ISOLATION AND CHARACTERIZATION OF AVIAN INFLUENZA VIRUS FROM AN OUTBREAK IN COMMERCIAL POULTRY IN PAKISTAN

Khushi Muhammad, Muhammad Akram Muneer and Tahir Yaqub
Microbiology Section, College of Veterinary Sciences, Lahore, Pakistan

ABSTRACT

The causative agent of an outbreak causing exceptionally high mortality (total birds died so far approximately 0.6 millions) in breeders, layers and broilers in suburb areas of Islamabad, was isolated. The samples prepared from each of the morbid sample of trachea, lung, head, spleen and liver, of the infected birds were inoculated in allantoic cavity of embryonated hen eggs. Each sample induced the embryonic death within 36 hours post-inoculation. The allantoic-amniotic fluid (AAF) from the chilled eggs haemagglutinated red blood cells (RBC) of chicken, sheep, rabbit, guinea pig, cow, parrot, pigeon, quail and sparrow. The haemagglutination (HA) of chicken RBC was not inhibited with antibodies of infectious bronchitis or Newcastle disease viruses. However, the HA was inhibited with convalescent sera from recovered birds. The clinical signs, postmortem lesions, and serological tests confirmed that the cause of the fatal outbreak was H7 type avian influenza virus.

INTRODUCTION

Poultry industry in Pakistan is being adversely affected by the occurrence and spread of infectious diseases such as Newcastle disease (ND), infectious bronchitis (IB), infectious bursal disease (IBD), Marek's disease, egg drop syndrome etc. (Jaffery, 1981). High density rearing, multi-age groups at the same farm, improper managemental practices, intake of mycotoxin-rich feed and existence of poultry farms in the vicinity of dwelling sites of the migratory birds, are helping in the wide spread occurrence of infectious disease problems.

During 1994-1995, an outbreak of apparently infectious origin in poultry breeder flocks with high mortality in the vicinity of Islamabad, Murree, and Abbatt Abad was recorded. The signs and symptoms, postmortem lesions and pattern of disease occurrence were suggestive of avian influenza, infectious laryngotracheitis (ILT), fowl cholera, salmonella, ND, IB etc (Muneer *et al.*, 1995). The objective of the present study was to isolate and characterize causative agent of the outbreak on the basis of its cultural and serological characters.

MATERIALS AND METHODS

Collection of morbid samples

A total of 1000 breeders, layers, and commercial broilers were necropsied and the morbid organs (trachea, lung, head, spleen and liver) were collected in sterile polythene bags and immediately transported to the College of Veterinary Sciences, Lahore for isolation and characterization of the causative agent.

Processing of samples

Each sample was weighed, admixed with equal quantity of normal saline, triturated and centrifuged at 400 G for 15 minutes. The supernatant was separated and the antibiotics (Combiotic 1 mg/ml, Nystatin 20 units/ml) were added to the samples. All the samples were stored at - 20°C till used for inoculation in developing chick embryos.

Inoculation of embryonated hen eggs

Sixty 10-day old embryonated hen eggs were obtained from M/S Hi-Bred Hatchery, Lahore and were divided into 6 groups. The eggs in groups A1, A2, A3, A4, A5 and A6 were injected with inoculum prepared from trachea, lungs, head, spleen, liver and control (which did not contain any virus), respectively (Allan *et al.*, 1978). All the eggs were incubated for 48 hours. These eggs were candled for development of any change in embryo at 12 hours intervals. All the dead and surviving embryos were stored at 4°C for 24 hours and the allantoic-amniotic fluid (AAF) was harvested in sterile containers.

Haemagglutination and haemagglutination inhibition assay

The HA of the infectious agent in AAF was determined using red blood cells from avian and mammalian species. The inhibition of HA activity of the AAF to chicken RBC was also observed with known negative, positive and test sera against ND and IB viruses, as described by Allan *et al.* (1978).

Table 1: Haemagglutination activity of the allantoic fluid of eggs inoculated with different samples

Source of inoculum	Group of the eggs*	HA titre
Trachea	A1	1:32
Lung	A2	1:64
Head	A3	1:64
Spleen	A4	1:64
Liver	A5	1:64
Control	A6	Nil
	(diluent : normal saline : 0.85% sodium chloride)	

HA : Haemagglutination activity * : Each group contained 10 embryonated eggs

Table 2: Haemagglutination activity of allantoic-amniotic fluid after treatment with newcastle disease virus and infectious bronchitis virus specific antisera

Serum	Haemagglutination inhibiting antibody titers of the sera (+)		
	4HA*NDV^X	4HA*IB^Y	4HA*AAF^Z
1	1:8	1:32	0
2	1:128	1:32	0
3	1:256	1:128	0
4	1:256	1:64	0
5	1:32	1:16	0
6	1:128	1:128	0
7	1:64	1:32	0
8	1:64	1:64	0

\+ The eight serum samples from birds experimentally vaccinated against ND and IB were diluted in three 96-well round bottomed immunoplates. In the plate X, Y, and Z, the 4 HA titer of ND (Tad), IB (intervet) and the AAF were added respectively. About 30 minutes post reaction, chicken RBC (0.5%) were added in the wells of each plate.

* 4HA 4 haemagglutinating unit titer.

X NDV Newcastle disease virus.

Y IB Infectious bronchitis virus.

Z AAF Allantoic fluid.

RESULTS AND DISCUSSION

This disease outbreak involved more than 22 layer, broiler and broiler breeder flocks in suburb areas of Islamabad, Murree, and Abbatt Abad. The average mortality in the affected flocks was more than 85%. The history, pattern of the disease, signs and symptoms and postmortem observations were indicative of a disease complex of various pathogens such as ND, IB, ILT, fowl cholera, and avian influenza (Muneer *et al.*, 1995). All the infected flocks were treated with heavy doses of broad spectrum antibiotics such as quinolones (Notril, Avitryl), gentamycin, plasmocolin, lincospectin etc. In addition, the flocks had received vaccinations against ND, IB, and ILT. All measures to control this malady failed. The inocula prepared from spleen, liver, trachea, lung and head samples of the infected birds induced death of chicken embryos within 36 hours post-inoculation. The allantoic fluid from dead embryonated eggs exhibited haemagglutination (HA) with red blood cells of chicken, sheep, rabbit, guinea pig, cow, sparrow, parrot, pigeon, and quails (Table 1; HA titre 1:64 for all species).

This work indicated that the AAF when treated with RBC of avian and mammalian species exhibited HA activity (HA titre 1 : 32). The HA activity was not influenced when AAF was passed through bacterial membrane filter (HA titre 1 : 32).

The results indicated that the causative agent of the disease was a virus with HA activity. The HA activity

was not influenced within 2 hours at room temperature (22°C) but showed spontaneous elution on overnight incubation at 37°C, reinforcing that the causative agent was a virus having haemagglutinin and neuraminidase molecules on its surface. Similar type of molecules have been described by Webster and Cambell (1972) and Hinshaw *et al.* (1985) in avian influenza and ND viruses. This finding further indicated that the virus was a member of paramyxovirus or orthomyxovirus group. The possibilities of incrimination of a virus of paramyxovirus group-1 (ND) or corona virus (IB) were ruled out by observing the inability of ND and IB antibodies to inhibit HA activity of the virus in the AAF (Table 2).

The subtypes of the orthomyxovirus and paramyxovirus were characterized using mono-specific antisera (Alexander, 1986; Beard, 1970; Stalknechit *et al.*, 1990). The laboratory findings confirmed that the convalescent sera collected from birds of the infected area contained antibodies to H7 but not to H1 or H10 of AI (Kammal, M. M. personal communication). This further confirmed that the causative agent of the outbreak was AIV-H7 type. There are 14 types of H antigen and nine types of neuraminidase (N) antigen in avian influenza virus. Each serotype contains any of the combination of H and N antigens (reviewed by Easterday and Hinshaw, 1991).

The hyperimmune serum raised against indigenous isolate (Murree-Ibrahim Farm-1: MIBF-1) was used to type the local isolates from the diseased birds in Islamabad area (From March to July, 1995). No mutation in the H antigen type of the virus was observed over a period of 6 months. The H antigen is a major determinant of the virus pathogenicity in birds (Bosh *et al.*, 1979).

In the light of these results, it is suggested that further spread of the disease be controlled by vaccinating the healthy birds in and around the infected areas and by practicing the strict biosecurity, cleanliness and sanitation prgorammes at the farms.

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