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Isolation, identification and molecular characterization of *Pasteurella multocida* isolates obtained from emu (*Dromaius novaehollandiae*) in Gujarat state, India

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ABSTRACT

A total of 168 samples (bone marrow, lung tissue, liver and blood clot) were collected from 42 dead emus suspected to have died from fowl cholera. Samples were subjected for cultural isolation on blood agar. Of these, a total of 22 isolates of *Pasteurella* spp. were isolated and characterized biochemically and identified as *P. multocida* by PCR. Isolates were later tested for capsular type by multiplex PCR assay and all were found to be of capsular type A. This is the first report on the isolation, identification and molecular characterization of *P. multocida* from emu in the state of Gujarat, India. This study has revealed that emus are also susceptible to *P. multocida* (fowl cholera) and can act as a potential carrier of the organism.

Key words: *Pasteurella multocida*, emu, PCR, capsular typing, fowl cholera

Introduction

P. multocida is a small, non-motile, gram negative coccobacillary shaped organism that is a commensal of the respiratory tract of many wild and domestic birds, and produces disease when the birds are under stress (HARPER et al., 2006). *P. multocida* carries different types of polysaccharides on its capsule and based on that *P. multocida* is grouped into A, B, D, E and F capsular types (CHRISTENSEN et al., 2005). *P. multocida* isolates obtained from avian species mostly belong to serotype A and cause avian pasteurellosis, which is

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also known as fowl cholera, with incidences of high mortality and morbidity in infected farms, causing significant economic losses all over the world (RHOADES and RIMLER, 1989).

A nucleic acid based diagnostic test has been found to be more sensitive and reliable than the conventional method of bacterial identification. The main advantage of nucleic acid based tests is that they reduce the time consumption and allow the detection of the organism's genome even if it is in minute quantities, thus increasing the sensitivity and specificity of the test (INNIS et al., 1990). PCR is one such test that can be used for the identification of organisms at any level, viz: strain, species, genus or all members of a domain, just by using a specific primer sequence.

Emu farming is similar to poultry farming, in which emus are domesticated. In recent years, commercial farming of emus has gained great importance in India and is considered to be one of the latest emerging species in Indian poultry diversification. With the increase in the numbers of farm-raised ratites throughout Australia, Asia, Africa, Europe and North America, there has been a world-wide increase in the spread of parasitic and microbial diseases associated with these birds. Emus are now used as commercial birds in many countries, including India. To date, very little literature is available concerning fowl cholera in emus, although a report of *P. multocida* infection (Fowl cholera) was recorded by OKOH (1980) and PRABHAKAR et al. (2012) in Ostrich and emu, respectively. There were reports of sudden mortality among emu birds maintained at a private emu farm near the town of Anand in Gujarat state, India. According to the history provided by the farm owner, there was respiratory distress and bleeding from the nasal orifices in some of the birds, while other birds showed no prior symptoms of any kind of diseased state. On post mortem, the birds revealed gross lesions of congestion of the trachea, lungs, liver, spleen and petechial, and ecchymotic hemorrhages on the epicardium, trachea, endocardium, and intestines. The initial few samples received at the Department of Veterinary Microbiology for microbial analysis yielded the growth of *P. multocida* organisms. So an attempt was made to isolate and determine the capsular type of strains of *P. multocida* isolated from the emus which had died due to suspected cases of fowl cholera.

Materials and methods

Sample collection. During this study, 168 samples were collected, namely, bone marrow, lung, liver, and heart blood clot. They were obtained from 42 dead emus originating from a private emu farm located near the town of Anand, Gujarat, India, with suspected *P. multocida* infection (Fowl cholera). They showed congestion of the trachea, lungs, liver, spleen and petechial, and ecchymotic hemorrhages from the epicardium, trachea, endocardium and intestines.

Bacterial strains. The reference strain of *P. multocida* capsular type B was obtained from the Vaccine institute, Gandhinagar, Gujarat, India. Known strains of *P. multocida* capsular type A, D, and F, which were isolated and maintained at the Department of Veterinary Microbiology, Veterinary College, Anand, Gujarat, India, were used as known strains during this study. Known strains of *E. coli, Salmonella* Pullorum*, Klebsiella pneumoniae* and *Pseudomonas aeruginosa* were obtained from the Department of Veterinary Microbiology, Veterinary College, Anand, Gujarat, India and used as the negative control in the present study.

Processing of samples. A loopful of triturated tissue sample was first inoculated in Brain Heart Infusion (BHI) broth at 37 °C for 24 hours, which was sub cultured on 5% blood agar containing neomycin 2.5 µg, potassium tellurite 2.5 µg, tyrothricin 10.0 µg and actidione 1 µg per ml of medium. Identification or confirmation of bacterial species was assessed by observation of the colonial morphology, Gram staining results and biochemical properties: catalase, growth on MacConkey agar, indol production, oxidase reaction, sugar fermentation of glucose, mannitol, mannose, sucrose, arabinose, dulcitol, inositol, lactose, salicin, galactose, maltose, sorbitol, trehalose and xylose (CARTER, 1967).

DNA extraction. Genomic DNA of all the isolates was extracted following the method described by WILSON (1987). Briefly, bacterial cells were lysed by EDTA (0.5 M), SDS (0.5%) (HiMedia, India) and proteinase K (20 ng/ml) (Fermentas, Thermo Fisher Scientific, USA) followed by phenol-choloroform-isoamyl alcohol extraction. The DNA was precipitated by addition of sodium acetate and absolute ethanol, and was then washed with 70% ethanol, dried at room temperature and resuspended in TE buffer (pH 8). The concentration of DNA was measured spectrophotometrically (Nanodrop 1000, Thermo scientific, USA) at 260 nm and adjusted to 30 ng/µl, which was later used as template DNA.

Pasteurella multocida specific PCR (PM-PCR). For the specific detection of *P. multocida* isolates, PCR was carried out using primer sets designed from the sequence of the KMT1 by TOWNSEND et al. (1998) (25 µL of PCR reaction mixture containing, 12.5 µL of 2x PCR master mix (Fermentas, Thermo Fisher Scientific, USA), 10 pmol of each primer (Eurofins MWG Operon, Germany), 90 ng (30 ng/µL) of DNA template. The reaction mixture was subjected to amplification in a thermal cycler (Veriti, Applied Biosystem, USA) according to the following program: initial denaturation at 95 °C for 4 min, followed by 30 cycles of denaturation at 95 °C for 1 min, annealing at 55 °C for 1 min, extension at 72 °C for 1 min and a final extension of 72 °C for 6 min. The amplified product was separated by agarose gel electrophoresis in 2% agarose gel at 5 V/cm for 1 hour and stained with ethidium bromide (1% solution). DNA fragments were observed

under UV transilluminator and photographed (SynGene, Gene genius bioImaging System, UK).

Capsular typing with multiplex PCR: Capsular typing of the isolates was carried out by multiplex PCR using capsular specific primers (TOWNSEND et al., 2001) (Table 1).

Table 1. Primers used for the detection of capsular type of *P. multocida* isolates

Gene designated	Primer sequence (5'- 3')		Size of amplified products (bp)	Reference
capA	Forward	TGCCAAAATCGCAGTCAG	1,044 bp	TOWNSEND et al. (2001)
	Reverse	TTGCCATCATTGTCAGTG		
capB	Forward	CATTTATCCAAGCTCCACC	760 bp	TOWNSEND et al. (2001)
	Reverse	GCCCGAGAGTTTCAATCC		
capD	Forward	TTACAAAAGAAAGACTAGGAGCCC	657 bp	TOWNSEND et al. (2001)
	Reverse	CATCTACCCACTCAACCATATCAG		
capF	Forward	AATCGGAGAACGCAGAAATCAG	851 bp	TOWNSEND et al. (2001)
	Reverse	TTCCGCCGTCAATTACTCTG		

50 μL PCR reaction mixture contained 1 U Taq DNA polymerase (Fermentas, Thermo Fisher Scientific, USA), 3.2 mM of each primer (Eurofins MWG Operon, Germany), 200 μM of each dNTP (Fermentas, Thermo Fisher Scientific, USA), 1x PCR buffer, and 2 mM $MgCl_2$ (Fermentas, Thermo Fisher Scientific, USA). Amplification was carried out for 35 cycles, each cycle consisting of DNA denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 30 s. The cycles were preceded by initial denaturation at 95 °C for 5 min and followed by final extension at 72 °C for 10 min. To confirm the targeted PCR amplification, 5 μL of PCR product from each tube was mixed with 1 μL of 6X gel loading buffer and electrophoresed on 1.5 % agarose gel at a 80 V for 30 minutes in 0.5X TBE buffer along with 100 bp DNA Ladder (GeneRuler- Fermentas, Thermo Fisher Scientific, USA) and stained with ethidium bromide (1% solution). The amplified product was visualized as a single compact band of the expected size under UV light, and documented by the gel documentation system (SynGene, Gene genius bioImaging System, UK).

Results

Isolation and identification. A total of 22 isolates from 42 suspected birds were identified as *P. multocida* on the basis of the conventional bacteriological technique. The isolated bacterial colonies on blood agar plates were small, glistening, mucoid and dew drop like, and appeared as Gram-negative coccobacilli when stained with Gram's stain. The isolates failed to grow on MacConkey agar and were found to be non-haemolytic on blood agar. Details of isolation from different tissues are given in Table 2.

Table 2. Detail of isolation of *P. multocida* from various tissue sample of 42 dead birds

Sr. No.	Identity number	Type of tissue sample			
		Bone marrow	Liver	Lung	Blood Clot
1	PAE-1	(+)	(+)	-	-
2	PAE-2	(+)	(+)	-	-
3	PAE-3	(+)	(+)	(+)	-
4	PAE-4	(+)	-	-	-
5	PAE-5	(+)	(+)	(+)	(+)
6	PAE-6	(+)	-	-	-
7	PAE-7	(+)	(+)	-	-
8	PAE-8	(+)	(+)	(+)	-
9	PAE-9	(+)	(+)	-	-
10	PAE-10	(+)	-	-	-
11	PAE-11	(+)	(+)	-	-
12	PAE-12	(+)	-	-	-
13	PAE-13	(+)	(+)	-	(+)
14	PAE-14	-	(+)	-	-
15	PAE-15	(+)	(+)	(+)	(+)
16	PAE-16	(+)	(+)	-	-
17	PAE-17	(+)	(+)	-	(+)
18	PAE-18	(+)	-	-	-
19	PAE-19	-	(+)	(+)	(+)
20	PAE-20	(+)	(+)	-	(+)
21	PAE-21	(+)	-	(+)	-
22	PAE-22	(+)	(+)	(+)	(+)
23	N	NA	NA	NA	NA
24	N	NA	NA	NA	NA
25	N	NA	NA	NA	NA
26	N	NA	NA	NA	NA
27	N	NA	NA	NA	NA
28	N	NA	NA	NA	NA
29	N	NA	NA	NA	NA

Table 2. Detail of isolation of *P. multocida* from various tissue sample of 42 dead birds (continued)

30	N	NA	NA	NA	NA
31	N	NA	NA	NA	NA
32	N	NA	NA	NA	NA
33	N	NA	NA	NA	NA
34	N	NA	NA	NA	NA
35	N	NA	NA	NA	NA
36	N	NA	NA	NA	NA
37	N	NA	NA	NA	NA
38	N	NA	NA	NA	NA
39	N	NA	NA	NA	NA
40	N	NA	NA	NA	NA
41	N	NA	NA	NA	NA
42	N	NA	NA	NA	NA

N: Negative for isolation, NA: Not Applicable, (+): Positve, - : Negative

Biochemical characterization. Biochemical tests revealed that all the isolates of *P. multocida* were found to be positive for oxidase, catalase, indole production, nitrate reduction; all the isolates were found negative for citrate utilization test. All the isolates successfully fermented glucose, arabinose, maltose, mannitol, mannose, sucrose, galactose, and sorbitol, but failed to act on dulcitol, inositol, salicin, xylose, trehalose and lactose.

PM-PCR. All 22 isolates, including the known strain, were subjected to PM-PCR and were found to yield an expected product of 465 bp in size. None of the other gram negative bacteria viz., *E. coli, Salmonella* Pullorum, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* yielded any amplified product on PCR (Fig. 1).

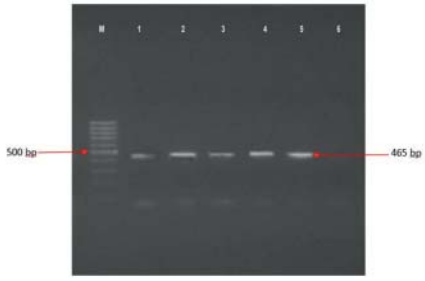

Fig. 1. Agarose gel showing PM-PCR amplified product of 465 bp for KMT gene using specific primer. LANE -M: ladder; 1-4: Isolates; 5: Positive control; 6: Negative control

Multiplex PCR assay for Capsular typing: Multiplex PCR was carried out for the detection of the specific gene responsible for the biosynthesis of the capsule yielded an amplified product of 1,044 bp similar to that of the known strain of Capsular type A, while the known strains of capsular type B, D and F yielded products of 760, 657 and 851 bp, respectively. The finding identified all the 22 isolates as capsular type A.

Discussion

The present finding of *P. multocida* infection in emus is probably the first report in the state of Gujarat, India. In the present study, PCR and multiplex PCR for capsular type detection was found to be a rapid and sensitive method, as reported earlier by other authors (TOWNSEND et al., 2001, MOHAMED et al., 2012). There is a paucity of literature citing the outbreak of *P. multocida* (Fowl cholera) infection in emus although it has been reported in ostrich (OKOH, 1980) and in emu (PRABHAKAR et al., 2012). 22 isolates were obtained from 42 emus which had died as suspected cases of *P. multocida* infection (fowl cholera). Fowl cholera generally occurs in water fowl and chickens, its occurrence in other avian species indicates its role as reservoir and they could transmit the disease to other susceptible flocks (TAYLOR, 1981). Based on the results of the sugar utilization test, isolates were found to be similar to that of group II, an unclassified biotype that failed to ferment dulcitol and xylose, and weakly fermented sorbitol as reported by HUNT GERARDO et al. (2001) isolated from dog and cat bite wounds. Similar sugar utilization characters were also reported by VARGA et al. (2013) for the isolates obtained from geese. As reported by MOHAMMED et al. (2012) PCR was found to be sensitive and effective for the rapid diagnosis of *P. multocida* infection. Multiplex PCR assay is very useful and quick for the capsular based molecular typing of the *P. multocida*. As reported by TOWNSEND et al. (2001) most fowl cholera outbreaks occur due to infection with *P. multocida* capsular type A. Detection of *P. multocida* infection in emus clearly indicates that it might have been transmitted to the emus through local or wild birds, or from the nearby established poultry farm as reported by BOTZLER (1991).

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SAŽETAK

Ukupno je 168 uzoraka (koštane srži, tkiva plućiju, jetre i krvnih ugrušaka) bilo prikupljeno od 42 emua uginula pod sumnjom na koleru peradi. Uzorci su bili nacijepljeni na krvni agar. Ukupno su bila izdvojena

22 izolata *Pasteurella* spp. te su biokemijski i lančanom reakcijom polimerazom identificirani kao vrsta *P. multocida*. Izolati su zatim bili pretraženi na kapsulni tip višestrukom lančanom reakcijom polimerazom te je ustanovljeno da su svi pripadali kapsulnom tipu A. Ovo je prvo izvješće o izdvajanju, identifikaciji i molekularnoj karakterizaciji vrste *P. multocida* izdvojenoj iz emua u državi Gujarat u Indiji. Istraživanje je pokazalo da je i emu prijemljiv na infekciju vrstom *P. multocida*, uzročnikom kolere peradi te može biti njezin kliconoša.

Ključne riječi: *Pasteurella multocida*, emu, lančana reakcija polimerazom, kapsulna tipizacija, kolera peradi