Characterization of Isolated Avian Influenza Virus

M. Numan*, M. Siddique‡, M. A. Shahid‡ and M. S. Yousaf†

Veterinary Research Institute, Lahore, Pakistan, ‡Department of Veterinary Microbiology, University of Agriculture, Faisalabad, Pakistan, †Department of Physiology and Biochemistry, University of Veterinary and Animal Sciences, Lahore, Pakistan

ABSTRACT

Non-vaccinated commercial layer farms against any subtype of avian influenza virus were visited, having respiratory and other problems confusing with avian influenza to collect tissue samples and swabs for isolation of the virus. Samples were processed and inoculated into embryonated chicken eggs. Harvested materials were subjected to haemagglutination test, agar gel precipitation test, and haemagglutination inhibition test for characterization of isolated virus. Paired serum sampling was done and haemagglutination inhibition test was performed for the determination of serum antibodies against avian influenza virus. The results showed that isolated virus was avian influenza virus subtype H_7.

***Key Words*:** Avian influenza, haemagglutination inhibition test, agar gel precipitation test

INTRODUCTION

Every year the global burden of influenza epidemics is believed to be 3-5 million cases of severe illness and 300,000 to 500,000 deaths (Anonymous, 2005). Avian influenza (AI) is a contagious viral disease, world wide in distribution. It affects the chickens of all ages with variable morbidity and mortality. With the highly pathogenic AI (HPAI) viruses, morbidity and mortality rates are very high (50-89%) and can reach 100% in some flocks (Capua et al., 2000). Influenza A and B viruses are enveloped viruses with a segmented genome made of eight single-stranded negative RNA segments of 890 to 2341 nucleotides each (Kamps et al., 2006). On the basis of the antigenecity of the surface glycoproteins, haemagglutinin (HA) and neuraminidase (NA), influenza A viruses are further divided into sixteen (H_1 to H_{16}) and nine (N_1 to N_9) (Fouchier et al*.,* 2005). New epidemic of influenza strains arise every 1 to 2 years by the introduction of selected point mutations within two surface glycoproteins: HA and NA. The new variants are able to elude host defenses and there is, therefore, no lasting immunity against the virus, neither after natural infection nor after vaccination, as is the case with small-pox, yellow fever, polio and measles (Holmes et al., 2005). Avian influenza of highly pathogenic (HP) type was first reported in Pakistan in 1995 (Naeem and Hussain, 1995). Since then the disease has been repeatedly reported from various poultry rearing areas at different locations throughout the country. In view of this situation a survey was carried out with the objectives of determining prevalence of AI in commercial layer flocks in few areas of central Punjab, heavily populated with layers, to see whether still disease is present in commercial layers or have been controlled.

**Corresponding author*
numan_uaf@yahoo.com

MATERIALS AND METHODS

Collection and Processing of Samples

The study was carried out in few areas of Punjab (including Faisalabad, Toba Tek Singh, Kamalia, Pir Mahal, Arifwala, Sahiwal, Sammundri, and Rajana), Pakistan, from December 2004 to February 2005. Cloacal swabs, faecal samples and morbid tissues were collected from typical diseased birds showing signs and symptoms of disease, and immediately dipped in the glycerol medium for transportation then shifted to –20°C for freezing in the laboratory. Acute phase serum samples were taken soon after onset of clinical signs and convalescent phase serum samples were collected 2 to 4 weeks later.

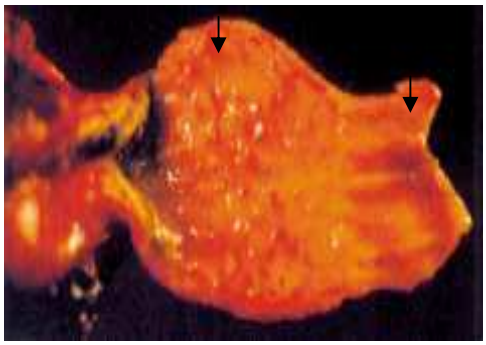

Figure 1 Petechial haemorrhages in the mucosa of proventriculus in poultry

A total of 14 suspected farms were visited. Ten samples (including cloacal/faecal swabs and tissue samples) were collected from each farm. Frozen cloacal/faecal swab/samples were thawed and centrifuged at 3000 rpm for 15 minutes, supernatant was collected. Frozen tissues were thawed and grinded in a sterile pestle and mortar with sterilized sand and a 10% suspension with transport medium were made. Homogenate was centrifuged at 3000 rpm for 15 minutes and supernatant was collected. To check bacterial and fungal contamination, antimicrobials were added in the supernatant.

Polyvalent serum for detection of nucleocapsid protein (NP) of avian influenza virus (AIV) field isolate as well as known AIV antigen for positive control were obtained from National Agricultural Research Centre (NARC), Islamabad. Avian influenza virus subtypes H_7N_3 and H_9N_2 as well as specific antisera against these subtypes were also obtained from NARC in the project of Food and Agriculture Organization (FAO).

Test Procedure

Nine-day-old embryonated chicken eggs (not vaccinated against any type of AIV) were procured form Poultry Research Centre, University of Agriculture, Faisalabad, Pakistan. These eggs were inoculated and allanto-amniotic fluid (AAF) was harvested (Anonymous, 2002). Typing was done on the basis of NP of the virus. For this purpose agar gel precipitation test (AGPT) using 0.9% nobel agar containing 8% NaCl in phosphate buffer saline (Beard, 1978). Haemagglutination inhibition (HI) test was conducted for each AGPT positive AAF to confirm subtype of the virus using specific antisera against H_7N_3 and H_9N_2 subtypes (Olsen et al., 2003).

RESULTS AND DISCUSSION

In the present flocks, under study, pattern of the disease, signs and symptoms and postmortem observations were indicative of a disease complex of various pathogens such as Newcastle disease (ND), infectious bronchitis (IB), coryza, and AI. The infected flocks were treated with heavy doses of broad-spectrum antibiotics like quinolone, gentamycine, penicillin, chloramphenicol etc. but none of these medicines was effective against the disease agent. Affected flocks had been vaccinated against ND,

infectious Bursal disease (IBD) and hydropericardium syndrome (HPS), all measures to control this disease were failed. It was noted that majority of those farms were seropositive which had a loose biosecurity practices at their farms.

Birds of the farms under consideration reflected abnormalities including coughing, sneezing, lacrimation and hens showed decrease in egg production. In few of the farms, birds showed huddling, ruffled feathers, depression, decreased activity and decreased feed and water consumption. Birds reflected a variety of lesions including swelling of the head, face, upper neck and feet as a result of subcutaneous oedema. Periorbital oedema and cyanosis of combs and wattles were also seen in many of the birds. Necrotic foci were also present in some of the affected birds. Haemorrhages on legs and in the mucosa of the proventiculus were also noticed in few of the dead birds (Figure 1).

Three eggs were used for each inoculum prepared from each farm. A total of 4 isolates showing haemagglutination (HA) activity with the chicken red blood cells (RBCs) were obtained and 3 passages in the eggs were taken from each sample to avoid giving false negative results, as in the experiments conducted by Khawaja et al. (2005) there were four isolates which could not be recovered in the 1st or in the 2nd passages but isolated in their 3rd passages (Table 1). These findings are also supported by Cox and Kawaoka (1998) who demonstrated that most type A influenza viruses that are originally isolated in eggs will grow well in the allantoic cavity after one or two passages.

The positive samples by HA test, were tested to confirm type of the virus by agar gel precipitation (AGP) test using AIV polyvalent antiserum and only one sample of Sammundri area was found positive by this test, showing precipitation band between the wells containing AI virus polyvalent serum and the virus test sample. These results were also supported by the findings of Naeem and Hussain (1995) and Naeem et al. (1999) in the chickens and in wild birds by Khawaja et al. (2005).

Haemagglutination inhibition test on the harvested fluid was conducted on AAF to confirm subtype of the virus using reference antisera against H_7N_3 and H_9N_2 subtypes. Positive HI results were shown only by the specific serum against H_7N_3. Specific serum against H_9N_2 was unable to inhibit HA activity of the virus. Furthermore, the fluid was also tested against ND specific serum to detect contamination of this fluid by ND virus, as similar signs and symptoms are produced by ND virus infected birds and ND virus also exhibits HA activity to chicken RBCs. Specific serum against ND virus was unable to inhibit HA activity of the AAF of isolate of Sammundri area. These results were in agreement with the findings of Palmer et al. (1975); Muhammad et al. (1997); Guo and Cheng (1999); Naeem et al. (1999); Muhammad et al. (2001); Bano et al. (2003) and Khawaja et al. (2005).

In this study the 1st harvested allantoic fluid of H_7 was having HA activity upto only 1st well (1:2) of serial two-fold dilution of the fluid. In 2nd passage the HA activity boosted upto the 4th well (1:16) and in the 3rd passage it was upto 9th well (1:512). Naeem and Hussain (1995) found HA activity of H_7N_3 isolate upto 9th well having a titre of 512 in the 1st passage and isolated subtype belonged to the HP subtype of the AIV. This difference of HA activity might be due to severity of the H_7 subtype more than that of this isolated H_7 subtype, because HP AI virus subtypes replicate at a very high speed than that of mildly pathogenic (MP) subtypes and ultimately having more titre. These findings suggested that the isolated subtype might be belonging to the MP subtype of the AIV. Similar findings have also been

documented by Cox and Kawaoka (1998) and Swayne and Halvorson (2003). The outbreaks in Pennsylvania during 1983-1984, in Mexico during 1994-1995, and in Italy during 1999-2000 showed that HP AI could emerge from MPAI outbreaks. In these instances, HPAI emerged after MPAI H_5 or H_7 viruses circulated widely in susceptible poultry flocks for several months as described by Halvorson et al. (1998). This illustrates the need for prompt responses to MP AI outbreaks. Prevention and control of mild influenza outbreaks are the most important steps to prevent outbreaks of HPAI.

Paired serum samples were subjected to HI test to see the difference in the titres of antibodies against AI. Sample of Sammundri area showed more than four fold increase in the serum antibodies as compared to the first serum sampling, while other seropositive flocks by the first sampling were not having such pattern. These results suggested that seroconversion had taken place for AIV subtype H_7 and birds were suffering from infection by AI. Furthermore, the deaths of about 3,000 birds out of 30,000 flock size within 36 hours and signs and symptoms also gave strong evidence of outbreak of AI. Naeem et al. (2003) observed same pattern of seroconversion to H_9N_2, and for H_7N_2 similar results have been found by Smith et al. (1980), similar recommendations to declare AI infection have also been found by Anonymous (2002).

The other non-vaccinated flocks which exhibited various titres of antibodies against AI indicated that in the past infection with AIV occurred but it remained un-noticed by the farmer and the concerning authority. These results are supported by the findings of Halvorson et al. (1992). They described that although there were not any clinical signs of AI but birds were giving positive results during routine serological monitoring. Might be virus attack to these flocks (seropositive flocks but not seroconverted), was of MP virus subtype, having no severe mortality but with a marked decrease in egg production. Warner et al. (2003) detected antibodies against H_7 as well as isolated H_7 of low pathogenic (LP) type from the affected flock of turkey. Similar results have been found in layer flocks in the present research work. Usually by 4 weeks after the initiation of the infection, virus can not be detected. This might be the reason of not isolation of the virus from remaining seropositive flocks, as their serum antibody levels against H_7 and/or H_9 were also low suggesting infection occurred much before the sampling done and at present their antibodies might be catabolized, so having low antibody titres. This statement was supported by the findings of Swayne and Halvorson (2003).

The other three isolates showing positive results in the HA test and negative in the AGP test were subjected to HI test using hyperimmune serum against ND virus. The results of HI test showed that out of these three isolates, two were of ND origin. Similar findings for the presence of ND virus, have been found by Rauf et al. (1986) in doves, parrots and quails, Singh et al. (1989) in pigeons and Arshad et al. (1994) in doves, parrots and quails, while remaining one isolate indicate either non-specific results or the presence of some haemagglutinating agents other than ND virus and AIV. The same has also been observed in captive birds by Ashton and Alexander, (1980) and in wild birds by Arshad et al. (1994).

In Thailand there is strong association between the H_5N_1 viruses and the abundance of free-grazing ducks and, to a lesser extent, native chickens and cocks. This is a critical factor in HP AI persistence and spread (Gilbert, 2006). Poorly controlled movement and lack of biosecurity caused AI to become endemic in some poultry populations, especially in Europe and few areas of Asia (Stubbs, 1948).

Vaccinated flocks cannot be considered influenza virus-free, but the use of vaccine typically reduces the amount of virus shed in experimentally vaccinated and challenged birds, thereby reducing shedding and potential transmission of the virus to other birds (Halvorson, 1987).

Table 1 Virus detection at passage level in various flocks

Farm No.	Virus detection at passage (P) Levels		
	P1	P2	P3
1	-	-	-
2	-	-	-
3	-	+	++
4	-	-	-
5	-	-	-
6	-	+	++
7	-	-	-
8	+	++	+++
9	-	-	-
10	-	-	+
11	-	-	-
12	-	-	-
13	-	-	-
14	-	-	-

In this scenario, the earlier identified presence of H_9N_2 and H_7N_3 in poultry in this country and in other countries in the region, poses a continuous threat for the emergence of more pathogenic strains of both avian and/or human influenza viruses. For this purpose there is a constant need to carry out a coordinated surveillance for influenza viruses both in birds and humans in the country.

REFERENCES

Anonymous, 2002. Global influenza programme. In: *WHO Animal Influenza Manual*, Department of Communicable Disease Surveillance and Control, Review. pp: 15-64.

Anonymous, 2005. Centers for disease control. Prevention and control of influenza recommendations of the advisory committee on immunization practices (ACIP). MMWR, 54: 1-40.

Arshad, M., A. R. Rizvi, M. Naeem, H. Afzal, and S. U. Rehman. 1994. Newcastle disease virus in feces of doves, parrots and quails. *Pakistan Veterinary Journal*, 14: 132-134.

Ashton, W. L. G. and D. J. Alexander. 1980. A two year survey on the control of the importation of captive birds into Great Britain. *Veterinary Record*, 6: 80-83.

Bano, S., K. Naeem and S. A. Malik. 2003. Evaluation of pathogenic potential of avian influenza virus serotype H_9N_2 in chickens. *Avian Diseases*, 47: 817-822.

Beard, C. W. 1978. Avian influenza antibody detection by immunodiffusion. *Bulletin WHO*, 42: 799-806.

Capua, I., F. Mutinelli, M. A. Bozza, C. Terregino, and G. Cattoli. 2000. Highly pathogenic avian influenza (H_7N_1) in ostriches (*Struthio camelus*). *Avian Pathology*, 29: 643-646.

Cox, N. J. and Y. Kawaoka. 1998. Orthomyxoviruses: Influenza. In: *Topley and Wilson*. L. Collier, A. Balows, M. Sussman and B. W. Mahy (ed). In: *Microbiology and Microbial Infections*, 9th (ed). Oxford University Press, Inc., New York. pp: 385-433.

Fouchier, R. A. M., V. Munster, and A. Wallensten. 2005. Characterization of a novel influenza A virus haemagglutinin subtype (H_{16}) obtained from black-headed gulls. *Journal of Virology*, 79: 2814-2822.

Gilbert, M. 2006. Free-grazing ducks and highly pathogenic avian influenza. Thailand. *Emergent Infectious Diseases*, 12: 227-234.

Guo, Y. and X. Cheng. 1999. Seroepidemiological survey for avian (H_9N_2) virus in humans, chickens, and pigs. *Chinese Journal*, 30: 105-108.

Halvorson, D. A. 1987. A Minnesota cooperative control programme. *Proceedings of the 2nd International Symposium on Avian Influenza. U.S. Animal Health Association: Richmond*. pp: 327-336.

Halvorson, D. A., V. Sivanandana, and D. Laver. 1992. Influenza in commercial broiler breeder. *Avian Diseases*, 36: 177-179.

Halvorson, D. A., D. D. Frame, K. A. J. Friendshuh, and D. P. Shaw. 1998. Outbreaks of low pathogenicity avian influenza in USA. *Proceedings of the 4th International Symposium on Avian Influenza. US. Animal Health Association: Richmond*. pp: 36-46.

Holmes, E. C., E. Ghedin, N. Miller, J. Taylor, Y. Bao, K. St. George, B. T. Grenfell, S. L. Salzberg, C. M. Fraser, D. J. Lipman, and J. K. Taubenberger. 2005. Whole-genome analysis of human influenza virus reveals multiple persistent lineages and reassortment among recent H_3N_2 viruses. *PLoS Biology*, 3: 1579-1589.

Kamps, B. S., C. Hoffmann, and W, Preiser. 2006. In: *Influenza report.* B. S. Kamps and G. R. Teron (ed). Flying Publisher Paris. pp: 17-47.

Khawaja, J. Z., K. Naeem, Z. Ahmed, and S. Ahmad. 2005. Surveillance of avian influenza viruses in wild birds in areas adjacent to epicentre of an outbreak in federal capital territory of Pakistan. *International Journal of Poultry Science*, 4: 39-43.

Muhammad, K., M. A. Muneer, and T. Yaqub. 1997. Isolation and characterization of avian influenza virus from an outbreak in commercial poultry in Pakistan. *Pakistan Veterinary Journal*, 17: 6-8.

Muhammad, K., I. Hussan, A. Riaz, R. Manzoor, and M. A. Sajid. 2001. Isolation and characterization of avian influenza virus (H_9 type) from outbreaks of respiratory syndrome in commercial poultry. *Pakistan Journal of Science and Research*, 53: 3-4.

Naeem, K. and M. Hussain. 1995. An outbreak of avian influenza in poultry in Pakistan. *Veterinary Record*, 137: 438-439.

Naeem, K., A. Ullah, R. J. Manvell, and D. J. Alexander. 1999. Avian influenza A subtype H_9N_2 in poultry in Pakistan. *Veterinary Record,* 5: 559-560

Naeem, K., M. Naurin, S. Rashid, and S. Bano. 2003. Seroprevalence of avian influenza and its relationship with increased mortality and decreased egg production. *Avian Pathology*, 32: 285-289.

Olsen, C. W., A. Karasin, and G. Erickson. 2003. Characterization of a swine-like reassortant H_1N_2 influenza virus isolated from a wild duck in the United States. *Virus Research*, 93: 115-121.

Palmer, D. F., M. T. Coleman, W. R. Dowdle, and G. C. Schild. 1975. In: *Advanced Laboratory Techniques for Influenza Diagnosis*. Immunology Series no. 6, Washington DC, US, Department of Health Education and Welfare. pp: 51-52.

Rauf, A., M. Ajmal, A. R. Rizvi, M. Naeem, and M. Arshad. 1986. Seroepidemiological survey of ND in doves, parrots and quails. *Journal of Animal Health and Production*, 6: 49-55.

Singh, G., A. P. S. Mangat, and B. S. Gill. 1989. Isolation of newcastle disease virus (Avian paramyxovirus-1) in India. *Current Science*, 58: 154-155.

Smith, V. W., W. Coakley, D. Maker, J. S. Mackenzie, and P. A. Lalor. 1980. The serological response of chicken to

an avian Influenza A virus administration by various routes. *Research in Veterinary Science*, 29: 248- 250.

Stubbs, E. L. 1948. Fowl pest. In: *Diseases of Poultry*. H. E. Biester, and L. H. Schwarte. 2nd (ed). Iowa State University Press: Ames, IA. pp: 603-614.

Swayne, D. E. and D. A. Halvorson. 2003. Influenza. In: *Diseases of Poultry*. Y. M. Saif. H. J. Barnes, J. R. Glisson, A. M. Fadly, L. R. McDougald, and D. E. Swayne 11th (ed). Iowa State Press. pp: 135-160.

Warner, O., E. Starick, and C. H. Grund. 2003. Isolation and characterization of a low-pathogenicity H_7N_7 influenza virus from a turkey in a small mixed free-range poultry flock in Germany. *Avian Diseases*, 47: 110.