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RESEARCH ARTICLE

Frequency Distribution of Opportunistic Avian Pathogens in Respiratory Distress Cases of Poultry

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ABSTRACT

Involvement of opportunistic pathogens in the respiratory distress cases of poultry was detected from the poultry flocks scattered in some districts of Punjab, Pakistan. Serum plate agglutination (SPA) test for serological screening of *Mycoplasma gallisepticum* (MG) and *Mycoplasma synoviae* (MS) were performed separately and it revealed 80% flocks were sero-positive among respiratory distress cases. Specimen samples including trachea (n=157), lungs (n=40), spleen (n=93), oral (n=201) and nasal swabs (n=147) were collected from both sero-positive and sero-negative flocks. On the basis of multiplex PCR, the percentage of opportunistic pathogens in sero-positive flocks was found maximum with Newcastle disease virus (28.6%), followed by *E. coli* (24.4%), *Avibacterium paragallinarum* (5.7%), Infectious bronchitis virus (4.7%) and Avian Influenza (1.6%), while in case of sero-negative flocks highest distribution was found in NDV (12.6%), followed by *E. coli* (8.4%), *Av. Paragallinarum* (3.8%), IBV (3.1%) and AIV (1.1%). The Frequency of respiratory tract pathogens including NDV and *E. coli* in combination with MG and MS was recorded significantly high.

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INTRODUCTION

Despite high annual growth rate, poultry sector is confronted with infectious diseases, among them respiratory tract pathogens are of major concern (Ali and Reynolds, 2000), which cause heavy economical losses both in terms of production and cost of treatment (Anonymous, 2010). Infected birds expressed respiratory and other lesions such as cough, respiratory distress, poor growth and production leading to high economic losses (Pang *et al*., 2002). Mycoplasma infections are important as they result in the reduction of egg production, increased embryo/chick mortality and poor feed conversion ratio. Mycoplasmosis is caused by multiple Mycoplasma spp. and due to its subclinical status, it is unable to be detected through routinely used diagnostic procedures, thus provide opportunity to other respiratory pathogens including bacteria and viruses to aggravate the situation (Mohana *et al*., 2011). The bacterial pathogens include *Mycoplasma gallisepticum, Mycoplasma synoviae, E. coli, Av. paragallinarum* while viral pathogens may include Newcastle disease virus (NDV), Infectious bronchitis virus (IBV) and Avian influenza virus (AIV). These pathogens may cause disease alone or in combination with infections of bacterial/bacterial, bacterial/viral and viral/viral infections (Ali and Reynolds, 2000). Present study has elaborated the frequency distribution of bacterial and viral opportunistic pathogens in poultry.

MATERIALS AND METHODS

Sample collection: Out of 141 farms monitored in different districts of Punjab, 34 farms were having respiratory distress. Blood samples were collected from 10% of infected flock (Ehtisham *et al*., 2011) for Serum Plate Agglutination (SPA), to screen MG and MS carrier birds using separate antigens as described by (Luciano *et al*., 2011). Specimen samples were collected including oral swabs, nasal swabs (live birds) trachea, lungs and air sac swabs (post mortem cases) from 34 poultry farms including breeder (2) layer (15) and broiler (17) flocks showing signs of respiratory tract infection for isolation and molecular identification of pathogens (Salisch *et al*., 1999).

Samples from SPA positive and SPA negative flocks were inoculated into the Frey's modified medium for the

isolation of *Mycoplasma* species (Kleven, 2008) and MacConkey's (Oxide) and blood agar (Oxide) for other bacterial pathogens (Blackall, 1999). The Mycoplasma isolates were identified using biochemical tests; glucose fermentation test, arginine hydrolysis test, casein hydrolysis test, film and spot assay (Bencina *et al*., 2006). For virus identification samples were inoculated into nine-day-old embryonated chicken eggs through allontoic cavity route and the isolated viruses were identified using Hemagglutination (HA) test, Hemagglutination Inhibition (HI) for ND and AI test and Indirect Hemagglutination (IHA) test for IB (Mahmood *et al*., 2004).

Molecular Identification

DNA/RNA isolation and Multiplex PCR: The isolates were confirmed through Multiplex-Polymerase chain reaction (mPCR). The DNA from the bacterial pathogens was isolated using Phenol chloroform method (Wang *et al*, 1997) while RNA from viral pathogens was isolated using TRIZOL® reagent method (Jackwood *et al*., 2007).

The mPCR was performed in two steps, in the first step RNA from viral pathogens were converted into cDNA while in the second step DNA/cDNA was amplified using already reported primers. For MG, lipoprotein was amplified using primer as detailed in table 4. The mPCR was performed using 50µl master mix (Invitrogen, USA) and 10pM of each primer in the thermal cycler (PeqLab, Germany). The thermal cycler conditions optimized includes initial denaturation at 94°C for 3 min followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 50°C for 45 sec and extension at 72°C for 60 sec while final extension was done at 72°C for 3 min. The amplified PCR product was separated on 2% agarose gel electrophoresis having 0.2 µg/ml ethidium bromide and visualized through Gel Documentation system (Dolphin Doc, USA) while 100bp ladder (Invitrogen, USA) to as DNA marker.

Statistical analysis: Kruskal-Wallis test was calculated for multiple comparisons using Minitab release 15 software while statistical significance was considered to be present when the P-values obtained were P<0.05.

RESULTS

Two breeder flocks showing respiratory signs were examined out of which one was found SPA positive and one SPA negative. In case of 15 layer flocks visited with respiratory distress problem, 13 were serologically positive and 2 were negative while from 17 broiler flocks, 13 were SPA positive and 4 were negative.

In the sero-positive flocks maximum isolates were recovered belongs to MG (39.6%), followed by NDV (28.6%), *E. coli* (24.4%), MS (8.8%), *Av. Paragallinarum* (5.7%), IBV (4.7%) and AIV (1.6%) as detailed in Table 1. Similarly the isolation of pathogenic organism from SPA –ve flocks showed highest percentage recovery of NDV (12.6%) and *E. coli* (8.4%) followed by other organism including MG (5.8%), MS (4.9%), *Av. paragallinarum* (3.8%), IBV (3.1%) and AIV (1.1%) as shown in Table 2. The statistical results showed the significantly dominant recovery rate of pathogenic organism was recorded from SPA positive flocks as compared to the sero-negative flocks.

Out of total 34 flocks visited the most frequently recovered pathogens belongs to *Mycoplasma gallisepticum* (31.8%) followed by Newcastle disease virus (24.9%), *E. coli* (20.7%), *Mycoplasma synoviae* (7.9%) *Av. paragallinarum* (5.3%), Infectious bronchitis virus (4.3) and avian influenza virus (1.5%) as detailed in Table 3. Kruskal-Wallis multiple comparisons results showed that MG, *E. coli* and NDV combination or mixed infection was significantly dominant among sero-positive birds with P>0.0001, 0.0009 and 0.0001 respectively while other isolates combination frequency was non-significant. The isolates were confirmed using mPCR yielding specific base pair product for each pathogen by agarose gel electrophoresis as mentioned in Table 4 (Fig. 1).

Table 1: Overall breed wise distribution of bacterial and viral isolation recovery from Serum Plate Agglutination +ve cases

Bird type	Sample (n)	MG (n)	MS (n)	*E. coli* (n)	*Av. Paragallinarum* (n)	ND (n)	IB (n)	AI (n)
Breeder	74	29	-	9	-	11	4	2
Layer	464	184	33	111	24	125	23	6
Broiler	336	133	44	93	26	114	14	6
Total/994	874	346	77	213	50	250	41	14
%		39.6	8.8	24.4	5.7	28.6	4.7	1.6

Table 2: Overall distribution of bacterial and viral isolation recovery from Serum Plate Agglutination -ve cases of breeder, layers and broilers

	Sample (n)	MG (n)	MS (n)	E. coli (n)	Av. Paragallinarum (n)	ND (n)	IB (n)	AI (n)
Breeder	84	5	4	3	-	10	3	1
Layer	55	5	3	6	1	10	2	-
Broiler	122	5	6	13	9	13	3	2
Total	261	15	13	22	10	33	8	3
%		5.8	4.9	8.4	3.8	12.6	3.1	1.1

Table 3: Overall comparative frequency percentage of bacterial and viral isolation recovered from breeder, layer and broiler flocks from sero-positive and sero-negative flocks

	Sample (n)	MG (n)	MS (n)	*E. coli* (n)	*Av. paragallinarum* (n)	ND (n)	IB (n)	AI (n)
SPA +ve	874	346	77	213	50	250	41	14
SPA –ve	261	15	13	22	10	33	8	3
Total	1135	361	90	235	60	283	49	17
%		31.8	7.9	20.7	5.3	24.9	4.3	1.5

Table 4: Primer sequences used to detect respiratory tract pathogens

Pathogen	Gene amplified	Primer sequence	Product size (bp)
MG[1]	lp	MG-F 5'-GGATCCCATCTCGACCACGAGAAAA-3' MG-R 5'-CCTTCAATCAGTGAGTAACTGATGA -3	732
MS[2]	16SrRNA	MS-F 5'- GAA GCAAATAGTGATATCA- 3' MS-R 5'- GTCGTCTCGAAGTTAACAA - 3'	207
NDV[3]	F	ND-F 5'- GGAGGA TGTTGGCAGCATT-3' ND-R 5'- GTCAACATATACACCTCATC-3'	320
IBV[4]	S-gene	IB-F 5'- CATAACTAACATAAGGGCA- 3' IB-R 5'- TGAAAACTGAACAAAAGACA-3'	1720
AIV[5]	HI	AI-F 5'- AGCAAAAGCAGGGGATAC-3' AI-R 5 -GTCTGAAACCAT ACCATCC-3'	1050

[1] Niscimento *et al*., 1991; [2] Lauerman *et al*., 1993; [3] Stauber *et al*., 1995; [4] Kwon *et al*., 1993; [5] Pang *et al*., 2002

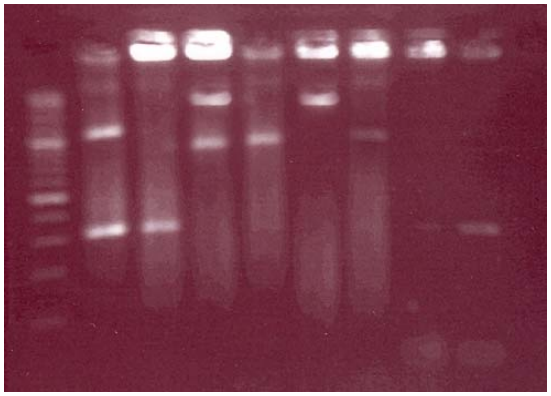

Fig. 1: The mPCR amplification of respiratory tract pathogens. From left to right, Lane 1: marker 100bp, lane 2: positive samples for Newcastle disease virus (320bp) and Avian influenza virus (1050bp), Lane 3: Newcastle disease virus (320bp), Lane 4: Mycoplasma gallisepticum (720bp) and Infectious bronchitis virus (1720bp), Lane 5 & 7: Mycoplasma gallisepticum (720bp), Lane 6: Infectious bronchitis virus (1720bp).

DISCUSSION

Mycoplasma gallisepticum and *Mycoplasma synoviae* are considered as significant poultry pathogens which cause heavy economic losses all over the world (Kleven, 2008). The most frequent sign and symptoms of respiratory distress recorded at the farms include, sneezing, coughing, nasal discharge, tracheal rales, however the severity of disease is greatly affected by the environmental conditions and Mycoplasma spp involved and other pathogens of respiratory tract infection. Mild or sub-clinical disease may be observed with simple MG involvement or in combination of MS while the severity of disease increases with the involvement of other pathogens (Roussan *et al*., 2008). Role of MG as immune-suppressive agent has long been described which may allow MG to interact with other *Mycoplasma* species and other pathogens including NDV, IBV and *E. coli* (Kleven, 2008). Other factors which may promote the respiratory distress cases includes poor sanitation, overcrowding and poor management (Chanie *et al*., 2009)

The serological study based on Serum plate agglutination test revealed the predominant involvement of Mycoplasma infections (79.4 %), although the test was nonspecific and may give rise false positive results due to its cross reactivity with Mycoplasma spp. as described by Kleven *et al*. (2000) but still recommended to be used for the screening of flocks and large population of birds worldwide. The serological result coincides with those of Ehtisham *et al*. (2011) and Hasan *et al*. (2002), who showed 76% sero-positivity for the Mycoplasma in Pakistan while 76% sero-positivity has been reported in Bangladesh (Sarkar *et al*., 2005) .

The multiplex PCR was optimized for successful detection of five of the respiratory tract pathogens including *Mycoplasma gallisepticum*, *Mycoplasma synoviae*, Newcastle disease virus, Infectious bronchitis virus and Avian influenza virus (Marois *et al*., 2000; Yousof *et al*., 2005; Seifi *et al*., 2010; Ehtisham *et al*., 2011). Several studies showed successful detection of individual pathogens which have been reported including multiplex PCR for the detection of *Mycoplasma* spp. (Wang *et al*., 1997) and multiplex RT-PCR for respiratory tract viruses (Pang *et al*., 2002).

The frequency of MG, ND and *E. coli* was found significantly higher as compared to other pathogens, similarly the recovery rate of these pathogens were significantly higher in sero-positive flocks as compared to the sero-negative flocks. The mixed respiratory tract infection was reported with the involvement of different pathogens mainly based on serological studies, while Hasan *et al*. (2002), recorded the involvement of six pathogens based on serology and culture isolation of *E. coli* and *Av. paragallinarum* showing similar type of pattern while Roussan *et al*. (2008) investigated the pathogenic involvement in Jordan showing similar results including high involvement of MG and NDV in the respiratory distress cases while mixed infections of different pathogens of respiratory tract were also reported. More severe disease may result with the interactions between MG, ND IBV and *E. coli* (Kleven, 2008). Involvement of MG with *H. gallinarum* (now *Av. paragallinarum*) was also recognized. The control program of MG was recorded based on serological screening using SPA and by enzyme-linked Immunosorbent assay. Confirmation of organisms can be done from SPA positive flocks.

Based on our findings it was concluded that the highest number of cases depicted in respiratory involvement were predominantly belongs to *Mycoplasma gallisepticum* which enhanced the involvement of *E. coli* and NDV along with *Av. paragallinarum* IBV and AIV in the exestuation of respiratory trouble in broiler layer and breeder flocks. It is recommended that Mycoplasma free flock must be maintained through early detection of respiratory pathogens particularly the MG status which may be accessed successfully through multiplex PCR technique.

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