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Molecular detection and antibiogram profile of *Pasteurella multocida* isolated from backyard poultry and turkeys from Puducherry, India

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ABSTRACT

Fowl cholera, caused by *Pasteurella multocida* serogroup A, is a highly infectious and economically significant disease affecting commercial and backyard poultry, including turkeys. In this comprehensive study, we examined ten birds (chickens and turkeys) with the history of high mortality and exhibited clinical signs such as nasal discharges, anorexia, and respiratory distress. During post-mortem examinations, multifocal necrosis of the liver, edematous lungs and pericarditis were observed. Microscopic examinations of Leishman-stained heart blood smears and liver impression smears revealed the presence of bipolar organisms. The tissue samples were subjected to bacterial isolation and identification using conventional biochemical tests, detecting five *P. multocida* isolates from chickens and five from turkeys. All ten isolates were further confirmed through Polymerase Chain Reaction (PCR) targeting the KMT gene, a species-specific primer for *P. multocida*, ensuring the accuracy of our findings. The antibiogram indicated that the isolates were susceptible to Enrofloxacin (100%), Tetracycline (80%), Gentamicin (60%), and Ciprofloxacin (40%), while all the isolates were resistant to Penicillin G (100%) and most were resistant to Sulphamethoxazole (90%). This study indicates that fowl cholera was prevalent in backyard chickens and turkeys in the Puducherry region, so backyard poultry farmers are encouraged to implement regular vaccination practices against fowl cholera.

Introduction

Pasteurella multocida, responsible for fowl cholera, is a highly contagious septicemic infection impacting poultry production worldwide. It is an economically important bacterial disease of commercial and backyard poultry, including turkeys. India has about 851.8 million poultry as per the 20th Livestock Census [1], which is estimated to be about 16.8% of the world's total poultry population. In rural areas, backyard poultry production is a primary source of eggs and meats. Free-range backyard poultry are an important livestock for many rural families worldwide. It also has an important role in providing family income by creating employment opportunities in rural areas, particularly among the landless, small, and marginal farmers in the Indian subcontinent.

Fowl Cholera is a severe disease of poultry that affects both domestic and wild avian species such as chickens, turkeys, geese, ducks, and waterfowls. It is present as a commensal in the nasal passage of healthy livestock and poultry and can cause disease under unfavourable conditions. The transmission of fowl cholera is mainly through the carrier birds [2]. The severity of the infection and its incidence are influenced by overcrowding, climate, nutrition, and concurrent diseases [3].

It can be of acute or chronic forms, with the clinical signs occurring very late in the infection. The symptoms include nasal discharges, facial oedema, blackening of the comb and wattles, ataxia, backward retraction of the head, fever, anorexia, depression, ruffled feathers, diarrhoea and an increased respiratory rate and high morbidity (up to 50%) and mortality (up to 10%) [4]. In the chronic form, the infection targets hock joints, foot

pads, respiratory organs, and other areas [5]. Typical PM findings of *Pasteurellosis* include pin-point hemorrhagic lesions in the epicardium, multifocal necrosis in the liver, congestion and consolidation of the lungs, congestion of the spleen and catarrhal enteritis [6].

P. multocida is a Gram-negative coccobacilli, non-motile, capsulated and non-spore forming bacteria occurring singly, in pairs, or occasionally as pleomorphic arrangement belonging to the *Pasteurellaceae* family. It can survive in carrier birds' larynx and pharynx for more than nine weeks.

The organism is classified into serotypes based on capsular and somatic (O) antigens. Based on Indirect Haemagglutination Assay (IHA), five capsular serotypes (A, B, D, E, and F) are found in *P. multocida*. Each serotype is specifically associated with the specific host [7], and Fowl cholera in avian species is associated with serotype A [8]. By somatic serotyping, 16 serogroups (1-16) were found based on an agar gel precipitation test [9]. The strain of *P. multocida* and host species affected highly varies, based on the virulence capacity of the organism. Laying flocks are more susceptible to fowl cholera than younger chickens.

Recent studies have reported that backyard poultry may act as carriers of *P. multocida* [2]. However, clinical conditions are not reported often, and the chronic signs are shown to be normal in this farming system [4]. Accurate and timely diagnoses are essential for controlling such infectious diseases in poultry. Therefore, diagnosing the *P. multocida* infection early in poultry is important to avoid huge mortality in the avian species. The information regarding *P. multocida* infection in backyard poultry and turkeys is scanty in India. Therefore, the objective of the present study was to detect *P. multocida* infection in backyard poultry and turkeys in Puducherry.

Materials and methods

Sample collection and processing

A total of 10 birds, including five backyard chickens (20 weeks of age) and five non-descript turkeys (16 weeks of age) from 5 different poultry farms in and around Puducherry, were presented for PM examination at Rajiv Gandhi Institute of Veterinary Education and Research (RIVER), Puducherry with the history of high mortality in the flocks. The clinical signs reported were nasal discharges, anorexia, and respiratory distress before death. The PM lesions included multifocal necrosis of the liver, edematous lungs, and pin-point hemorrhagic pericarditis. Heart blood, pieces of liver, and lungs were collected aseptically and transported to the laboratory for microscopic examination followed by isolation and identification of the bacterial pathogens.

Microscopic examination

Leishman's staining technique was used to stain the heart blood smear and the impression smear from the lung and liver, which were examined under 100X oil immersion microscopy to detect bipolar organisms.

Isolation and identification of *P. multocida*

The pooled samples of heart blood, lung, and liver were subjected to inoculation onto 5% Sheep blood agar and incubated at 37ºC for 24 to 48 hours in a candle jar for the isolation of *P. multocida*. Growth on blood agar in the form of non-hemolytic dewdrop colonies was subjected to Gram staining. The Gram-negative cocco-bacilli were taken up for further identification based on colony morphology on Muller Hinton agar and MacConkey's agar, followed by microscopic examination and biochemical tests. The biochemical tests include catalase, oxidase, indole, methyl-red, voges-proskauer test, citrate, urease, and sugar fermentation tests specifically for *P. multocida* [10].

PCR assay for detection of *P. multocida*

The template DNA was extracted from bacterial colonies by boiling and snap chilling methods, as described by the previous study [11]. The OIE-recommended primers for the detection of *P. multocida* were used in this study (Table 1) [12, 13]. The PCR assay was optimized with 12.5µl reaction mixture containing 2.5µl of DNA template, 6.25µl of 2X Taq DNA Polymerase Master Mix RED (Amplicon), 1µl each of forward and reverse primers (10 pmol/µl) and the rest of the volume is made by adding nuclease-free water. The cycling conditions were as follows: initial denaturation at 95°C for 6 min; 35 cycles of denaturation at 95°C for 2 mins, annealing at 56°C for 45 sec, and elongation at 72°C for 1 min and a final elongation step at 72°C for 10 min. The PCR amplification was done in an automated thermal cycler (Eppendorf Master Cycle, Germany). PCR products and 100bp DNA ladder were electrophoresed in 1.5% agarose gel containing ethidium bromide (0.5µg/ml). PCR products were visualized under a UV transilluminator.

Table 1. Primer pairs for the detection of *P. multocida*

Target gene	Primer Sequence (5'-3')	Amplicon size
KMT1SP6	GCTGTAAACGAACTCGCCAC	460 bp [12, 13]
KMT1T7	ATCCGCTATTTACCCAGTGG	

Antibiotic Sensitivity Test (ABST) of *P. multocida* isolates

All the *P. multocida* positive isolates were subjected to antibiotic sensitivity tests using eight antibiotic agents by the disc diffusion method [14]. The antimicrobial agents used were Enrofloxacin (EX, 5µg), Tetracycline (TE, 30µg), Chloramphenicol (10µg), Sulphamethoxazole (SXT, 5µg), Ciprofloxacin (CIP, 5µg), Gentamicin (GEN, 10µg) and Penicillin –G

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(10µg). The inoculated plates were incubated at 37°C for 24 hrs. The interpretation of the zone of inhibition was read as per Clinical Laboratory Standard Institute (CLSI) guidelines [15].

Results and discussion

This study collected post-mortem samples from 10 birds, including five backyard chickens and five non-descript turkeys. The gross lesions included multifocal necrosis on the liver, hyperaemic and oedematous lesions in the lungs and pin-point haemorrhagic pericarditis (Fig. 1). Bipolar organism (safety pin appearance) was detected from heart blood smear and impression smear of liver and lung by using Leishman's staining technique (Fig. 2). On bacterial isolation, small, dewdrop, non-hemolytic colonies, were found on sheep blood agar (Fig. 3). Based on cultural, morphological and biochemical characteristics, ten *P. multocida* isolates were obtained (Table 2). The results of morphological, cultural and biochemical tests were in agreement with the previous studies [16, 17].

P. multocida-specific PCR is a sensitive, specific, and rapid method for detecting *P. multocida* in birds [12, 13]. In our study, all ten isolates (100%) were further confirmed by PCR using species-specific primer targeting the KMT1 gene for *P. multocida* with a product size of 460 bp, as recommended by OIE (Fig. 4). Similarly, the previous studies reported the prevalence of *P. multocida* with 94 (n=94, 100%) and 20 (n=20, 100%) isolates from cases of avian Pasteurellosis affecting chickens from different geographical locations of India and Bangladesh respectively [18, 19]. In contrast, previous studies reported the presence of *P. multocida* with four isolates from 35 dead chickens (11.43%) in Bangladesh [3] and 21 isolates from 275 backyard chickens (7.6%) in Upper Egypt [4].

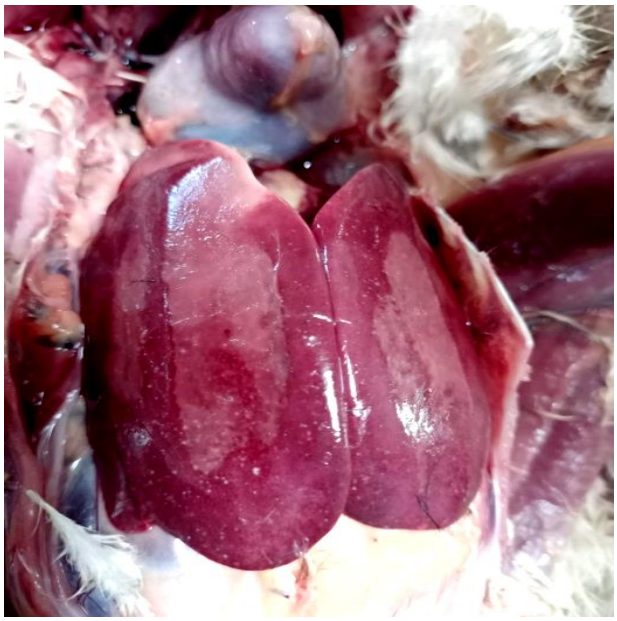

Fig 1. Multifocal necrosis on the liver

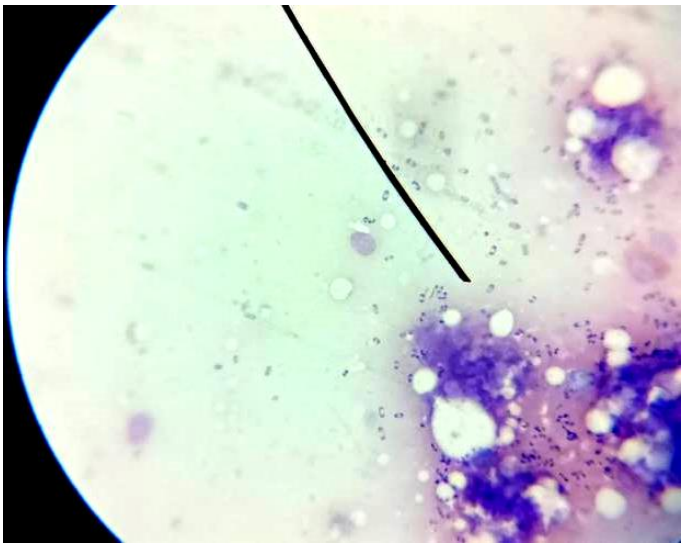

Fig 2. Safety pin appearance of bipolar organism (*P. multocida*) in liver impression smear stained by Leishman's technique

Fig 3. Small, dewdrop, non-haemolytic colonies on sheep blood agar

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Table 2. Morphological and biochemical characterization of the *P. multocida* isolated from backyard chickens and turkeys

Gram staining	Motility	Capsule	Growth aerobically	Biochemical tests							SFT			
				C	O	I	MR	VP	C	U	Glu	Suc	Lac	Man
Gram negative coccobacilli	-	+	+	+	+	+	-	-	-	-	+	+	-	+

C: *Catalase,* ***O:*** *Oxidase,* ***I:*** *Indole test,* ***MR:*** *Methyl red test,* ***VP:*** *Voges-Prausker test* ***C:*** *Citrate utilization test,* ***U:*** *Urease;* ***SFT:*** *Sugar Fermentation Tests (****Glu****- Glucose,* ***Suc****- Sucrose,* ***Lac****- Lactose,* ***Man****- Mannitol)*

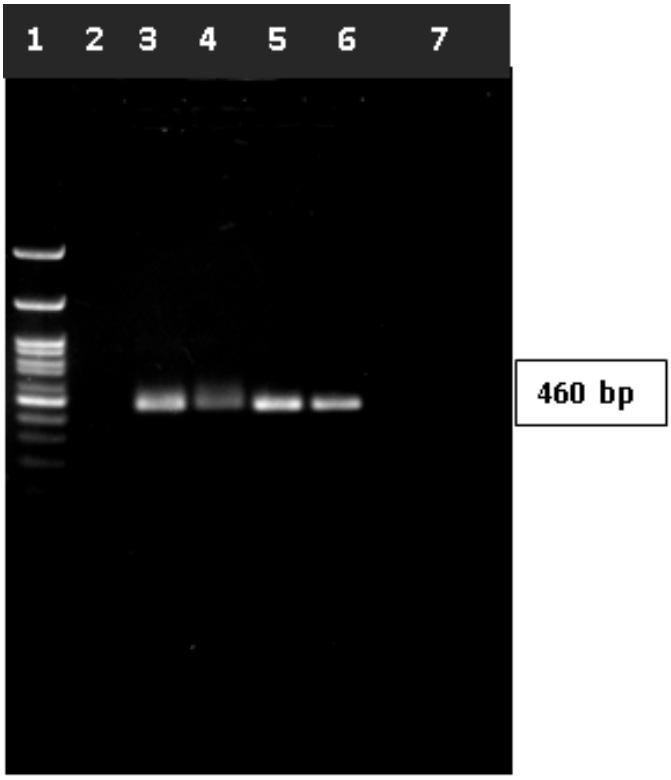

Fig 4. Agarose gel electrophoresis showing the results of PCR amplified product of *kmt* gene of *P. multocida* with size 460 bp. Lane 1- 100 bp ladder, 2 & 3 Negative and Positive control, Lane 4, 5 and 6 *P.multocida* positive field isolates.

Antibiotic sensitivity test of the *P. multocida* isolates revealed that the organisms were sensitive to Enrofloxacin (100%), Tetracycline (80%), Gentamicin (60%), Ciprofloxacin (40%) and showed resistant to Penicillin G (100%) and Sulphamethoxazole (90%) (Table 3). Similar findings were reported in the previous study, with the isolated strains showing resistance (85.7%) to Penicillin G, Streptomycin and Sulfadiazine [20]. The previous study by Bauer also showed the antibiotic sensitivity of *P. multocida* in turkeys, in which the isolates were sensitive to Enrofloxacin, Amikacin, Ampicillin, Ceftiofur, Cephalothin, Gentamicin, and Oxacillin [21]. Varying degrees of sensitivity to Florfenicol, Ciprofloxacin and Trimethoprim-Sulfamethoxazole were observed in Upper Egypt [4], but all these isolates showed resistance (100%) to Tetracycline and Amoxicillin, and 40 % were resistant to Doxycycline.

Table 3. Antibiogram results of the *Pasteurella multocida* isolated from backyard chickens and turkeys

Antibiotics	Sensitive	Intermediate	Resistant
Enrofloxacin	100% (10)	-	-
Tetracycline	80% (8)	20% (2)	-
Gentamicin	60% (7)	-	40% (4)
Sulphamethoxazole	-	10% (1)	90% (9)
Chloramphenicol	20% (2)	60% (6)	20% (2)
Ciprofloxacin	40% (4)	50% (5)	10% (1)
Penicillin –G	-	-	100% (10)

Conclusion

Post-mortem examinations, microscopy, bacterial cultures, biochemical tests, and PCR assays of the bacterial isolates confirmed that the cause of the disease in the turkeys and chickens was *Pasteurella multocida*. The isolates of *P. multocida* in this study were found to be 100% sensitive to Enrofloxacin, Tetracycline, and Gentamicin. However, these isolates exhibited resistance to commonly used antibiotics such as Sulphamethoxazole and Penicillin-G. Therefore, rapid and accurate diagnosis through PCR, early initiation of treatment based on antibiogram results, and implementing good management practices may help reduce the spread of fowl cholera and decrease mortality rates among backyard birds. Backyard poultry farmers should also be encouraged to adopt regular vaccination practices against fowl cholera.

Ethical approval

Ethical approval is not required since this study does not involve any animal experimentation.

Disclosure statement

The author(s) reported no potential conflict of interest.

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