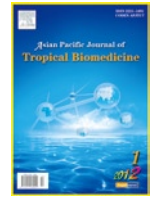




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Pathogenicity of avian influenza virus H5N1 2007 isolates from Pakistan

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ABSTRACT

Objective: To assess the pathogenicity and to categorize the high pathogenic avian influenza (HPAI) H5N1 of four isolates from different outbreaks during 2007. **Methods:** Primary assays of pathogenicity test were used such as mean death time (MDT), intracerebral pathogenicity index (ICPI) and intravenous pathogenicity index. **Results:** The MDT of isolate 1, 2, 3 and 4 was 35 h, 36 h, 38 h and 35 h respectively in ten-day old chicken embryonated eggs. The ICPI in one day old chick for all isolates were more than 0.7 and the intravenous pathogenicity indexes in six-week old specific pathogen free chicken for all isolates were more than 0.7 and 1.2. **Conclusions:** It is the first report of pathogenicity assessment of H5N1 virus circulating in Pakistan and all isolates were categorized to HPAI viruses through primary methods of pathogenicity test.

1. Introduction

'Fowl plague' is referred to infection with virulent serotype influenza A virus of Orthomyxoviridae family and subdivided into 16 different HA subtypes. This type of viruses poses the most significant threat to human and animal health and causes unprecedented poultry outbreaks and human infections in Asian, European, and African countries[1,2]. The increased pathogenicity in chickens may be associated with disruption of the thermoregulation system and innate immune responses due to highly rapid replication of the virus in macrophages and vascular endothelial cells[9]. Avian influenza viruses A detection and full spectrum identification was remained an important task for influenza surveillance, outbreak control, and animal and public health[10]. Recently, AI virulent serotypes belong to H5 or H7 subtypes and most of these isolates have been of low virulence. The molecular basis of pathogenicity produces a great understanding for determine the pathogenicity of serotype virulence for birds, but still need primary methods such as the inoculation of a minimum of eight susceptible 4–8 week old chickens with infectious virus for pathogenicity assessment[2]. Currently, the primary methods such as mean death time (MDT), intracerebral

pathogenicity index (ICPI) and intravenous pathogenicity index (IVPI) for pathogenicity assessment cannot be neglected along with molecular methods[2,3]. In early 2006, high pathogenic avian influenza (HPAI) H5N1 was detected in many countries included Pakistan at the end of the 2007[4,5]. In this scenario, overwhelming attention has been given to avian influenza virus (AIV) H5N1 isolates diagnosis, epidemiology, vaccines and control in Pakistan[5]. Moreover, some studies were carried out for subtyping, molecular characterization and physico-chemical properties of 2007 isolates[6]. In contrast, the pathogenicity of AIV e.g. mechanisms and intensity of virulence of serotypes received little attention. This study was designed to assess the pathogenicity of H5N1 2007 isolates of Pakistan by methods of MDT, ICPI and IVPI.

2. Materials and methods

In this study, four isolates from different outbreaks were propagated in 9 to 11-day old chicken embryonated eggs as described previously[5]. The mean death times (MDT) of all isolated were calculated from freshly infected allantoic fluid with HA titer $>1/16$ ($>2^2$ or $> \log^2 4$ when expressed as the reciprocal). Allantoic fluid was diluted 1/10, 1/100, 1/1000, ..., 1/100000000 in sterile isotonic saline. Eight groups of ten day old embryonated eggs were inoculated with 0.1 mL of each dilutions of virus by the chorioallantoic sac route.

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Moreover, 0.1 mL of the diluent was also inoculated into two embryonated eggs as control. Eggs were incubated at 37 °C and candled at 6 hour intervals for 5 days. Eggs were scored as alive or dead, and the time of death recorded. All the isolates which killed embryonated eggs between 96 h after inoculation were categorized high pathogenic[3,8].

For the assessment of the pathogenicity of H5N1 by ICPI[2], briefly, fresh infected allantoic fluid from each isolates with a HA titer $>1/16$ ($>2^2$ or $> \log^2 4$ when expressed as the reciprocal) was diluted 1/10 in sterile isotonic saline and 0.05 mL of the diluted virus was injected intracerebrally into ten specific pathogen free (SPF) day old chicks. Moreover, 0.05 mL of the diluent was also inoculated into two chicks as control. They were examined every 24 h for 8 days. Each normal, sick and dead bird was scored 0, 1, 2 respectively, at each observation. The judgment of sick and severely sick birds was subjected on clinical assessment. ICPI was the mean score per observation over the ten day period. An index of 2.00 means that all birds died within 24 hours, and an index of 0.00 means that no bird showed clinical sign during the 8-day observation period. The virus has an ICPI in day old chicks 0.7 or greater was consider high pathogenic.

IVPI of four H5 different isolates were calculated by standard methods described elsewhere[3]. Briefly, fresh infected allantoic fluid with a HA titer $>1/16$ ($>2^4$ or $> \log^2 4$ when expressed as the reciprocal) were diluted 1/10 in sterile isotonic saline. Ten SPF six week old chicks were inoculated with 0.1 mL virus intravenously. Moreover, 0.1 mL of the diluent was also inoculated into two chicks as control. The birds were examined for clinical signs at intervals of 24 h for 10 days. Each normal, sick, severely sick and dead bird was scored 0, 1, 2 and 3 respectively, after

each observation. The judgment of sick and severely sick birds was subjected on clinical assessment, e.g. respiratory involvement, depression, diarrhoea, cyanosis of the exposed skin or wattles, oedema of the face and/or head, nervous signs. Normally, 'sick' birds were presented only one of the above clinical signs; whereas 'severely sick' birds presented more than one of the above signs. The IVPI was the mean score per bird per observation over the 10-day period. Isolates were categorized high pathogenic, non-pathogenic and intermediate pathogenic by IVPI equal 2.0–3.0, 0.0–1.0 and 1.2–1.4 respectively[3]. Moreover, every isolate with IVPI 1.2 or greater was considered high pathogenic according to European Union definition[2,3].

3. Results

All isolates were categorized as high pathogenic AIVs according to their MDT, ICPI and IVPI (Table 1).

The mean death time in ten day old embryonated chicken eggs inoculated with isolate 1, 2, 3 and 4 was 35 h, 36 h, 38 h and 35 h respectively. The average mean death time of four isolates was 36 h (Table 1).

The intracerebral pathogenicity index in day old SPF chicks of isolate 1, 2, 3 and 4 was 1.95, 1.90, 1.95 and 2.00 respectively. The average ICPI of all isolates was 1.95 (Table 1). All isolates were categorized high pathogenic with intracerebral pathogenicity index (ICPI) more than 0.7 in day old chicks. The intravenous pathogenicity index in 6 week old SPF chicks of isolate 1, 2, 3 and 4 was 2.80, 2.75, 2.85 and 2.70 respectively. The average intravenous pathogenicity index of all isolates was 2.775 (Table 1). The IVPI of individual and mean of all isolates were higher than 1.2. So,

Table 1

Characteristics of the avian influenza (H5N1) isolates in 2006 outbreaks in Pakistan.

Isolate No.	HA titer	IVPI	ICPI	Pathogenicity in chickens		
				Time (h)	No. of death / No. inoculated	Pathogenicity
1	28	2.80	1.95	35	8/8	High
2	28	2.75	1.90	36	8/8	High
3	28	2.85	1.95	38	8/8	High
4	28	2.70	2.00	35	8/8	High
Means	–	2.775	1.95	36	All	–

MDT– Mean death time; IVPI– Intravenous pathogenicity index; ICPI– Intracerebral pathogenicity index.

these all isolates were categorized HPAI viruses according to European Union definition.

4. Discussion

Highly pathogenic avian influenza term relates to the assessment of virulence in chickens. It is used to describe a disease of fully susceptible chickens with clinical signs such as ocular and nasal discharges, coughing, sneezing and dyspnoea, swelling of the sinuses and/or head, apathy, reduced vocalisation, marked reduction in feed and water

intake, cyanosis of the unfeathered skin, wattles and comb, incoordination and nervous signs and diarrhea[2].

AIV is considered high pathogenic on the bases of standard intravenous pathogenicity index test and molecular classification[14]. Every day, new discoveries on AIV host range and impact on affected species are reported. The research is advancing scientific understanding on the emergence of evolving pandemic HPAI viruses. It is also stirring countries and the world at large to become prepared to face the future challenges of the next influenza pandemic[7].

Generally in avian natural reservoir, avian influenza

viruses have low pathogenicity and cause subclinical infections of the intestinal or respiratory tract. Some viruses have gained virulence by mutation after transmission and adaptation to susceptible gallinaceous poultry. HPAIV cause high mortality in susceptible poultry and lead to tremendous economic losses^[4].

Highly pathogenic avian influenza H5N1 strain has involved in the culling of millions of birds with tremendous economic loss and still a serious threat to poultry industries worldwide^[13,15]. In early 2006, HPAI H5N1 was detected in twenty European, twelve Middle–East countries including Pakistan and seven African countries from dead poultry and wild birds. It remained endemic in many European, African and Middle–East countries^[4] including Pakistan at the end of the 2007^[5]. They were controlled by enforcing biosecurity measures, controlling infected poultry movements, using inactivated vaccines and introducing a comprehensive artificial intelligence surveillance network throughout the country^[3,5]. It is a first report related to assessment of pathogenicity of H5N1 isolates of Pakistan to assess their virulence by MDT, ICPI and IVPI.

AIV pathobiological changes vary with host species and virus strain. High pathogenic virus causes a severe, systemic disease with high mortality in chickens. The recent H5 and H7 HPAI viruses have increased pathogenicity for chickens as evidenced by shorter MDT^[11]. H5N1 HPAI viruses have been shown to have a MDT of around 44 h^[8]. Similarly, four isolates of H5N1 have mean death time in ten days old embryonated chicken less than 44 h. The average MDT of four isolates were also less than 44 h. These all were categorized HPAI H5N1 virus according to their MDT.

H5N1 HPAI have ICPI in day old SPF chicks of all isolates more than 0.7^[2]. Similarly, four isolates of H5N1 have ICPI more than 0.7 in day old chicks and were categorized as HPAI virus.

H5N1 HPAI serotypes having mortality more than 75% or IVPI more than 1.2 were considered highly pathogenic within ten days observation period. According to European Union definition, this assay is a means of testing the level of pathogenicity of an isolate by observing clinical signs in 6 week old SPF infected birds over a ten day period^[2,3]. Similarly, four isolates of H5N1 have IVPI higher than 1.2. Hence, all these isolates were categorized as HPAI viruses according to their IVPI.

Previously demonstrated low pathogenic virus with laboratory assessment may be converted into 'potentially pathogenic' viruses by a single point mutation^[2]. However, pathogenicity of avian influenza virus A Chicken/ Pakistan/ 1/ 95 (H7N3) was assessed and found high pathogenic in 2–7 day old chicken^[12]. But avian influenza H5N1, a causative agent of many epidemics in Pakistan, was never investigated for pathogenicity with primary method of pathogenicity assessment. First time in this study, the pathogenicity of H5N1 virus circulating in Pakistan was tested and found to be high pathogenic by primary assays for pathogenicity assessment. It emphasized a need of continuous serological and virological surveillance of low pathogenic AIV in this region to control the disease due to its ability to mutate and to gain the ability of high pathogenic. Moreover, these finding highlighted the importance and future challenges related to new epidemics of avian influenza virus in AIV endemic and previously disease free countries.

Conflict of interest statement

We declare that we have no conflict of interest.

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