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RESEARCH ARTICLE

Role of *Mannheimia* (*Pasteurella*) *haemolytica* in Severe Respiratory Tract Infection in Commercial Poultry in Pakistan

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ABSTRACT

Mannheimia haemolytica is the causative agent of several economically significant diseases of cattle and sheep, however, its role in causing infection in poultry is limited as secondary pathogen, co-infecting with viral pathogens like infectious bronchitis virus (IBV), infectious laryngotracheitis virus (ILTV) etc. The present study reports first time in this country regarding the isolation of *M. haemolytica* from adult commercial poultry flocks, initially reported with severe respiratory distress. Necropsy findings were quite similar to those found in Fowl cholera infection. The samples were also processed for the detection of any avian respiratory viruses in addition to testing for bacterial presence. The lab investigations led to the detection of *M. haemolytica* from the clinical samples and subsequent use of norfloxacin, selected on the basis of sensitivity pattern of the organism, resulted in curing of the flock. To further confirm the pathogenic potential of this organism, 7-day old broilers were challenged with the new isolate. The bacterium *M. haemolytica* was subsequently recovered from the birds which died within 3 weeks post-infection, confirming the pathogenic potential of this organism in poultry. The drug susceptibility pattern of the recovered bacterium was similar to the original isolate. This study further identifies the role of different management factors leading to infection of birds with *M. haemolytica* and suggests appropriate control measures in poultry flocks.

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INTRODUCTION

Mannheimia haemolytica, a gram negative, non-motile coco-bacillus, is usually present as commensal in the upper respiratory tract of various animal species and can act as an opportunistic pathogen, causing mild to severe respiratory infections under stress conditions (DeRosa *et al.*, 2000). *M. haemolytica*, the inhabitants of tonsillar cells as commensal in cattle can act as the primary bacterial pathogen in the bovine respiratory disease complex by sliding down to the lower respiratory tract (Klima *et al.*, 2014; Boukahil and Czuprynski, 2015). The epidemiology studies of M. haemolytica regarding the bovine respiratory disease complex revealed that it is complicated and contain many factors to be studied (Burciaga-Robles *et al*., 2010; Desmolaize *et al*., 2011). *M. haemolytica*, constitutes chicken and animals usual flora of the respiratory tract and plays a drastic role of opportunist at times of stress by causing the respiratory disease (Taylor *et al*., 2010). *Mannheimia* belongs to the family Pasteurellaceae along with various other genera including *Actinobacillus, Haemophilus, Pasteurella, Phocoenobacter*, *Gallibacterium*, *Lonepinella* and others (Christensen *et al*., 2003). This bacterium was first named as *Bipolare multocidum* by Theodor Kitt in 1885 was renamed as *Pasteurella* by Count Trevisan to honor the work of Louis Pasteur on fowl cholera. Later on in 1932 it was renamed as *Pasteurella haemolytica* and divided into A (13) and T (4) serotypes (Jaworski *et al*., 1998). Afterwards, T serotypes were reclassified as *Pasteurella trehalosi* in 1990 and the A serotypes as *M. haemolytica* except A-11 serotype which was classified as *M.*

glucosida. Even though *Pasteurella haemolytica* has been classified into three distinct species, they are still considered as a single species for serotyping and identification purpose (Odugbo *et al*., 2003). The name *Mannheimia* was given in tribute to a German biologist, Walter Mannheim for his contribution in the taxonomic understanding of *Pasteurellaceae* family (Angen *et al*., 1999). The role of *M. haemolytica* is well understood in ruminants as a highly pathogenic organism, being responsible for severe respiratory tract problems particularly in cattle, sheep and goats. On the contrary, its identification as secondary or co-pathogen in chicken affected with respiratory viral pathogens like IBV and ILTV and as a primary respiratory pathogen reported in poultry recently after its isolation from clinically ill and dead chicken in Nigeria (Antiabong *et al*., 2006). A number of viral and bacterial respiratory pathogens cause huge economic losses to the poultry industry around the globe every year in the form of production losses, high mortalities, vaccination and medication costs. Isolation of a gram negative, non-motile, hemolytic coco-bacillus from chicken showing clinical signs similar to fowl cholera besides heavy load of round worm infestation in all the necropsied bird prompted us to further investigate the biological aspects of this pathogen.

MATERIALS AND METHODS

Dead birds (n=22) along with blood samples (n=86) were received at the National Reference Lab for Poultry Diseases, NARC, Islamabad, for laboratory investigations from two adult broiler breeder flocks (nearly 50,000 population) with the history of nearly 4% mortality, 60% off fed birds and significant decline in egg production. The birds were necropsied and tissue samples from liver, spleen, intestine, cecal tonsils, trachea, lungs and heart were collected for further lab investigation. All the tissue samples were processed for virus isolation by inoculating into nine (09) day embryonated chicken SPF eggs following standard procedures. These tissues were also processed for detection of major respiratory pathogens like Newcastle Disease (NDV), Infectious Bronchitis Virus (IBV), Avian Influenza Virus (AIV), and *Mycoplasma gallisepticum* (*MG*), through Polymerase Chain Reaction (PCR). Bacteriological evaluation was also done by inoculating a sterile wire loop in the pre-steered liver, heart and spleen samples into Brain-Heart Infusion broth (Oxoid, UK) and Tetrathionate broth (Oxoid, UK), used as pre-enrichment media for *Pasteurella multocida and Salmonella pullorum/ gallinarum* (*SPG*) respectively, and incubating at 37^{o}C for 24 hrs in shaking incubator fixed at 110 rpm. Similarly, same samples were also cultured directly onto 5 % sheep blood agar, MacConkey agar and brilliant green agar (Oxoid, UK). Qualitative composition of the microbial flora was determined on the basis of morphology and primary diagnostic tests (Smith, 1974). For identification purpose the pure colonies of 18-20 hrs were suspended in sterile distilled water (3-4 ml) and inoculated in the analytical profile index 20E strips (API-20E, Biomerieux Inc, USA) followed by its incubation at 37^{o}C for identification. Sulfur Indole Motility Media (Oxoid, UK) and Hanging drop method was used to check motility of the isolate and pure colonies were stained using Gram's staining technique (Bryan Merkey *et al.,* 2013). The antibiotic sensitivity assay was performed on a pure colonies by disc diffusion method using Muellin Hinton medium(Jorgensen, 1993). The antibiotic discs of amoxicillin (10µg), ciprofloxacin (5µg), norfloxacin (10µg), enrofloxacin (5µg) chloramphenicol (30µg), gentamicin (10µg), colistin (10µg), oxytetracycline (30µg), erythromycin (15µg) and sulphamethoxazole (25µg) (Oxoid, UK) were used in this regard. The interpretation was made using standard methods (Jorgensen, 1993). The earlier collected blood samples were used for the detection of antibodies against MG, SPG using commercially available stained antigen (Intervet, Netherlands). Pathogenicity testing was done by inoculating 2 x 10^7 CFU per ml of pure culture through sub-cutaneous route into ten 7-day-old broiler chicken, while a group of 05 chicks were kept unchallenged as a control. These birds were kept in chicken isolator for 04 weeks. The birds were observed twice daily for any clinical symptom & mortality etc. Postmortem of the birds dying during the course of experiment or those showing respiratory signs were performed to record any lesion developed and to collect the samples for further lab investigations. The Tissue samples were tested for the detection of AIV, NDV, IBV and MG through RT-PCR and also processed for bacteriological evaluation, as carried out previously. Here 75% birds died in 3rd week post challenge and rest in the 4th week post challenge. There was no mortality in the control group.

RESULTS

The major lesions noticed upon necropsy of broiler breeders were similar to those found in fowl cholera such as cyanotic combs and wattles, hyperemic viscera, congested lungs, air sacculitis, petechial hemorrhages on heart, areas of mild focal necrosis on hypertrophied liver, splenomegaly, egg peritonitis besides heavy load of round worm infestation in all the necropsied birds. Virological evaluation was negative both through *in ovo* inoculation and by PCR against AIV. IBV, NDV, MG. The bacteriological evaluation showed circular odorless colonies with narrow zone of β-hemolysis on 5 % sheep blood agar and visible growth as lactose fermenting colonies on MacConkey agar upon direct inoculation as well as that subculture from BHI broth. However, no significant growth was found from the culture propagated in Tetrathionate broth indicating non-existence of *Salmonellae*. The isolated bacteria were non motile and gram negative coccobacilli, quite similar to *Pasteurella multocida* in morphology. The biochemical test showed reaction of catalase positive, oxidase positive, mannitol positive, urease negative, Voges–Proskauer (VP) negative and non-acid production from D-mannose [Table 1]. The isolates showed maximum sensitivity to norfloxacin (32mm) and minimum sensitivity to Erythromycin (16mm) [Table 2]. The analytical profile index (API-20E, Biomerieux Inc, USA) gave threshold values of 0000124 with >90 % identification for *M. haemolytica*. The serum plate agglutination test reflected negative results for presence of antibodies against MG and SPG. The

Table 1: Biochemical characters of the isolated *Mannheimia haemolytica* compared with the descriptions of Smith (1974) and Bisgaard (1977)

Tests performed	*P. haemolytica* (Smith, 1974)	*P. haemolytica* (Bisgaard, 1977)	*M. haemolytica* (Present Study)
Motility	-	-	-
Catalase	+	+	+
Oxidase	+	+	+
H_2S/TSI	+/-	-	+
Hemolysis	+	+	+
MacConkey Growth	+	-	+
VP (sodium pyrovate)	-	-	-
Urease (Urea)	-	-	-
Arginine	No information	-	-
Inositol	+	+/-	-
Indol	No information	-	-
Gelatin	-	-	-
L-Tryptophan	No information	No information	-
Beta-galactosidase	+	+	+
Mannitol, acid	+	+	+
Sorbitol, acid	+	+/-	-
Glucose, air	-	-	-
Mannose, acid	+/-	-	-
Rhamnose, acid	+/-	-	-
L-Lysine	No information	-	-
Ornithine	+/-	-	-
D-Sucrose	No information	+	+
D-Melibiose	No information	-	-
Amygdalin	No information	No information	-
L-Arabinose	+/-	-	-

Table 2: Antibiotic sensitivity pattern (diameter of inhibition - mm) of primary and secondary isolates

Antibiotic	Primary Isolates	Recovered Isolates
Norfloxacin	32	30
Ciprofloxacin	32	26
Oxytetracycline	28	24
Colistin sulphate	26	26
Chloramphenicol	28	22
Enrofloxacin	30	26
Sulphamethazole	24	24
Amoxicillin	20	18
Erythromycin	16	12

experimentally inoculated chicks died 2-3 weeks post-challenge, showing mild respiratory signs. Upon necropsy these dead birds showed nodular lesions on enlarged liver along with splenomegaly, congested lungs and mild tracheitis. The bacteriological evaluation subsequently revealed *M. haemolytica* having non motile coco-bacilli and nearly similar antibiotic sensitivity pattern.

DISCUSSION

M. haemolytica is a commensal of upper respiratory tract of various animals including poultry. It has proven role in causing pneumonic pasteurellosis and shipping fever in various animals including bovine, caprine and ovine species (Bavananthasivam *et al.*, 2012; Dassanayake *et al.*, 2010). Its role in producing disease in poultry is considered as secondary pathogen especially as co-infecting agent during viral infections with ILTV, IBV etc, involving respiratory tract in poultry hence aggravating the situation (Hodgson *et al*., 2005; Hafez, 2011). The present study in fact identifies the *M. haemolytica* as a primary source of disease which has been reported to cause a severe respiratory distress in the adult poultry birds besides resulting in significant mortality and loss of production in mature chicken. This finding is contrary to the previous reports, representing *M. haemolytica* as a secondary or co infecting pathogen in poultry. Based on the existing knowledge about different farming practices in this country it is further hypothesized that any production/management stress, high concentration of ammonia in the farm, heat stress or even high worm infestation may have some role in enhancing the pathogenesis of this bacterium (Theurer *et al.*, 2013). In the current situation it appears that heavy round worm infestation along with high production levels in the affected flocks may have generated the required stress to activate the multiplication of *M. haemolytica* in this flock, bringing it down with the clinical condition narrated above. Furthermore, although the ammonia concentration in the poultry house is required to be limited to or even lesser then 25 parts per million (ppm), and because of the fact that continuous exposure to ammonia concentrations as low as 10 ppm can damage the respiratory system of birds and increase the risk of infectious diseases (Madrid *et al.*, 2012; Nimmermark *et al.*, 2009). However no strong evidence is available to incriminate the higher levels of ammonia in the affected flocks in this scenario. As regards the role of round worm infestation in poultry, it is another proven stress factor in adult and laying birds which results in anemia, poor feed conversion ratio (FCR) and poor response to the administration of various live and killed vaccines. Furthermore, the pathogenic potential of *M. haemolytica* is further supported by the ability of disease reproduction this organism upon its inoculation and re-isolation from the affected organs in this study. Furthermore, as the drug sensitivity pattern of this organism indicates its broad susceptibility to different antibiotics, its presence is usually goes un-noticed due to non-judicial usage of different antibiotics in poultry farming across the country. It is, therefore, anticipated that *M. haemolytica* isolated here may have also been previously involved in different infections, as opportunistic pathogen; however, because of routine use of antibiotics in poultry the burden of such bacteria would have been unknowingly under control. In recent system of environmentally controlled housing for commercial poultry farming, where the un-necessary use of drug is somewhat discouraged, role of such bacteria may become more significant in causing the conditions similar to those reported in this study. It may also be further suggested that to avoid un-necessary usage of antibiotics for the treatment of sick poultry and the danger for its transfer to food for human consumption, proper culture and drug sensitivity may be employed prior to the selection of such drugs for use of treating poultry. As regards the molecular diagnostic methods, there are studies showing that the ratio of the *M. haemolytica* positive samples were 70% through PCR as compared to the conventional methods of bacterial culture and isolation which suggests that the growth of *M. haemolytica* is hampered by the other bacteria (Kugadas *et al.*, 2014). This aspects of molecular detection needs to focused in the future for the differential diagnosis of the *M. haemolytica* from other respiratory pathogens.

Conclusion: With the emergence of intensive commercial poultry farming in the world, a variety of new pathogens have emerged in nature as well. Furthermore, co-infection of various organisms, especially bacteria and few respiratory avian viruses in poultry have generated interest of diagnostician to understand such co-infecting

pathogens for devising appropriate strategies to overcome their circulation. In this scenario, the knowledge of the circulation of a new bacterium, *M. haemolytica* as a causal agent of respiratory tract infection in poultry under stressful farm management conditions is expected to help the clinicians and diagnosticians to apply the available tools for differential diagnosis of this infection and devise appropriate response strategy for its proper treatment, accordingly.

Author's contribution: AA and KN designed and conceived the research and provided consultation. AA and KN wrote the manuscript. AA, AG, NS, SR, ARM and RA edited the manuscript. The NS and MAA provided reagents and materials. All authors have read and approved the final manuscript.

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