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SUSCEPTIBILITY OF DESI AND COMMERCIAL LAYER BREEDS TO LOW PATHOGENICITY AVIAN INFLUENZA VIRUS INFECTION

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ABSTRACT

Host resistance in Desi and commercial layer breeds was studied in this study. It was evaluated by measuring sensitivity of RBCs to LPAIV induced hemagglutination by using RBCs of two breeds and nine other avian species. Moreover, egg infectious dose 50 (EID_{50}) along with mean death time (MDT) in embryonated eggs, intracerebral pathogenicity in day-old chicks, and intravenous pathogenicity, in four weeks old chickens, of LPAIV H_9N_2 in two breeds were also compared. Using same viral suspension and RBCs from various avian species including three chicken breeds, a statistically non-significant variation was found among chicken breeds, while on species level there were significant differences in H_9N_2 titers. The viral titer for same virus stock solution in Desi embryonated eggs was found $10^{11.24}$ EID_{50} / ml while in commercial layer embryonated eggs it was found to be 10^{11} EID_{50} / ml. The mean death time in Desi layer was 96.3±13.8 hours while in commercial layer it was found to be 106.6±19.3 hours. Intracerebral Pathogenicity Index (ICPI) of the virus in Desi chicken was found to be 0.213 and in commercial layer it was 0.216. Intravenous Pathogenicity Index (IVPI) of 0.137/3 was found in Desi chicken while it was 0.1/3 in commercial layer. The H_9N_2 infection resulted in reduction in body weight, feed intake, water intake and a rise in rectal temperature. In conclusion, the AIV H_9N_2 isolate was found to be of low pathogenic in nature and it produced variable pathogenicity in Desi and commercial layer breeds pointing towards a genetic basis of resistance against LPAIV infection.

Keywords: Disease resistance, LPAIV, H_9N_2, Avian influenza, Desi, Layer.

INTRODUCTION

Low pathogenic avian influenza (LPAI) is a highly contagious viral disease affecting chickens of all ages with variable morbidity and mortality. LPAI occurs naturally in wild birds and can spread to domestic poultry. The infected flocks experience 10-20 percent mortality but due to secondary bacterial infections, it may increase from 30 to 80 percent (Naeem, 1999). In most cases it exhibits no signs of infection or only minor symptoms in birds. The LPAIV H_9N_2 was first isolated in the year 1995 in Pakistan in poultry and since then frequent outbreaks have been reported (Muneer, 2008). However, due to poor biosecurity and intensive farming, this subtype has become endemic. Vaccination is often resorted to but its efficacy is compromised by constant strain variations due to mutations.

Genes responsible for genetic resistance to scrapie, Infectious Bursal Disease, Avian lymphoid leukosis, and Marek's disease have been discovered, while the observation that some chickens can survive following challenge even with HPAI deserves further attention. However, it has been found that present commercial lines in poultry industry do not possess sufficient biodiversity to enable selection for resistance against infectious diseases. Thus, indigenous non-commercial poultry due to constant pressure of infectious agents may have greater frequency of resistance genes (Cheng, 2010). However, measuring disease resistance against low pathogenicity viruses like LPAIV H_9N_2 poses difficulty as mortality is not a common feature.

The LPAI infections are common in commercial poultry while backyard poultry are mostly raised free-range in villages and are therefore more likely to contact with infected wild birds (Cecchi *et al*., 2008). Village poultry sold in urban live bird markets can introduce the avian influenza viruses to commercial poultry (Abbas *et al*., 2011). We have for the first time used various pathogenicity tests which are used for measuring virus pathogenicity, for quantifying and comparing host resistance. The susceptibility of a commercial layer and Desi poultry to LPAIV infection was evaluated by comparing sensitivity of RBCs to LPAIV induced hemagglutination, and susceptibility of embryos, day-old chicks, and adults to the H_9N_2 strain of LPAIV.

MATERIALS AND METHODS

Virus isolation and culture: Morbid organs (trachea, lungs and spleen) were collected from diseased commercial poultry flocks in Rawalpindi showing symptoms of LPAI. These were ground with sterile sand in a mortar and pestle. The homogenized material was centrifuged in at 4^0C, 5000 rounds per minute (rpm) for

15 minutes. The supernatants were separated and filtered through syringe derived filter of 0.2 µm porosity. The filtrate was mixed with antibiotics (Penicillin10,000 IU/ml, Gentamycin 1mg/ml, Streptomycin 10,000 µg/ml) and antifungal agents (Amphotericin B). The virus was isolated by injecting 0.2ml of inoculum into allantoic cavity of nine-day old embryonated chicken eggs. The amnio-allantoic fluid (AAF) was harvested after the death of embryos 96-106 hours post-inoculation. The AAF was cultured on blood agar medium to confirm its sterility. Hemagglutination (HA) assay was performed to check viral titer. Hemagglutination inhibition (HI) assay was performed on AAFs for confirmation/ identification of isolated H_9N_2 subtype of LPAIV as described by Alexander and Chettle (1977). The serum samples of chickens used for IVPI experiment were checked by HI assay to ensure that these were free of anti-H_9N_2 antibodies.

HA titration by using RBCs of various avian species/breeds: The sensitivity of red blood cells (RBCs) of avian and mammalian species to LPAIV H_9N_2 induced agglutination was evaluated. For the purpose, blood samples from three individuals of each avian species including chicken (Desi, layer and broiler) ostrich, duck, peacock, pigeon, and turkey were used. HA titration was performed as is already described (OIE, 2012 and Webster & Krauss, 2002).

Egg infectious dose $_{50}$ (EID_{50}) and Mean Death Time (MDT) determination: EID_{50} /ml and MDT of the LPAIV H_9N_2 were determined in 09 days old embryonated eggs of Desi and commercial layer chicken by the method as described in the OIE manual (2009). Briefly, fresh AAF having LPAIV subtype H_9N_2 was serially (10 fold) diluted in sterile normal saline to give dilutions ranging from 10^{-1} to 10^{-15}. A volume of 0.1 ml of each dilution (10^{-5} to 10^{-15}) was inoculated through chorio-allantoic sac (CAS) route in embryonated chicken eggs (5 eggs were used for each dilution). The eggs were incubated at 37ºC, were candled every eight hours daily for next 7 days and the time of embryo mortality was noted. All eggs showing embryo mortality were chilled for three hours, AAFs were harvested and tested by HA assay. Embryos not showing mortality up to 7 days post-inoculation were also opened and tested for LPAIV H_9N_2 by HA test. Calculation of EID_{50} was performed by Reed and Muench (1938) formula.

Intracerebral pathogenicity index (ICPI): ICPI was determined in day-old chicks by intracerebral injection of LPAIV H_9N_2 as per the standard procedure mentioned in Terrestrial Manual of OIE (OIE, 2012). Fresh infective allantoic fluid obtained after passaging the AIV H_9N_2 in embryonated eggs with a HA titer >1/16 was diluted 1/10 in sterile isotonic saline. Next, 0.05 ml of the diluted virus was injected intracerebrally into each of ten chicks (of both types of poultry) between 24 to 40 hours after hatch. While sterile normal saline solution was also intra-cerebrally inoculated into ten chicks (of both types of poultry), which served as negative controls. The birds were examined every 24 hours for an observation period of eight days. At each observation, the birds were scored: 0 if normal, 1 if sick, and 2 if dead. The ICPI was calculated as the mean score per bird per observation over the 8-day period.

Intravenous pathogenicity index (IVPI): Desi and layers (commercial name Babcock-300) were raised for four weeks and found sero-negative for H_9N_2 by HI test were used for determination of IVPI. Fresh infective allantoic fluid with a HA titer >1/16 (or >log24 when expressed as the reciprocal) was diluted 1/10 in sterile isotonic saline. 0.2 ml of the diluted virus was injected intravenously into eight 4-week-old susceptible chickens (of both breeds). Moreover, control groups comprising eight birds of each Desi or commercial breeds were also injected with sterile normal saline solution. Birds were examined at 24-hour intervals for 10 days. At each observation, each bird was scored 0 if normal, 1 if sick, 2 if paralyzed and 3 if dead. The IVPI is the mean score per bird per observation over the 10-day period. An index of 3.00 means that all birds died within 24 hours, and an index of 0.00 means that no bird showed any clinical sign during the 10-day observation period (OIE, 2012).

Evaluation of clinical and necropsy findings during LPAIV infection of chickens: The chicken, intravenously injected with LPAIV, were observed for clinical signs and as none of the birds died during the experiment, all birds were euthanized and dissected at the end of experiment to study the lesions produced during the course of disease.

Statistical Analysis: Analysis of variance (One way ANOVA) was performed to find out the significant differences among the data obtained for mean death time and HA titration, using the computer package Student Edition of Statistics (SXW), version. 8.1 (copy right 2005, Analytical Software, USA). The least significant differences of mean (LSD = 0.05) test was used to compare the significant differences between the groups

RESULTS

HA titration of LPAIV H_9N_2 using RBCs of various avian breeds and species: The HA titers of various avian species including chicken, ostrich, duck, peacock, pigeon, and turkey were analyzed and results are depicted in Fig. 1. The mean HA titer values for chicken breeds (Desi, layer and broiler) were 8±0, 8±0 and 7±0 respectively. It was observed that there was no significant difference (P>0.05) among the chicken breeds (i.e. Desi, layer and broiler). The mean HA titer values for duck,

pigeon, ostrich, peacock and turkey were noted as 9.67±0.67, 9.67±0.33, 9.00±0.58, 4.33±0.33 and 4.33±0.33, respectively. The mean HA titer value of duck and pigeon was significantly (P<0.05) higher followed by ostrich, than that of Desi, Babcock-300, broiler, peacock and turkey. The HA titers in peacock and turkey were found to be similar (P>0.05) but were lower than the other species.

MDT and EID_{50} determination: The MDT of embryos of Desi and Babcock-300 chicken was recorded and the results are presented in Fig. 2. The mean death time of LPAIV H_9N_2 inoculated Desi and layer (commercial name Babcock-300) embryos was 96.3±13.8 and 106.6±19.3 hours respectively (Figure 2). The MDT in layer chicken was significantly (P<0.05) higher than that of Desi chicken. The EID_{50} / ml of virus stock solution in Desi embryonated eggs was $10^{11.24}$ / ml while in Babcock-300 embryonated eggs it was found to be 10^{11} /ml.

ICPI and IVPI determination: The ICPI of Desi and layer (commercial name Babcock-300) was analyzed and results are depicted in the table 1. ICPI in Desi chickens was found to be 0.213 and in layer it was 0.216 (Table 1). While no morbidity or mortality was found in the control groups of day old chicks intracerebrally injected with sterile normal saline solution.

The intravenous pathogenicity index of Desi and layer chickens was analyzed and results are depicted in the table 2. IVPI in Desi chicken was found to be 0.137 and while it was 0.1 in layer (Table 2). No morbidity or mortality was observed in the control group of 4-week old chickens intravenously injected with sterile normal saline solution.

Clinical and necropsy findings during LPAIV infection of 4-week chickens: During experiment infected birds of both breeds showed clinical signs such as ruffled feathers, depression, diarrhea, respiratory rales and edema of head or face. Moreover, body weight, feed intake and water intake by H_9N_2 virus infected chickens were found reduced PI. At the completion of experiment, birds of both breeds were euthanized on 11th day post-inoculation. The major necropsy findings in both breeds were mild congestion / hyperemia on trachea, lungs, and kidneys.

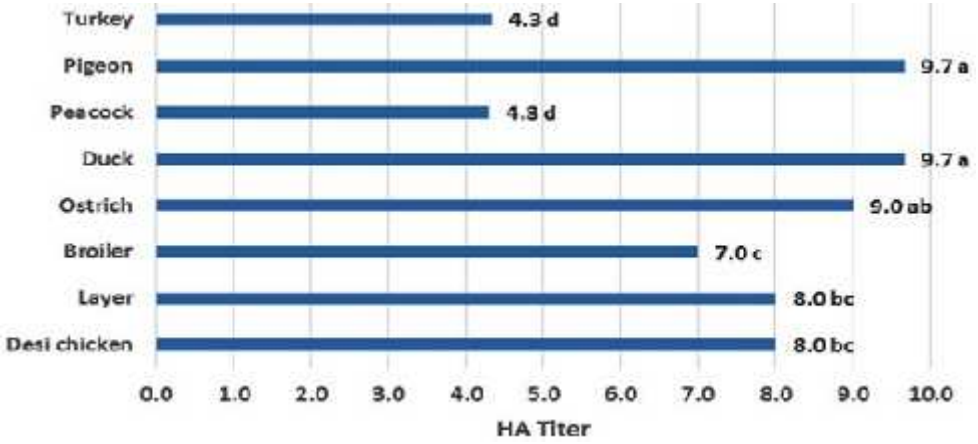

Fig. 1. HA titration of H_9N_2 using RBCs of various avian species

Means having superscript with similar letters on columns are not significantly different from one another.
LSD (0.05) = 1.1173 SE± = 0.5270

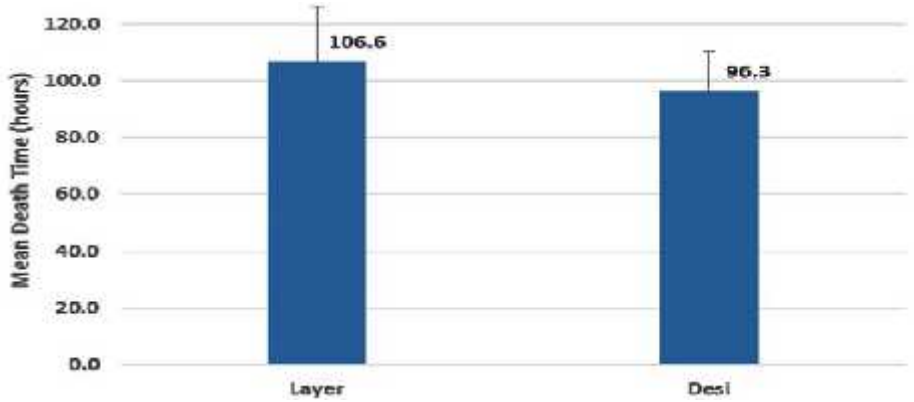

Fig. 2. Mean time of death (hours) in commercial layer and Desi breeds

Significance level (p<0.05) = 0.0357

Table 1. Intracerebral Pathogenicity index for H_9N_2 virus infected day-old chicks

Groups	Clinical signs	Days								Total	Weight	Score
		1	2	3	4	5	6	7	8			
Layer	Normal	16	17	20	20	16	16	15	16	136	0	0
	Sick	4	3	3	3	1	0	0	0	14	1	14
	Dead	0	0	0	0	2	3	3	3	11	2	22
												36/166= **0.216**
Desi	Normal	17	17	19	17	16	17	15	18	136	0	0
	Sick	3	3	2	2	2	2	0	0	14	1	14
	Dead	0	1	1	1	1	2	2	2	10	2	20
												34/160= **0.213**

Table 2. Intravenous Pathogenicity index for H_9N_2 virus infected chickens.

Groups	Clinical signs	Days										Total	Weight	Score
		1	2	3	4	5	6	7	8	9	10			
Layer	Normal	8	7	6	6	6	7	8	8	8	8	72	0	0
	Sick	0	1	2	2	2	1	0	0	0	0	8	1	8
	Paralyzed	0	0	0	0	0	0	0	0	0	0	0	2	0
	Dead	0	0	0	0	0	0	0	0	0	0	0	3	0
														8/80= **0.1**
Desi	Normal	8	7	5	6	6	6	7	8	8	8	69	0	0
	Sick	0	1	3	2	2	2	1	0	0	0	11	1	11
	Paralyzed	0	0	0	0	0	0	0	0	0	0	0	2	0
	Dead	0	0	0	0	0	0	0	0	0	0	0	3	0
														11/80 = **0.137**

DISCUSSION

The outbreaks of low pathogenic avian influenza virus (LPAIV) H_9N_2 are common in Pakistan and this subtype is now endemic in commercial poultry farms (Aamir *et al*., 2007; Naeem *et al*., 1999). Thus breeding for disease resistance may provide a difficult but a good long term solution for disease control. However, testing of breed resistance to LPAIV infection is not possible by conventional methods using challenge and mortality pattern analysis as these are viruses of extremely low virulence and do not cause mortality. We have thus used for the first time various pathogenicity tests, which are conventionally used for measuring pathogenicity of various AIV strains, for testing resistance of various host breeds to H_9N_2 virus.

Nine days old embryos of layer chicken were found less susceptible to H_9N_2 sub-type of AIV than embryos of Desi chicken. The MDT for layer chicken embryos was found 106.6±19.3 hours and that of Desi chicken embryos was 96.3±13.8 hours (Fig. 1). Moreover, it is worth noting that average weight of eggs of layer chicken was 51.6 grams while that of Desi chicken was 28.7 grams. A difference embryo size between two breeds may also have an effect on susceptibility. Chai (2007) determined MDT of 25 isolates of LPAIV H_9N_2 obtained from Henan province of China during 1998-2005 and reported that pathogenicity varied between among 25 H_9N_2 subtypes with MDT ranging between 66.5 hours to 103.2 hours. Moreover, 2.5 times higher H_9N_2 virus titers were found upon titration on Desi embryonated eggs than on commercial layer chicken eggs. Both the lower MDT and higher EID_{50} point towards greater susceptibility of embryos of Desi chicken to LPAVI H_9N_2.

The ICPI for AIV H_9N_2 was measured on both layer and Desi day-old chicks. The ICPI for H_9N_2 isolate was found 0.216 in layer chicks while in Desi chicks it was 0.213 (table-1). Results do not show any significant differences in both breeds. These results are similar to the findings of Chai (2007) who found determined ICPI of 25 isolates of AIV H_9N_2 in same breed of chicken which was in the range of 0.238 to 0.437. Moreover, in our studies we also determined the IVPI of H_9N_2 using 4-week old chicken two different poultry breeds. We found an IVPI of 0.137 and 0.1 /3.0 respectively in Desi and commercial layer breeds (table-2). According to the criteria set by WHO (Capua, 2009) this subtype belongs to LPAIV as its IVPI score is <0.7. The IVPI score of 0.13 found in Desi chicken in our study is similar to that of Subtain *et al.* (2011) who found it about 0.12 in broiler chickens. It appears that H_9N_2 viruses isolated from Pakistan are almost similar in their pathogenicity. Variation in pathogenicity of H_9N_2 isolates have been reported by

various researchers. Nagarajan *et al.* (2008) and Slemons *et al*. (1991) reported that IVPI of H_9N_2 virus shows in the range of 0.0 to 0.49/3.0. Similarly, Chai (2007) determined the IVPI of 25 Chinese isolates of H_9N_2 which ranged from 0.34 to 0.51.

The chickens intravenously inoculated with H_9N_2 showed diarrhea, respiratory rales and slight depression. These signs are similar to with the findings of the Vasfi Marandi *et al.* (2000) and Davison *et al*. (1999) who similarly reported diarrhea and depression in experimental birds. In our study, no clinical signs of respiratory involvement were noted except mild tracheal rales which are in partial in agreement with the findings of Mutinelli *et al*. (2003) who reported mild respiratory signs like mild tracheal rales, coughing and sneezing in broiler chickens, post-inoculation with H_9N_2. This may be due difference in resistance to LPAIVs between layer and broiler chicken and also due to possible relative difference of pathogenicity of isolates/strains. No natural mortality occurred in AIV H_9N_2 infected birds of both breeds. All birds were euthanized on 11^{th} days post-inoculation. Similarly, Subtain *et al.* (2011) and Iqbal *et al.* (2013) have also reported no deaths after intravenous and intranasal inoculation of AIV H_9N_2, respectively. Iqbal *et al.* (2013) have reported slightly higher virus excretion and more severe clinical signs in three week old layers than in jungle fowl. However, age and route of H_9N_2 virus inoculation, difference of isolate, strain of layer and purity of Desi breed may have resulted slight variation from our results. The postmortem lesions during necropsy were similar in intensity in both the commercial layer and Desi chicken.

Conclusion: Present study concludes that HA titers of LPAIV H_9N_2 do not vary upon using blood of various poultry breeds but use of other avian species' blood gives variable titers. Conventional virus pathogenicity tests like MDT, ICPI and IVPI may give variable results due to breed variation and therefore may be used evaluate breed resistance to avian influenza. Lower MDT and higher IVPI point towards higher susceptibility of Desi chicken to LPAIV H_9N_2.

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