ISOLATION AND CHARACTERIZATION OF AVIAN INFLUENZA (H_9N_2) VIRUS FROM AN OUTBREAK AT POULTRY FARMS IN KARACHI

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ABSTRACT

Avian influenza virus (AIV) type H_9 N_2 was isolated from poultry flocks which were suffering from acute respiratory illness in Karachi area. High haemagglutination inhibition (HI) titers against AIV virus type H_9N_2, ranging from 6.38 to 7.81, in the convalescent sera of birds were demonstrated. The infected flocks had mortality between 30 to 80 per cent. Those flocks also had considerable of HI titres against infectious bronchitis (IB) virus stains D274 and D1466, against which they were never vaccinated. It is believed that AI H_9 N_2 virus in collaboration with IBV and some unidentified bacterial species caused high mortility in the infected flocks.

Key Words: Avian influenza, Hoemagglutination inhibition, Karachi.

INTRODUCTION

Avian influenza (AI) has struck the poultry flocks in Pakistan, on a number of occasions since 1994. The AI outbreak of 1994-95 caused by a highly pathogenic strain of AIV H_7N_2 resulted in high economic losses primarily in breeding stocks in the northern region of Pakistan (Naeem and Hussain, 1995). A severe syndrome involving the respiratory and gastro-intestinal tract causing a severe illness with very high mortality (30-80 %) was reported from the commercial broiler and layer poultry farms in Karachi during 1998 and 1999.

The purpose of this investigation was to: (a) study the clinico-pathological picture of the infected flocks (b) isolate the infectious agent(s) involved in disease syndrome and (c) investigate the seroconversions against various pathogens of poultry.

MATERIALS AND METHODS

Isolation of viruses from 100 morbid samples were attempted in 9- days old developing chicken embryo using sterile equipment all the morbid tissues were cut into small pieces. A 10 per cent homogenate of each organ was prepared in sterile nutrient broth and clarified at 1500xg for 15 minutes. The supernatants were collected in sterile test tubes and used for virus isolation. Before inoculation of chicken embryos, each millilitre of the supernatant was treated with 1000 IU penicillin, 200 µg streptomycin, 100 µg gentamycin and 100 IU mycostatin. The inocula from trachea lungs, air sacs and nasal washings were injected individually and in pooled form.

A total of 90, nine-day-old embryos collected from of M/S Top Line Poultry Breeding Company, Lahore were brought to Microbiology Laboratory, College of Veterinary Sciences, Lahore, where each of the embryos was candled for its livability. Each active embryo was inoculated with 0.1 ml of antibiotic treated inoculum via the allantoic sac under aseptic conditions, and incubated at 99°F upto 96-hours postinoculation (PI). Un-inoculated and only the diluent-inoculated control embryos were also incubated with the homogenate-inoculates. Each embryo was candled at 12, 24, 36, 48, 60,72 and 96 hours PI. Embryos indicating mortality within 25 to 96 hours PI were chilled at 4°C for 12 hours to avoid blood contamination while harvesting the allantoic fluid (AF). The AF from chilled embryos was aseptically harvested, and the chorio-allantoic membranes were preserved after being checked for hemagglutination (HA) activity. The collections of AF and Amniotic fluid (Amn-F) positive for HA activity were pooled together, clarified at 1000 x g for 15 minutes in a refrigerated centrifuge, and treated with gentamycin (100 ug/ml) and mycostatin (100 ug/ml) and reinoculated in the 9-day-old embryos. The AF and Amn-F from the re-inoculates were harvested at 60 PI hours (after chilling the eggs at 4°C for 12 hours) checked for spontaneous HA activity and pooled and stored at -20 °C.

Known H_9 N_2 and H_7 N_2 AIV lyophilized antisera and antigen for use in HA/HI antigens obtained from Animal and Poultry Research, Laboratory Weybridge, UK, were used for typing of the isolate. The hemagglatinating new isolate from the infected broiler and layer flocks was repeatedly inoculated at 4-days intervals to rabbits to obtain hyperimmune serum. Four HA units of the isolate were used in HI tests conducted with known H_9 N_2 and H_7 N_2 antisera. The acute and convalescent period sera obtained from 16 infected broiler and layer flocks were also tested for HI antibody using the known H_9 N_2 and H_7 N_2 AIV-antigens; and the present isolate according to procedure described by Alexander and Chettle (1977).

Hemagglutination Test (HA) was conducted in the round bottom 96-well microtitre plates (Gibco Labs, Grand Island, NY, USA) according to the procedure described by Alexander and Chettle (1977). Briefly, 50 µl of newrtfgvb54 isolate were serially diluted from 1:2 to 1:1024 dilutions and to each well 50 µl of 0.5 per cent chicken RBC suspension in PBS, pH 7.2 were added and the results were recorded after incubating the plates at room tempurature for 20-30 minutes.

When the RBCs had settled as button at bottom of micro well 12; and when HA activity in well 1 was clear. The HA titre of the test antigen was reciprocal to the highest dilution indicating HA activity.

HI Test:

For each serum sample, HI test was conducted in duplicate. Briefly, 25 µl amount of serum sample was serially diluted in PBS pH 7.2 from (dilutions 1:2 to 1:1024) micro-wells 1 through 10. Well 11 was used as the antigen control and well 12 as the erythrocyte control. A 25 µl amount of AIV antigen (H_9 N_2) containing 4HA units per 50 µl was added to wells 1 through 11 and the plates were incubated at rooms temperature for 45 minutes. A 50-µl amount of 0.5 percent chicken erythrocyte suspended in PBS, pH 7.2 was added to each well and the results were recorded after incubating the plates at room temperature for 30 minutes. The HI titre was reciprocal of the highest dilution of serum inhibiting haemagglutination.

Exclusion serology:

Sera samples collected from the flocks which had experienced sickness during past one-month were examined for HI antibody to IBV (M.41, D.274, D.1466 types), NDV, AIV (H_9 N_2, H_7 N_2) and *Mycoplasma gallisepticum* (MG; Table 2). MG antibodies were also examined using serum plate agglutination test. The *Salmonella pullorum* (SP) antibodies were detected using serum plate test with the SP antigen obtained from the Intervet Labs, USA.

RESULTS AND DISCUSSION

Observations on Diseased Flocks:

A total of 16 broiler (n=11) and layer (n=5) farms housing over 1,50,000 birds in Kathore and Angara Goth areas of Karachi were examined. Mortality at broiler farms ranged between 30-80 percent. Major signs of disease were swollen heads and infraorbital sinuses, purulemnt occulo nasal discharge, gasping for air, dyspnoea and reluctance to move. The postmortem examination of dead birds indicated tracheitis with mucous accumulation in trachea, congestion of lungs with presence of caseated pus, thickened air-sacs with foci of infection, necrotic foci on liver, perihepatitis and enteritis. Colisepticemia was observed in few cases. Spleen and proventriculus had slight petechiation. Bursae were atrophied. Thymus was inflammed and the cecal tonsils were hemorrhagic. Hydropericardium was seen only at one broiler farm. Birds at those infected farms had been vaccinated against NDV, IBV, IBDV (intermediate plus strains) and Angara disease virus. Almost every flock had been treated with antibiotics like oxytetracycline, gentamycin, neomycin, quinolones, plasmacolin, erythromycin, colistin, co-trimoxazole, ampicillin, chloramphemicol, cephalosporins, etc.

Layer flocks suffered mortality between 10-22 per cent. Infected chicks looked depressed, had oculo-nasal discharge, bilateral swelling of orbital sinuses and heads; and were gasping for air. Postmortem lesions described for broilers were also noted in the dead and moribund layer birds. The lesions recorded in the infected and dead birds were similar to those observed by Swayne (1997) upon experimental inoculation of AIV (H5 N2) to 4 weeks old chickens.

Isolation of Pathogen's:

The inocula from trachea, lungs, air sacs, and nasal washings, were pooled. The inoculation of pooled suspension from those organs into 9-days chicken embryos did not cause any visible pathology to the inoculates upto 96 hours PI, except a slight generalized surface congestion. However, the amnlo-allantoic fluid (AAF) from the inoculates did indicate slight hemagglutination activity on spot agglutination testing with the 5 per cent chicken RBC's. Such HA activity was also visible in the AF from the embryos inoculated with the individual suspensions prepared from trachea, lungs, air-sacs spleen, and rectal swabs. The AAF from the embryos inoculated with liver or kidney homogenates did not indicate any HA activity. No visible lesions on the inoculates were observed upto 96-hours PI during first passage of the agent. Upon second

passage in 9-days embryos the mortality of the inoculates was visible from 36 through 72 hours PI. The AF and Amn. fluid indicated good HA activity upon with chicken RBCs. No stunting or dwarfing of embryos was observed upon first or second passaging of the isolate even upto 144 hours PI. The HA titre in pooled AF and amn-F on first passage was 8; at second passage it was 32, and at third passage was 128, indicating that the virus titre increased upon further passaging. The AF had higher HA activity than amn-F. the 10 inoculates, 1,2, 1 and 2 embryos died at 36, 48, 60, and 72 hours PI. There was no mortality from 73 hours to 96 hours PI. The highest HA titre of 64 was recorded in the AF harvested at 48, 60 and 72 hours PI. At 72 hours PI. the HA titre in AF and Amn. F was comparable (titre=64). A decrease in HA titre than that at 72 hours PI was noted at 96 hours P.I. (titre=32).

Isolation of AIV using 9-11 days chicken embryos has been reported by Perdue *et al.* (1989). The haemagglutination activity of AIV is well-documented (Allan and Gough, 1974). In the present work PI presence of AIV in various embryonic tissues was demonstrated using hemagglutination test and the specific antibodies to the isolate were demonstrated using HI test (Allan and Gough, 1974).

Flocks in acute phase of illness had quite low log2 GMT HI titres against both the AIV types. The maximum GM HI titre in acute illness in 43 days old broiler flock against H_7 N_2 was 4.06 and against H_9 N_2 titre was 4.75. The layer flock indicating the acute illness had GM HI titre of 3.25 and 4.63 against H_7 N_2 and H_9 N_2 types on day 3 post natural infection. Although low HI titres against both the AIV types were detectable in acute phase of illness. The titres against H_9 N_2 AI virus were generally higher than those against H_7 N_2 AIV. (Table 1). All the flocks in convalescent period indicated higher GM log^2 HI H_9 N_2 AIV titres than those observed for H_7 N_2. The highest GM HI titre in convalescent sera was noted in a 246-days old layer flock (GMT=7.81) on 21st days post development of initial signs of disease. Of the total 6 flocks, in acute disease only 3 could be examined in their convalescent phase. In flock A the AIV H_9 N_2 GMT of 4.25 at the age of 22 days rose to 6.98 at 39th days; in flock C the H_9 N_2 GMT of 4.19 at the age of 28 days rose to a GMT of 7.37 on 20 days post-first recording the GMT; and in layer flock L the AIV H_9 N_2 GM HI antibody titre of 4.63 at day 27 increased to 8.13 at day 63. Although a slight increase in AIV H_7 N_2 GMT titres was also registered (Flock A from GMT 3.69 to 4.13; flock C from GMT 3.81 to 4.75; flock L from GMT 3.25 to 4.81) this increase was lower than that against AIV H_9 N_2. The known positive control sera from Animal and Poultry Laboratory UK had HI titre of (log2) 2.56 against H9N2; and a titre of 128 against H7N2 AIV.

The NDV, IBV and MG HI antibody in the vaccinated flocks were determined using the techniques of Alexander and Chettle (1977). Antibody to MG were also determined by the serum plate test (Table 2). The acute and convalescent period serum NDV HI titres in flocks in acute stage of illness ranged between 4.4 to 7.00; and titres of flocks in convalescent period ranged between GM titres of 4.75 to 7.60. The MG-HI titre in flocks E, I and M were 24, 29 and 32, respectively. An interesting finding was the presence of considerable HI

Table 1: AIV HI antibody titre in sera of commercial broiler and layer flocks.

Flock[a]	Age	Phase of illness	log2 GMT	
			H7 N2	H9 N2
A. Broiler	22 days	Acute	3.69	4.25
B. Broiler	33 days	Acute	3.06	4.43
C. Broiler	28 days	Acute	3.81	4.19
D. Broiler	37 days	Acute	3.63	4.38
E. Broiler	43 days	Acute	4.06	4.75
F. Broiler	46 days	Convalescent[b]	4.56	6.38
G. Broiler	34 days	Convalescent	4.13	6.98
H. Broiler	48 days	Convalescent	4.75	7.37
I. Broiler	44 days	Convalescent	4.06	7.00
J. Broiler	51 days	Convalescent	5.38	7.38
K. Broiler	43 days	Convalescent	4.00	7.81
L. Layer	27 days	Acute	3.25	4.63
M. Layer	32 days	Convalescent	4.56	7.31
N. Layer	63 days	Convalescent	4.81	8.13
O. Layer	231 days	Convalescent	4.13	7.63
P. Layer	246 days	Convalescent	5.06	7.81

[a]None of the flocks had been vaccinated against H_7 N_2 or H_9 N_2 AIV types
[b]Convalescent sera were collected between 15-20 days post initial signs of illness.

antibody titres against IBV D-1466, and D-274 types for which vaccine was never administerial. GM HI titres against D-1466 IBV in flocks A. B, C. D. E and L in acute phase of illness were 4.56, 5.66, 5.46, 6.00, 4.82, and 5.00, respectively. The GM HI titres against D-274 IBV in the flocks A, B, C, D, E and L in acute phase of illness were 5.00, 5.50, 6.25, 4.75, 4.62 and 4.75, respectively. In convalescent period flocks F, G, H, I, J, K, M, N, O and P had GM HI titres of 6.8, 5.50, 6.00, 4.64, 5.46, 5.72, 4.48, 5.66, 4.78, and 5.86, respectively, against IBV D-1466; and HI titres of 6.56, 5.55, 4.86, 3.92, 7.55, 7.00, 5.58, 4.76, 5.88, and 6.00, respectively against IBV D-274 types. Muneer *et al.* (1999) also reported quite high GM HI titres against D-274, and D-1466 IBV types in the unvaccinated chickens. Although no stunting and dwarfism were observed in the embryos inoculated with the isolate; the presence of considerable IBV HI antibody titres against D-1466 and D-274 call for more investigations in theflocks in Karachi area. Six flocks (A, B, C, E, I, and M) were MG positive on serum plat testing and four flocks (3 broilers: one layer flock) were positive for SP antibody on plate testing.

Isolation of AIV types such as H_5 N_2 (Swayne 1997), H_7 N_3 (Selleck *et al.*, 1997), H_7 N_2 (Muhammad *et al.*, 1997), H_{13} N_2 (Laudert *et al.*, 1993), H_5 N_1 (Alexander *et al.*, 1993) H_3 N_8 (Selleck et al 1994), H_6 N_5, H_8 N_4, H_{12} N5, H_1 N_5, H_8 N_3, H_8 N_5, H_8 N_6, and H_8 N_9 (Shieh *et al.*, 1992), from various avian species in various countries has been documented. In 1994-95, an outbreak due to AIV was recorded in the parent, and commercial layer and broiler flocks in suburbs of Islamabad, Murree and Mansehra area. Over 0. 8 million birds were lost in that outbreak. The isolate from that outbreak was characterized as AIV H_7 N_2 by different investigators (Naeem and Hussain, 1995; Yaqub *et al.*, 1996; Muhammad *et al.*, 1997). The authors also isolated H_9 N_2 AI_2V from a 6-week-broiler flock of over 25000 birds from Karachi area in 1998. However, that virus did not cause mortality upon inoculation into 4-week-old unvaccinated broilers

Table 2: Analysis of serum antibody against, NDV, IBV, MG and *Salmonella pullorum*.

Flock	Age (days)	Illness period	NDV, GM HI titre	IBV-M41GM HI titre	Status of MG Antibody	HI Status of *Sal. pullorum*, AT
A	22	Acute	6.00	5.11	Negative	Negative
B	33	Acute	7.00	6.05	Negative	Negative
C	28	Acute	6.00	6.06	Negative	Positive
D	37	Acute	5.50	5.05	Negative	Negative
E	43	Acute	4.40	4.00	Positive	Negative
F	46	Convalescent	4.75	5.81	Negative	Negative
G	34	Convalescent	5.83	6.83	Negative	Negative
H	48	Convalescent	7.00	7.25	Negative	Negative
I	44	Convalescent	6.00	7.50	Positive	Negative
J	51	Convalescent	5.5	7.00	Negative	Positive
K	43	Convalescent	7.5	7.5	Negative	Positive
L	27	Acute	6.00	6.5	Negative	Positive
M	32	Convalescent	7.00	5.5	Positive	Negative
N	63	Convalescent	7.5	6.5	Negative	Negative
O	231	Convalescent	7.6	7.00	Negative	Negative
P	246	Convalescent	7.5	7.25	Negative	Negative

A= 02/12 Sera were MG antibody positive with serum plate agglutination test.
B= 04/12 sera were MG antibody positive with serum plate agglutination test.
C= 07/12 sera were MG antibody positive with serum plate agglutination test.
E= 03/20 sera positive with MG plate test
I= 02/12 sera positive with MG plate test
M=07/18 sera positive with MG plate test
G= 12/12 sera positive for SP antibody.
J= 02/12 sera positive for SP antibody.
K= 05/12 sera positive for SP antibody.
L= 03/12 sera positive for SP antibody.

(Unpublished data) despite the fact that over 20 percent mortality was observed in the broiler flocks upon natural infection with AIV H_9 N_2. The broiler and layer flocks yielding AIV H_9 N_2 in the present investigation had also suffered a very high mortality. Eckroade *et al.* (1984) also reported that their isolate (AIV H_5 N_2) did not cause death in the experimental birds upon initial inoculation. However, their subsequent isolates (AIV H_5 N_2) from the disease outbreaks in October 1983 were high pathogenic as those isolates killed over 75 per cent of the inoculated chickens. It is possible that present AIV H_9 N_2 isolate has become more pathogenic due to mutation (Hinshaw *et al.*, 1984; Murphy *et al.*, 1984; Austin *et al.*, 1986; Kida *et al.*, 1987), and this Virus in collaboration with some bacterial/viral factors has caused high mortality.

Role of factors like raising of broiler flocks in poultry estates; housing of flocks of different age groups and breeds; changing weather conditions especially those in September through December (significant day and night temperature variations at least by 10°C) indiscriminate use of antibiotics; mycotoxins in feed; concurrent bacterial and viral infections; migratory and wild birds and animals; poor biosecurity, poor quality of vaccines and vaccination programs; visits of vaccination crews, service personnels, etc. as potentiators in epidemiology and pathogenicity of AIV in poultry flocks in Pakistan is to be thoroughly investigated. There is also need for more experimentation of isolate on its transmission and immunizing potential.

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