS) were added and agglutination was monitored after incubation at room temperature for 45 min. The HI titer was defined as the reciprocal of the highest serum dilution completely inhibiting agglutination.

Reverse Transcriptase-Polymerase chain reaction (RT-PCR) analysis: RT-PCR analysis was used for confirmation of the virus inactivated by BEI treatment at different time points by the primers specific for H5. The viral RNA of AIV was extracted by QIAamp® viral Mini kit (Qiagen, USA) following the manufacturer's instructions. The first amplification reaction was carried out with one-step RT-PCR (AccessQuick™ RT-PCR system, Promega, USA) by the forward primer of 5′-GACTCAAATGTCAAGAA CCTTTA-3′ and the reverse primer of 5′-CCACTTATTTCCTCTCTGTTTAG-3′ (Payungporn et al., 2004). The PCR reaction followed the reverse transcription reaction at 48°C, 60 min, 1 cycle; initial denaturation at 95°C, 2 min, 1 cycle; denaturation at 95°C, 30 sec; annealing at 55°C, 30 sec and extension at 72°C, 1 min. The PCR was processed for 40 cycles with the final extension step at 72°C, 10 min (Amonsin et al., 2006). The amplification products were visualized by means of ethidium bromide staining after electrophoresis in a 1.5% agarose gel.

Results

Virus inactivation: The two concentrations of BEI, 0.01 and 0.001 M, were tested for their ability to inactivate AIV at 37°C using a different incubation period. The pH of BEI-allantoic fluid solution was found to have a small range of variation on the hemagglutination activity in each experiment after virus inactivation at 0, 4, 6, 8 and 24 hr respectively (Table 1).

Table 1 Effect of 0.01 and 0.001 M BEI on HA titers and pH of AIV in allantoic fluid.

BEI	Replication	Inactivation time (hr)	HA titer	pH
0.01 M	1	0	2^7	7.1
		4	2^7	7.6
		6	2^7	7.7
		8	2^7	7.8
		24	2^7	7.8
	2	0	2^7	7.1
		4	2^7	7.6
		6	2^7	7.8
		8	2^7	7.8
		24	2^7	7.9
	3	0	2^6	7.1
		4	2^6	7.5
		6	2^6	7.6
		8	2^6	7.6
		24	2^6	7.8
0.001 M	1	0	2^7	7.1
		4	2^7	7.2
		6	2^7	7.4
		8	2^7	7.5
		24	2^7	7.6
	2	0	2^7	7.1
		4	2^7	7.2
		6	2^7	7.4
		8	2^7	7.5
		24	2^7	7.6
	3	0	2^6	7.1
		4	2^6	7.2
		6	2^6	7.3
		8	2^6	7.4
		24	2^6	7.6

Virus infectivity: At a concentration of 0.01 M BEI incubation for 4 hr, AIV still showed infectivity due to the dead of embryos and the positive results of HA and HI tests having been found. Conversely, the inactivation of 0.01 M BEI at 6, 8 and 24 hr, AIV was completely destroyed (Table 2). The infectivity of AI H5N1 inactivated by 0.001 M BEI for 4, 6 and 8 hr revealed an ability to infect chicken embryos. Due to the death of embryos and also HA and HI tests were found positive on the collected allantoic fluids. By contrast with, inactivation of 0.001 M BEI for 24 hr, the AIV was completely inactivated which was proven by the live status of the embryos and the negative results of the HA and HI tests, respectively.

Table 2. Effect of 0.01 and 0.001M BEI on HA titers, and the infectivity of AIV in allantoic fluid.

BEI	Replication	Inactivation time (hr)	Number of dead embryo/ inoculated egg	HA titer	Virus isolation
0.01M	1	0	NT	NT	NT
		4	5/5	2^8, 2^8, 2^8, 2^8, 2^8	+
		6	0/5	-	-
		8	0/5	-	-
		24	0/5	-	-
	2	0	NT	NT	NT
		4	5/5	2^6, 2^6, 2^6, 2^6, 2^3	+
		6	0/5	-	-
		8	0/5	-	-
		24	0/5	-	-
	3	0	NT	NT	NT
		4	0/5	-	-
		6	0/5	-	-
		8	0/5	-	-
		24	0/5	-	-
0.001M	1	0	NT	NT	NT
		4	5/5	2^6, 2^6, 2^6, 2^5, 2^5	+
		6	5/5	2^5, 2^5, 2^4, 2^4, 2^4	+
		8	5/5	2^5, 2^5, 2^5, 2^5, 2^5	+
		24	0/5	-	-
	2	0	NT	NT	NT
		4	5/5	2^6, 2^6, 2^6, 2^6, 2^6	+
		6	5/5	2^6, 2^6, 2^6, 2^6, 2^6	+
		8	5/5	2^7, 2^6, 2^6, 2^5, 2^5	+
		24	0/5	-	-
	3	0	NT	NT	NT
		4	5/5	2^9, 2^9, 2^8, 2^7, 2^6	+
		6	5/5	2^9, 2^9, 2^8, 2^7, 2^7	+
		8	5/5	2^9, 2^8, 2^8, 2^7, 2^6	+
		24	0/5	-	-

NT: no test, +: positive (virus isolated), -: negative (no virus isolated)

Virus detection by RT-PCR: The RT-PCR reaction using a specific primer set of glycoprotein H5 of AIV H5N1 could detect the presence of AIV H5N1. At a 0.001 M BEI incubated for 24 hr, the H5 glycoprotein gene could not be detected. In contrast with BEI incubation for 2, 4, 6 and 8 hr, the H5 glycoprotein gene could be detected. This means AIV could not be inactivated with 0.001M BEI during 2-8 hr of incubation. At a 0.01 M BEI concentration, the H5 gene of AIV H5N1 could not be detected for 4, 6, 8 and 24 hr of inactivation times by RT-PCR but the H5 gene could be detected at 2 hr of 0.01M BEI inactivation with AIV H5N1. This suggests that AIV H5N1 could be inactivated with 0.01M BEI during 6-24 h of incubation (Fig. 1).

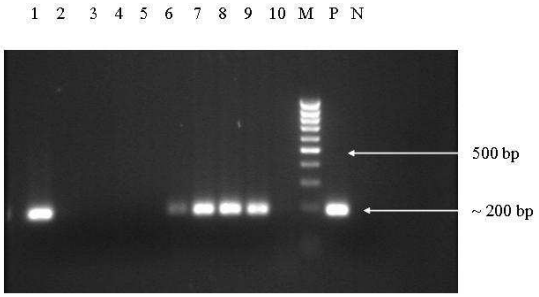

Figure 1. The detection of H5 gene by RT-PCR. Lane 1-5: 0.01 M BEI inactivated to AIV for 2, 4, 6, 8 and 24 hr, respectively. Lane 6-10: 0.001 M inactivated to AIV for 2, 4, 6, 8 and 24 hr, respectively. Lane M: 100 Basepair marker. Lane P: positive control. Lane N: negative control.

Discussion

The aim of this experiment was to determine the AIV inactivation by BEI. AI H5N1 can be inactivated by BEI will respect to the concentration and inactivation times. AI H5N1 was completely destroyed by 0.001M BEI when the allantoic fluid

containing AIV was incubated for 24 hr but the AIV could not be inactivated when incubated with 0.001M BEI for 2-8 hr because AIV can kill embryonated eggs and a positive result was found by RT-PCR, in accordance with the report of King (King, 1991). Comparison of virus isolation and RT-PCR, RT-PCR revealed higher sensitivity, specificity and efficacy than virus isolation at 95, 98 and 97%, respectively (Atmar et al., 1996). Furthermore, RT-PCR was a useful tool for virus detection when there was a small amount of RNA in submitted samples from 1 ng to 0.1 fg (Lee et al., 2001). However, 50% tissue culture infective dose, 50% egg infective dose and real time RT PCR is required for proving the viability of virus.

At a concentration of 0.01 M BEI, the virus was inactivated when incubated for 6-24 hr. BEI can inactivate AI H5N1 but the antigenicity can still be conserved and no effect on HA titer is found. Ethylenimine is the chemical reagent in the aziridine group that is active at the site of nucleic acid on viral RNA but does not react with other proteins that are present in the virus (Brown et al., 1998). Thus, protein in the BEI-virus suspension affects the BEI efficacy on virus inactivation (Hanson, 1982). The reaction of aziridines is more effective when incubated at 37°C compared with the reaction at 25°C because the chemical agents more quickly become inserted into viral particles when the temperature is rising (Burrage et al., 2000). At temperature lower than 37°C, the concentration of BEI should be increased (Bahnemann, 1990).

According to our experiments, 0.001 M BEI can inactivate AI H5N1 when the incubation time is at least 24 hr but at a concentration of 0.01 M AI H5N1 will be completely destroyed when incubated for 6-24 hr. The present study revealed that the hazard or virulence of infectious AIV in allantoic fluid can be eliminated or destroyed by treatment with BEI, without adverse effect on the virus antigen (Bahnemann, 1990; Habib et al., 2006) because activated sites occur on the RNA, and minor activities have been found on proteins)Brown et al., 1998).

Acknowledgement

This research was financially supported by grant no. BT-B-01-MG-09-4909 from the National Center for Genetic Engineering and Biotechnology (BIOTEC) and the Graduate School, Chulalongkorn University, Bangkok, Thailand. We thank Dr. Arunee Chaisingh, Department of Livestock Development, for kindly providing antiserum against AIV H5N1 and Dr.Taweesak Tirawatanapong, Faculty of Medicine, Chulalongkorn University for supplying equipments for virus purification.

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