

Short Communication

ISOLATION AND MOLECULAR CHARACTERIZATION OF *PASTEURELLA MULTOCIDA* FROM COMMERCIAL LAYER FLOCKS SUFFERING FROM RESPIRATORY SYNDROMES

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ABSTRACT

A total of five sick birds showing respiratory syndromes from each of the five commercial layer flocks of Karachi, Lahore, Vehari and Toba Tek Singh were selected and slaughtered. Liver, heart and long bones from each of the birds were collected and inoculated on brain heart infusion blood agar (BHI-BA). Inoculated agar plates were incubated at 37°C for 24 hours. Small pin head sized non hemolytic colonies having bipolar gram negative coccobacilli were observed. The isolates did not grow on MacConkey's agar and showed positive reaction to indole and H₂S production. The DNA of each bacterial isolate was amplified using universal and capsular serotype specific primers of *P. multocida*. The isolates showing amplicons of 590 and 1048bps, respectively were declared as *P. multocida* type A. These isolates were recovered 100, 60, zero and 20% from Karachi, Lahore, Vehari and Toba Tek Singh, respectively. It was concluded that clinical signs, post mortem changes, cultural and microscopic characteristics, biochemical profile and molecular characterization confirmed the causative agent of the disease as *P. multocida* serotype A.

Key words: Fowl cholera, Layer farms, Polymerase chain reaction, Brain heart infusion blood agar, *Pasteurella multocida*.

INTRODUCTION

Respiratory syndrome is a common problem of most of the commercial poultry farms all over the world. It is caused by either of the bacteria such as *Pasteurella multocida*, *Mycoplasma gallisepticum*, *Haemophilus paragallinarum*, etc or viral diseases such as Newcastle disease, Avian Influenza, Infectious Bronchitis, etc. *P. multocida* is the cause of fowl cholera (FC). FC is one of the contagious bacterial diseases of domestic and wild avian species. Acute form of the disease is characterized by nasal discharge, facial edema, blackening of comb and wattles, ataxia, back ward retraction of head, rise in temperature, off feed and water, dull, depressed with high morbidity (up to 50%) and mortality (up to 10%). In chronic form, the infection localizes hock joints, foot pad, respiratory organs, etc. (Shah *et al.*, 2008).

P. multocida is gram negative, cocco-bacilli, capsular, non-spore former, non motile, aerobe, oxidase positive and non-hemolytic on blood agar. It does not grow on MacConkey's agar but grow on potassium cyanide containing media. It ferments glucose, fructose, mannose, sucrose and galactose (Boyce *et al.*, 2000; De-Alwis, 1999; Pedersen *et al.*, 2003; Tabatabaei *et al.*, 2007).

It remains viable for more than nine weeks in larynx and pharynx of carrier birds (Christiansen *et al.*, 2000). The organism is divided into serotypes on the basis of capsular and somatic (O) antigens. The serotypes are named as A:1, A:3 & A:4 and are common in avian species all over the world (Mohamed *et al.*, 2012; Zahoor *et al.*, 2014). Common serotype of *P. multocida* associated with the disease is A:1 in Asian countries when characterized through polymerase chain reaction (Dziva *et al.*, 2008; Kumar *et al.*, 2004; Townsend *et al.*, 2001).

P. multocida is extensively involved in respiratory syndrome of commercial layers all over the world particularly in countries having hot humid environment but information regarding molecular characterization of indigenous isolates of *P. multocida* is scanty. This study is therefore, designed to isolate *P. multocida* from the layer flocks showing respiratory syndrome and their characterization through conventional and molecular techniques.

MATERIALS AND METHODS

Sources of samples: A total of five sick birds from each of the five commercial layer flocks showing respiratory syndrome at Karachi (Surjani Town), Lahore, Vehari and

Toba Tek Singh were selected and slaughtered. Liver, heart and long bones were aseptically collected and transferred to properly labeled screwed capped sterile plastic containers having peptone water. The samples were placed in a refrigerator at 4°C till further required (Naz *et al.*, 2012).

Isolation and identification of causative agent: Bone marrow, heart blood and liver was inoculated on Brain Heart Infusion (BHI) blood agar and incubated at 37°C for 24 hours. The bacterial isolates were identified on the basis of cultural, morphological and biochemical characteristics (Cheesbrough, 2006; Shigidi and Mustafa, 1979).

Molecular characterization

The pure growth of the bacteria was processed for molecular characterization through polymerase chain reaction (PCR) using universal and capsular serotype specific primers of *Pasteurella multocida* (Table 1) following Townsend *et al.*, (2001; 1998). PCR was performed on 25ul of PCR reaction mixture along with sample DNA. The mixture was composed of PCR buffer-10X (2.5 ul), DNTPs-10 mM (0.5 ul), MgCl₂-25 mM (1.5ul), KMT1T7- 10pmol/ul (0.5ul), KMT1SP6-10pmol/ul (0.5ul), Taq DNA Polymerase-3U/ul (0.3ul), Template DNA (5 ul) and distilled water (14.2ul). Initial denaturation was made at 95°C for 5 min while final denaturation was done at 95°C for 1 min. Annealing temperature was adjusted to 58°C for 1 min. Extension was made at 72°C for 1min. Final denaturation, annealing and extension were repeated 30 times. After that Final extension was done at 72°C for 6 min (Dutta *et al.*, 2001).

Agarose gel electrophoresis

The PCR product (5ul) along with 100 bp DNA ladder was processed for electrophoresis on 1.5% agarose gel (Bhimani *et al.*, 2014). The gel was stained using 1% ethidium bromide solution, visualized and photographed using gel documentation system (Bio Rad, USA).

RESULTS AND DISCUSSION

Commercial layer flocks showing respiratory signs were recorded at Lahore, Vehari, Toba Tek Singh and Karachi, Pakistan. The mortality on the farm was 10% and egg production loss was 30%. The birds were depressed, anorexic with ruffled feathers, mucous discharge from mouth and nares, inflamed joints, cyanotic comb and wattles. On postmortem examination, cloudy air sacs, petechial hemorrhages on serosal membranes and focal hepatic necrosis were observed. Large amount of viscous fluid in proventriculus and intestine, hemorrhages in sub-epicardium, sub-serosal layer of hollow organs and lungs were recorded. Respiratory signs hit many types of domestic as well as

wild birds throughout the world (Rhoades and Rimler, 1984). Researchers have observed the swollen liver containing multiple, small focal areas of necrosis in the acutely affected birds. Large amounts of viscid mucus in the digestive tract particularly in the pharynx, crop and intestine. Catarrhal exudates in esophagus and respiratory tract and caseous material in air sacs are also common. Petechial and ecchymotic hemorrhages are reported in 90% birds (Prantner *et al.*, 1990; Hungerford, 1968; Rhoades, 1964). In chronic cases, combs and wattles show cyanotic appearance while edematous swelling in face and joints are common (Rhoades and Rimler, 1989).

The material from liver, heart and bone marrow showed bacterial growth on blood and tryptic soya agar within 24 hours incubation at 37°C. In septicemic cases, the organisms are recovered from heart blood, trachea, nasal discharge, lungs, joints, liver and spleen. Liver is usually the organ of choice for bacterial culture but bone marrow from the ulna is suitable for culture from scavenged carcasses (Kamran *et al.*, 2014). Each of the isolates showed grey, viscous, mucoid, translucent and non-hemolytic colonies on blood agar after 24 hours incubation at 37°C but failed to grow on MacConkey's agar. Generally, description of colonies is made from 18-24 hrs cultures incubated aerobically at 37°C on enriched media containing serum, blood or dextrose starch (Boyce *et al.*, 2002; Moore *et al.*, 1994; Rhoades and Rimier, 1989). Most of the strains produce large watery mucoid colonies belonging to *P. multocida* (Carter, 1967). Each of the isolates was Gram negative coccobacilli. The bacteria in the *Pasteurella* genus are gram negative and bipolar coccobacilli (Tabatabaei *et al.*, 2007).

Each of the isolate was pathogenic and reactivated by inoculating in rabbits. Death was induced in susceptible rabbits within 18 hours post injection of 24 hours bacterial growth in BHI broth. The bacteria were re-isolated from liver, spleen and bone marrow of the experimentally inoculated rabbits. Tissue impression smears prepared from heart blood and liver when stained with Geimsa stain showed bipolar characteristic. The blood smears from infected field animals usually show bipolar organisms (Das and Bhagwan, 1997). The isolates were positive to oxidase, H₂S production, catalase, urease, indole production, nitrate reduction, citrate utilization, gelatin liquefaction and glucose, arabinose, maltose, lactose, dulcitol, inositol and sucrose fermentation (Table 2).

The DNA of each bacterial isolate was amplified using universal and capsular serotype specific primers. The isolates showing amplicons of 590 and 1048bps were declared as *Pasteurella multocida* type A (Figure 1). The bacterial isolates from outbreaks of layer flocks showing respiratory signs belong to capsular serotype A while, other serotypes such as B, D and E of *Pasteurella* species do not cause infection in avian species (Mohamed *et al.*,

2012; Glisson *et al.*, 2008; Shivachandra *et al.*, 2005; Wilson *et al.*, 1993).

P. multocida type A isolates were recovered from 100, 60, zero and 20% flocks of Karachi, Lahore, Vehari and Toba Tek Singh, respectively. Wet land ecosystem and hot humid environment in Karachi could be supporting factor for high prevalence of the fowl cholera in Karachi area. High density rearing of birds in hot humid environment and lack of vaccination are plausible factors support the prevalence of the disease (Hoffman and Stover, 1942).

It is concluded that clinical signs, post mortem changes of the sick birds, cultural and microscopic characteristics, biochemical reactions and molecular weights of PCR products of the bacterial isolates confirm that causative agent of the disease is *P. multocida* serotype A. It is therefore, recommended that in future vaccine should be prepared from *P. multocida* type A to induce immunoprophylaxis against the disease in different areas of Pakistan.

Table 1. Sequences of primers

Universal primer	Forward Primer	KMT1 KMT1T7	ATCCGCTATTTACCCAGTGG
	Reverse Primer	KMT1SP6	GCTGTAAACGAACTCGCCAC
Serotype specific primer	Forward Primer	A Cap Gene	GATGCCAAAATCGCAGTCAG
	Reverse Primer	A Cap Gene	TGTAGCCATCATTGTTCAGTG

Table 2. Percentage of *Pasteurella multocida* type A positive isolates

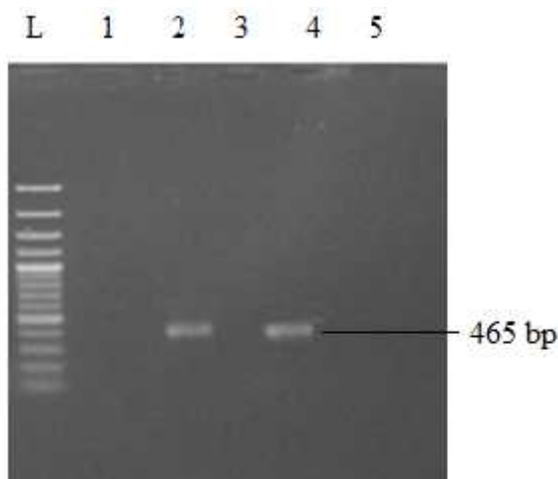
Sources of isolates (n=20)	Flock #	Samples showing typical features (#)		Biochemical reactions***	PCR Results of <i>Pasteurella multocida</i>		Prevalence (%)
		Typical colonies*	Microscopic character**		Universal primer	Capsular primer (A)	
Karachi	1	5	5	5	5	5	100
	2	5	5	5	5	5	100
	3	5	5	3	3	3	60
	4	5	5	4	4	4	80
	5	5	5	5	5	5	100
Lahore	1	4	4	4	4	4	80
	2	5	5	5	5	5	100
	3	5	5	5	5	5	100
	4	0	0	0	0	0	0
	5	0	0	0	0	0	0
Vehari	1	0	0	0	0	0	0
	2	0	0	0	0	0	0
	3	0	0	0	0	0	0
	4	0	0	0	0	0	0
	5	0	0	0	0	0	0
T. T. Singh	1	5	5	5	5	5	100
	2	0	0	0	0	0	0
	3	0	0	0	0	0	0
	4	0	0	0	0	0	0
	5	0	0	0	0	0	0

*Aerobic, visible growth in 24 hours at 37 °C, small pin head sized, smooth, non hemolytic on BHI-B agar, no growth on MaConkey,s agar

** Gram negative, coccobacilli bipolar, pleomorphic, non spore former

*** The isolate of each of the typical colony showed positive reaction to oxidase, H₂S production, catalase, indole production, nitrate reduction, glucose and sucrose fermentation. Moreover, each of the isolate showed negative reaction to urease, citrate, gelatin liquefaction, arbinose, maltose, salicin, lactose, dulcitol and inositol fermentation.

T.T. Singh Toba Tek Singh



Gel showing PCR product of *P. multocida* using universal primers.

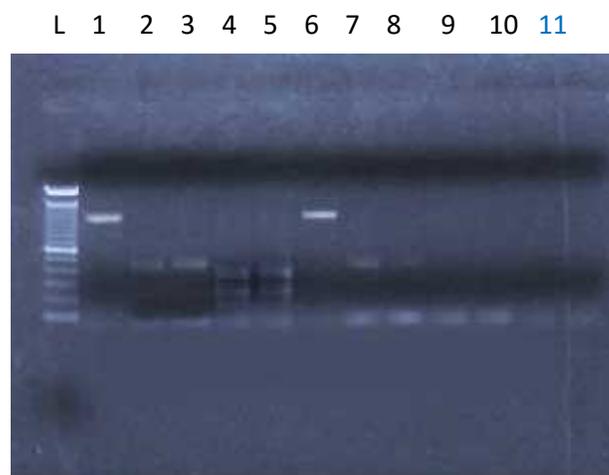
Lane L: Ladder, Lane 1: negative control and Lane 2, 4: amplified product of 465 bp

Figure:1

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Gel showing PCR product of *P. multocida* using serotype A specific primers.

Lane L: Ladder, Lane 10 & 11 negative control, Lane 2,3,4,5,7,8 & 9 suspected test samples, Lane 1 & 6 amplified product of 1048 bps

Figure: 2

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