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RESEARCH ARTICLE

Isolation of *Mannheimia haemolytica* from Layer Hens Showing Respiratory Signs

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A B S T R A C T

Mannheimia haemolytica (M. haemolytica) causes shipping fever and several other respiratory diseases in bovine but its involvement in diseases of *Gallus gallus domesticus* is limited to secondary infections with limited data available from Pakistan. Samples from layer hens showing respiratory clinical signs were processed for isolation and identification of causative agent through biochemical and molecular tests and the isolates were further tested for pathogenicity index. Phylogenetic studies were also done to establish the phylogeny of circulating species. Our study led to the isolation of a Gram-negative, non-motile, coccobacillus from the clinical samples and was identified as *M. haemolytica* which showed 99% homology with the *M. haemolytica* serotype A2 of bovine origin upon sequencing of its 16S rDNA gene. A single, smooth colony of this bacterium inoculated in five, 7-day-old embryonated eggs *via* the yolk sac route induced hemorrhages and 100% mortality. These results were further supported by the recovery of bacterium *M. haemolytica* from 8 week-old layer hens showing mild clinical signs within 1-week post challenge with the isolate. Our study provides evidence that *M. haemolytica* may cause clinical disease in *Gallus gallus domesticus* under stressful conditions and adds to the existing knowledge for differential diagnosis of respiratory pathogens in poultry.

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INTRODUCTION

Respiratory diseases are continuing to cause heavy economic losses in the poultry industry due to high production losses, mortality and medication costs. Respiratory diseases are multifactorial problems in poultry, with viral and bacterial respiratory pathogens often concurrently present and most probably influencing one another (Marien *et al*., 2007; Siddique *et al*., 2016; Fatima *et al*., 2017; Zaheer *et al*., 2017). A community of mucosal dwelling microorganism colonize healthy upper respiratory tract including both commensals and potential pathogens kept under control by the host immune system. Bacterial pathogens are playing a crucial role in causing respiratory diseases in domestic poultry. Bacterial pathogens may play a primary or secondary role in diseases. Usually, primary viral or environmental damage to respiratory tissue attract bacteria to colonize the respiratory system (Antiabong *et al*., 2005; Ashraf *et al*., 2011; Simon *et al*., 2016).

Mannheimia haemolytica (previously known as *Pasteurella haemolytica*) is an opportunistic pathogen of ruminants and belongs to family *Pasteurelleaceae*. It is a weakly haemolytic, gram-negative coccobacillus, which causes broncho-pneumonia, commonly called "shipping fever" under stress and immunosuppressive conditions. Based on capsular antigen typing, *M. haemolytica* includes twelve serotypes (A1, A2, A5-A9, A12-14, A16 and A17) (Theurer *et al*., 2013; Taylor *et al*., 2014). Although all these serotypes can cause disease but the most common are serotypes A1 and A2. Serotype A2 causes disease in sheep and is less-well characterized while serotype A1 causes pasteurellosis in cattle and has been the subject of extensive study. Gene cloning and DNA sequence analysis have facilitated identification and characterization of potential virulence factors of *M. haemolytica*. *M. haemolytica* has been identified as causative agent of severe respiratory tract problems in cattle, sheep and goat but it is usually found as a secondary or co-pathogen in poultry along with other

respiratory pathogens. Although it has been reported from chicken in Nigeria and Pakistan (Antiabong *et al*., 2005; Ali *et al*., 2015) but its role as a primary pathogen is not well studied in chicken. Following study investigates and presents the isolation of *M. haemolytica* bacterium from 52-wk-old, layer hen presenting characteristic clinical signs of fowl cholera.

MATERIALS AND METHODS

52-wks-old layer hens showing respiratory clinical signs (swollen wattles and sinuses, mucous discharge from mouth, respiratory gurgles (rales) and greenish diarrhea were submitted to the laboratory to confirm a suspected infection by *Pasteurella multocida* and the likely association with *E. coli*. Moreover, decrease in egg production and mortality (12%) was also observed. Hens were from a 3000 layer hens flock, raised in a conventional floor bedding system, and located adjacent to a cattle and buffalo dairy form. However, chickens had access to open ranges, the farm was multi-age, and biosecurity with regard to keeping wildlife out was minimal. A total of 12 birds were analyzed for necropsy and bacterial study.

Bacteriologic samples: The birds were necropsied and mucous material from mouth and sinuses and lesions were observed. Swabs were collected aseptically from different organs (liver, spleen, cecal tonsils, oviduct, trachea, lungs and heart) during necropsy examination. The swabs were shipped to the laboratory under cold chain and plated on brain heart infusion (BHI) plates. The plates were incubated in air at 37°C overnight. Colonies showing typical appearance of *M. haemolytica* were picked and two sequential single colony subcultures were performed. The same samples were also cultured directly onto 5% sheep blood agar, *Salmonella shigella* agar, MacConkey's agar and Eosin methylene blue agar (Oxoid, UK) for the detection of other bacteria.

Biochemical characterization and Gram staining: Morphological and primary diagnostic tests were used to determine qualitative composition of microbial flora (Angen *et al.*, 1999). 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 37ºC for identification. Hanging drop method and Sulfur Indole Motility Media (Oxoid, UK) was used to check motility of the isolate and pure colonies were stained using Gram's staining technique (Ali *et al.*, 2015).

PCR amplifications and phylogenetic analysis: For further laboratory investigation, tissue samples were taken from lungs, heart, trachea, oviduct, liver, spleen and cecal tonsils were detected. Polymerase Chain Reaction (PCR) was used to detect major respiratory pathogens such as NDV, IBV, AIV, MG, and *M. haemolytica* in all tissue samples. For molecular diagnosis of *M. haemolytica*, hemolytic colonies were obtained on agar blood plates. Genomic DNA was obtained as described previously (Hervas *et al*., 1996) and rDNA amplification was performed using the F (5′-AGAGTTTGGATCMTGGCT CAG-3′) and R (5′CGGTTACCTTGTTACGACTT-3′) primers under the following conditions: one cycle of denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30s and annealing at 55°C for 30s, and extension (72°C for 2 min); and a final extension at 72°C for 7 min. The PCR products were visualized by separation in a 1.5% agarose gel and ethidium bromide staining. The PCR products were purified and sequenced for phylogenetic reconstruction using BioEdit and MEGA 6 software (Tamura *et al*., 2013).

Pathogenicity: Pathogenicity index was determined by infecting embryonated eggs and chicken with isolated *M. haemolytica*. Small, glossy, odorless hemolytic colonies were identified as *M. haemolytica* based on biochemical characteristics, molecular diagnosis and sequencing. A single colony of *M. haemolytica* from a blood agar plate was inoculated via yolk sac into each one of five, 7-day-old, chicken embryonated eggs, and embryo mortality was checked every day. Negative control embryonated eggs were only inoculated with PBS (without bacteria). In order to know the pathogenic potential of the *M. haemolytica*, sixteen healthy chickens (8 wks old) were procured from local farm and divided into negative control and challenged groups containing 8 birds each. The chicken was inoculated with 0.5 ml suspension of *M. haemolytica* containing approximately 10^8CFU/ml, through intramuscular route. As a negative control, each bird was inoculated intramuscularly with PBS (0.5ml). Chickens were maintained in isolation units with feed and water *ad libitum* and were checked every day for the clinical signs of fowl cholera, i.e. diarrhea, mucous discharge from mouth, or prostration.

RESULTS

Necropsy findings were similar to fowl cholera in diseased layer hens such as cyanosis of combs and enlarged wattles, mucous discharge from mouth, congested lungs, petechial haemorrhages on heart, focal necrosis of liver and peritonitis (Figure 1).

Biochemical characterization: Small, glistening, odourless hemolytic colonies were found on sheep blood agar plates after overnight incubation at 37ºC. The bacteria were found to be haemolytic on blood agar, and did grow on McConkey's agar, but not on Eosin methylene blue agar, and Salmonella shigella agar. All the isolated organism in this study were found to be gram-negative cocco bacillary shape in Gram staining method and showing bipolar characteristics in Leishman's staining method The *M. haemolytica* organism gave positive result for catalase test by producing bubbles and oxidase test by changing its color. On the other hand, no reaction was seen with MR and VP tests. *M. haemolytica* isolate fermented sucrose, maltose, raffinose, sorbitol and mannitol but not trehalose and arabinose (Table 1). The analytical profile index (API-20E, Biomerieux Inc, USA) gave threshold values of 0000124 with >99% identification for *M. haemolytica.* The serum plate agglutination test reflected negative results for presence of antibodies against MG, SG and HP. The other micro-

organisms that were isolated from those birds, besides *M. haemolytica*, included *E. coli*, *Proteus* spp., and *Gallibacterium anatis* (Table 2). Twelve bacterial isolates were characterized by biochemical tests, PCR and 16S rRNA sequencing.

Molecular identification: The PCR technique was used as an initial strategy for molecular characterization and identification of the isolated strain. Sequence analysis of this product showed 99% identity with rDNA from *M. haemolytica* of bovine origin serotypes A2. The sequence data was submitted to NCBI database with accession number KU051693. *M. haemolytica*, Phylogenetic tree construction based upon 16S rRNA gene sequences is a common tool that has been used for classification of the *Pasteurellaceae* (Theurer *et al*., 2013; Taylor *et al*., 2014) including the creation of the genus *Mannheimia* (Fig. 2 & 3).

Pathogenicity determination: A single, smooth colony of *M. haemolytica* inoculated in five, 7-day-old chicken embryonated eggs via the yolk sac route induced petechial hemorrhages and 100% mortality by 48 hr post-inoculation while control negative group embryos remained healthy and viable. Bacteria recovered from the yolks of dead embryos showed identical biochemical characteristics to the bacteria used to inoculate the embryonated eggs. Chickens inoculated via the intramuscular route showed mild clinical signs e.g. anorexia, gasping, mucous discharge from mouth and greenish diarrhea as the sole clinical sign of an infectious process similar to fowl cholera three days post-inoculation (12/12). Clinical signs disappeared within 1 week post challenge and no mortality observed. Re-isolation of the inoculated *M. haemolytica* was positive (trachea, lungs, heart, liver and cecal tonsils) but it did not produce any characteristic lesion in all the necropsied chickens at the end of the challenge experiment (8 days post-inoculation). There were only petechial hemorrhages and mild congestion of trachea and lungs. The chicken in control negative group remained healthy and active throughout the experiment period.

Table 1: Biochemical characters of the isolated *Mannheimia haemolytica* compared with the descriptions of Bisgaard (1977) and Angen *et al.* (1999)

Tests performed	*P. haemolytica* (Bisgaard, 1977)	*P. haemolytica* (Angen *et al.*, 1999)	*M. haemolytica* (Present Study)
Motility	-	-	-
Catalase	+	+	+
Oxidase	+	+	+
H2S/TSI	+/-	-	+
Haemolysis	+	+/-	+
MacConkey growth	+	-	+
MR	-	-	-
VP	-	-	-
Indole	-	-	-
Urease (Urea)	-	-	-
Arginine	+	+	+
Beta-galactosidase	+	+	+
Mannitol	+	+	+
Sorbitol	+	+/-	+
D-Glucose	-	-	+
Maltose	+	+	+
Trehalose	Not known	-	-
Mannose	-	-	-
D-Sucrose	+	+	+

Table 2: Chicken bacteriologic analysis

Tissue samples	Bacteriologic identification	Number of birds
Infraorbital sinuses	*Gallibacterium anatis* biovar haemolytic	1 /12
Trachea	*M. haemolytica*	12 /12
	Proteus	4 /12
	Gallibacterium anatis biovar haemolytic	2 /12
Lungs	*M. haemolytica*	1 1/12
	E. coli	3 /12
Heart	*M. haemolytica*	11 /12
Spleen	*None*	12 /12
Cecal tonsils	*E. coli*	2 /12
	Proteus	4 /12
Liver	*M. haemolytica*	9 /12
Oviduct	*M. haemolytica*	12 /12

DISCUSSION

M. haemolytica has long been recognized in animals in context to its role in causing pneumonic pasteurellosis and shipping fever. It has proven that *M. haemolytica* is a commensal of upper respiratory tract of various animals including poultry and seeks opportunity of causing infections (Dassanayake *et al*., 2010; Bavananthasivam *et al*., 2012). The presence of hemolytic microorganisms that are classified into the *Mannheimia* genus and isolated from birds, has been previously described as secondary pathogen especially as co-infecting agent during viral infections (e.g NDV, IBV, AIV), involving respiratory tract in poultry and hence aggravating the situation (Hodgson *et al*., 2005; Hafez 2011). In addition, avian hemolytic *M. haemolytica* isolates causing respiratory distress and septicemia have been obtained from the upper respiratory tracts of chicken recently (Ali *et al*., 2015). In present study, we report role of bovine origin *M. haemolytica* in not only causing respiratory distress but also disrupting viability and production potential in laying hens. Chickens inoculated with this isolate showed mucous discharge from mouth as the sole clinical sign of an infectious process similar to fowl cholera. The mouth discharge disappeared within 1 wk post-inoculation. Re-isolation of the inoculated *M. haemolytica* was positive but it did not produce any characteristic lesions in all the necropsied chickens at the end of the challenge experiment, indicating that birds could manage this microorganism when it was present alone. However, when defensive mechanisms were abated by the presence of other microorganisms, *M. haemolytica* could colonize the respiratory tissues of chickens as an opportunistic pathogen, which indicates that the bird is a reservoir for new infectious events in animals, which are its natural host. A bovine production unit is adjacent to the egg layers production housing. It is tempting to speculate that birds acquired *M. haemolytica* infection via water or air contamination originating from this bovine production unit. It is further hypothesized that any production/ management stress, mycotoxin toxicity, 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; Umar *et al*., 2015). In the current situation it appears that heavy production, management stress, mycotoxin toxicity or vaccination stress may have generated the required stress to activate the multiplication of *M. haemolytica* in this flock, bringing it down with the clinical condition

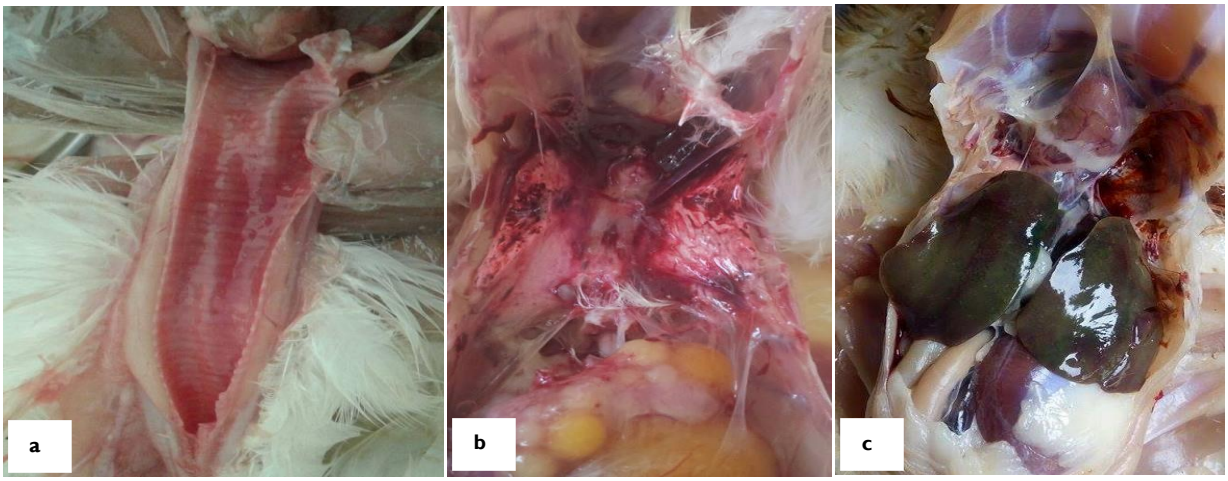

Fig. 1: Necropsy findings (a) tracheitis (b) congested lungs with mild airsacculitis (c) Greenish colour liver (hepatitis).

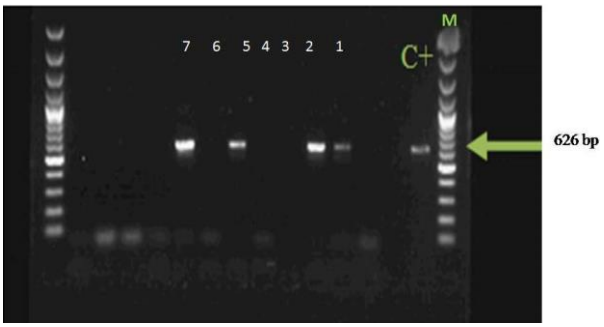

Fig. 2: Agarose gel electrophoresis for detection of *M. haemolytica.* M=100 bp ladder; C+: Positive control for *M. haemolytica;* Lane (1), (2), (5) and (7): Positive samples for *M. haemolytica;* Lane (3), (4) and (6): negative samples for *M. haemolytica.*

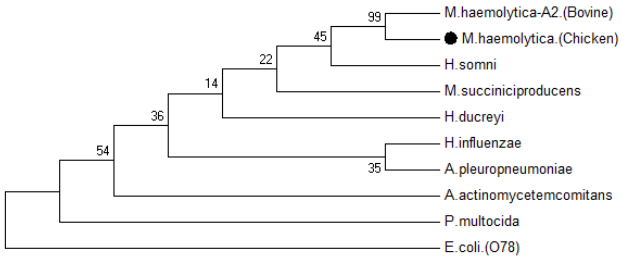

Fig. 3: Phylogenetic tree for *Mannheimia haemolytica* (type A2) detected in the current study and other related isolates of *Pasteurellaceae* species in gene bank. MEGA 6 was used to make tree, using sequences from nine sequenced *Pasteurellaceae* species. *E. coli* was used as an out group. Numbers at nodes represent bootstrap values (out of 100).

narrated above. Host specificity is the limiting barrier that any microorganism must defeat to colonize a host; however, when this restriction is overcome, a pathogen-opportunistic microorganism can infect a new host, resulting in disease. Bovine are considered the specific host of *M. haemolytica* (Klima *et al*., 2014) but this organism has also been isolated from respiratory infections associated with respiratory distress in chicken (Hodgson *et al*., 2005; Hafez 2011; Ali *et al*., 2015). Isolation of microorganisms from non-natural hosts has been reported for different pathogens (Moyaert *et al*., 2007; Aubin *et al*., 2013; Perez *et al*., 2014) and this could be an evolutionary strategy that confers a survival advantage to a microorganism or the possibility of infecting new, susceptible hosts. Furthermore, immunosuppressive effects of mycotoxins, wet litter and ammonia can increase the risk of infectious diseases by damaging anatomical barriers of respiratory system (Nimmermark *et al*., 2009; Madrid *et al*., 2012; Umar *et al*., 2015). Bacterial infections have primarily been controlled with antibiotics and the use of antibiotics containing growth promoters kept the burden of such bacterial infections under control for many years. The use of growth promoting antibiotics is being reduced worldwide because of concern for the spread of antimicrobial resistance. One of the negative consequences associated with the prohibition of antibiotic growth promoters in commercial poultry production is the increase in the infection of opportunistic bacteria like *M. haemolytica*. In current scenario, role of such bacteria may become more significant in causing the conditions similar to those reported in this study. Viral and bacterial co-infections have forced scientist to understand such co-infecting pathogens in experimental poultry models to find new strategies to control their circulation in domestic and wild poultry. This is the first study that has found the bovine strain of *M. haemolytica* in chickens. However, it is not clear if the chicken received contaminated water or feed and consequently harbored the strain in their body. Overall, this study has provided proof that bovine can spread *M. haemolytica* and that can be a potential source of infection in poultry under favorable condition.

Authors contribution: SU, SA, and MAAS designed, planned and executed the study, AR analysed the data and managed the manuscript, SU, MLS analysis of data and write up of the manuscript.

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