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Molecular detection and characterization of *Pasteurella multocida* from migratory ducks in the Cauvery delta region of Tamil Nadu

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Abstract
The present investigation was performed to find out the etiological agent in duck mortality in migratory flock by conventional and molecular methods. Around 150 ducks aged 10 months were maintained in Neivasal village, Orathanadu taluk, Thanjavur district. Sudden mortality with the history of dullness, depression and mucous discharge from the mouth were observed in the flock during the month of March 2017. On postmortem examination, necrotic foci in liver and hemorrhages in heart were found. Liver, heart and bone marrow samples from 5 dead birds were collected aseptically for bacteriological isolation and identification. Microscopic examination of colonies on sheep blood agar revealed gram negative, non-motile and non-spore forming coccobacilli or rod. Biochemically, the isolate was found to be positive for catalase, oxidase and indole. Further, Polymerase Chain Reaction (PCR) using *Pasteurella multocida* specific primers derived from KMT1 gene was employed and the isolate was confirmed as *Pasteurella multocida* by PCR. All 15 samples were found to be positive for *Pasteurella multocida* by conventional and molecular tests. Later, the isolate was subjected to sequence analysis and characterized as *Pasteurella multocida*. It is concluded that the disease outbreak in migratory ducks was diagnosed as *Pasteurella multocida* by conventional and molecular methods and phylogenetically closely related to duck isolates of Kerala.

Keywords: Migratory duck, *Pasteurella multocida*, PM-PCR and phylogenetic analysis

1. Introduction
Fowl cholera caused by *P.multocida* which is a highly contagious and severe disease of poultry. *P. multocida* is a gram negative, non-motile, non-spore forming coccobacilli that act as commensals in the respiratory tract of many avian species and produces disease in birds under stress [1]. It is distributed worldwide and causes huge economic losses to poultry rearing countries [2]. It is associated with high morbidity and mortality particularly chicken and ducks. Clinical signs include fever, depression, mucous discharge from the mouth, ruffled feathers and cyanosis of comb and wattles [3, 4]. It causes variety of diseases in livestock namely hemorrhagic septicemia in cattle and buffaloes, atrophic rhinitis in pigs, pneumonic and septicaemic Pasteurellosis in sheep and goats and snuffles in rabbits [5]. *P.multocida* is categorized into five serogroups A, B, D, E and F based on the presence of capsular antigens [6]. It has been reported that serogroup A, rarely F and D cause avian pasteurellosis [7]. It was further classified into 16 serotypes based on LPS antigens [8].
Carrier birds also involve in the transmission of fowl cholera [9]. *P. multocida* also act as causative agent for duck mortality [10, 11, 12]. Cross transmissions of *P. multocida* may occur between ducks and chickens and vice versa [13]. In India ducks are traditionally reared by poor farmers, agricultural landless and marginal farmers in nomadic style [14].
Clinical diagnosis of disease was done by herd history, typical signs, morbidity, mortality and gross pathological lesions. Laboratory diagnosis of the disease can be made based on isolation and identification of the causative bacterium by cultural and biochemical methods. Samples include heart, liver, spleen, bone marrow may yield pure culture of *P. multocida* [4].
In recent years, nucleic acid based diagnostic test have proved beneficial in overcoming some limitations of conventional method of bacterial identification [15]. Nucleic acid based assays allow the detection of organism's genome directly from clinical samples even if it is in small quantities [16].

Molecular technique is the effective tool for accurate and early diagnoses of any infectious disease since the conventional cultural and biochemical tests are time consuming and also less sensitive as compared to molecular technique.

The aim of the present study was to isolate and identify the causative agent of duck mortality by conventional and molecular techniques. In addition, the isolate was also characterized by phylogenetic analysis.

2. Materials and Methods

2.1 Sample Collection

Around 150 ducks aged 10 months were maintained in Neivasla village, Orathanadu taluk, Thanjavur district, Tamil Nadu. Sudden mortality with history of dullness, depression and mucous discharge from the mouth were observed in the flock during the month of March 2017. On postmortem examination, liver, bone marrow and heart samples were collected aseptically from 5 birds suspected died of avian Pasteurellosis. For bacterial isolation and identification, the samples were immediately transported to the Department of Veterinary Microbiology, Veterinary College and Research Institute, Orathanadu, Thanjavur district, Tamil Nadu.

2.2 Bacteriological Examination

The samples were inoculated into 5 % sheep Blood agar, MacConkey agar, Brain heart infusion agar and incubated aerobically for 24 hours at 37^0C. Small narrow zone hemolytic colonies were observed in blood agar and colonies were subjected to gram staining and routine biochemical tests such as IMVIC tests, sugar fermentation tests, catalase and oxidase test were carried out [17].

2.3 DNA Extraction

DNA was extracted from the suspected colony by boiling lysis method. One or two colonies from the suspicious cultures were picked up and suspended into 1.5 ml of micro centrifuge tube containing 50 µl of molecular grade water. The tube was placed in a boiling water bath for 10 minutes followed by snap chilling and centrifugation at 13000 rpm for 5 minutes. Without disturbing the pellet, the supernatant was removed and stored at -20^0C until for further analysis.

2.4 Polymerase chain reaction (PCR)

Polymerase chain reaction was performed using *P. multocida* species specific primers KMT ISP 6 and KMT 177 designed by Townsend *et al*. (1998) to amplify KMT1gene. The PCR protocol was followed as; 95 ^{0}C for 15 minutes; 40 cycles of 94 ^{0}C for 30 seconds, 60 ^{0}C for 30 seconds, 72 ^{0}C for 30 seconds and final extension of 72 ^{0}C for 10 minutes [18]. The analysis of PCR product was carried out in 1.5 per cent agarose gel stained with ethidium bromide (0.5µg/ml) and documented under Gel documentation system. The KMT1 gene amplicons were purified by using Hiyield plus PCR purification kit (cat#QPP100-Real biotech Corporation, Taiwan) following manufacturer's instruction [19].

2.5 Phylogenetic analysis

Purified KMT1 gene amplicons was subjected to sequencing by Sanger dideoxy sequencing method in an automated sequencer. The multiple sequence alignment was carried out using the Mega 5.2 software to generate sequence analysis data. The parameters used for sequence analysis were multiple sequence alignment (Clustal W). The phylogenetic tree was developed using Neighbour Joining (NJ) algorithm using bootstrap values and distance in Mega 5.2 software. Homology searches were performed with the NCBI database and BLAST.

3. Results

On Leishman's staining, tissue impression smears revealed characteristic bipolar organisms suggestive of *Pasteurella* species. In blood agar, the isolated bacteria produce dew drop, mucoid and non haemolytic colonies. It is failed to grow in MacConkey agar. Gram staining of the organism revealed Gram negative coccobacillary rods. Biochemical tests gave positive for indole production, nitrate reduction, oxidase and catalase production which correspond to characters of *P.multocida* [4, 17, 20]. Further, it was confirmed by *P.multocida* species specific PCR which gave positive results for 460 bp gene fragment of KMT 1 gene using primers as per Townsend *et al*.[18] shown in the Fig 1. The details of isolation of *P.multocida* from different tissues of ducks were shown in Table 1.

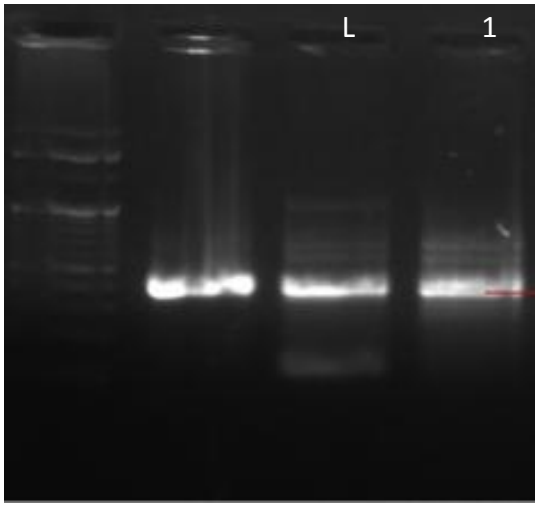

Fig 1: L -100bp ladder, 1, 2 and 3 positive samples with a band at 460bp with *P. multocida* specific primer.

Table 1: Details of isolation of *P. multocida* from different tissues of ducks

S. No	Tissues	No. of Samples	Biochemical tests			PCR	Percentage (%)
			Catalase	Oxidase	Indole		
1.	Liver	5	5	5	5	5	100
2.	Heart	5	5	5	5	5	100
3.	Bone Marrow	5	5	5	5	5	100

The partial coding sequence (cds) of KMT1 gene of this isolate was submitted to GenBank and the accession number MF627691 was obtained for KMT1 gene of OND duck strain. The phylogenetic tree showed a close cluster with *P.multocida* isolates from Duck, Kerala and avian vaccine isolates from Iran as shown in the figure 2. Sequence analysis of the duck isolate showed 99-100% sequence homology with other *P.multocida* isolates available in NCBI database.

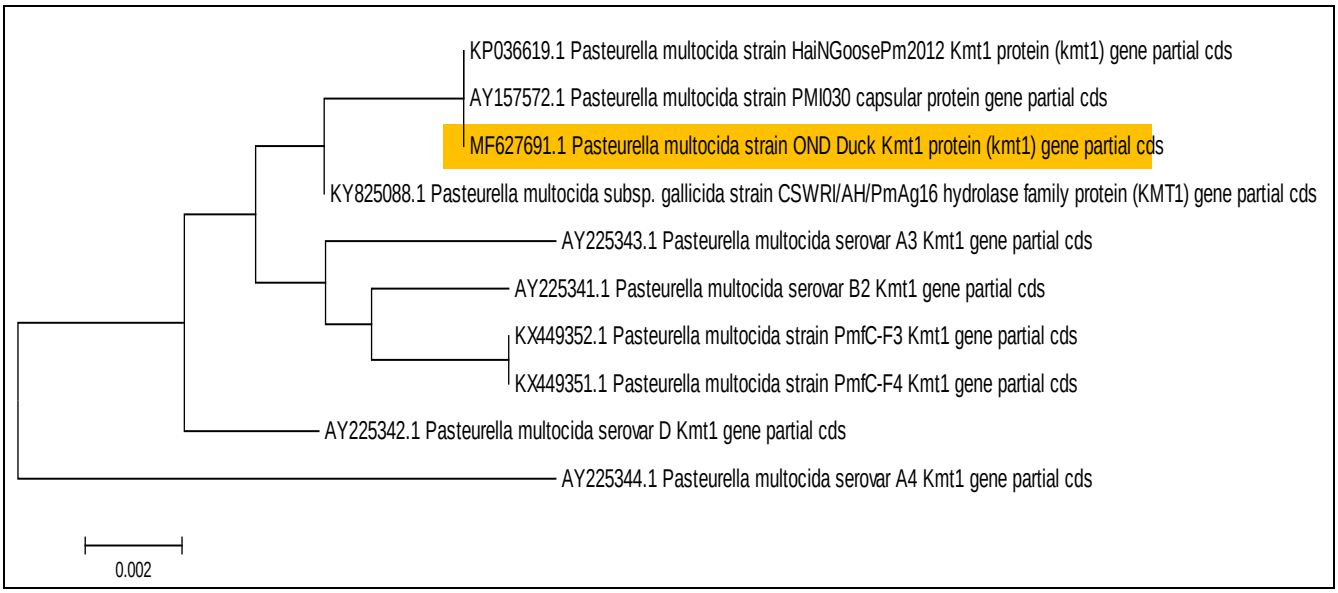

Fig 2: Phylogenetic tree based on partial Kmt1 gene sequences of *Pasteurella multocida* isolate.

4. Discussion

Pasteurella multocida is an important opportunistic respiratory pathogen which causes high morbidity and mortality in domestic birds and wild animals of tropical climate. *P.multocida* infection has also been reported in ostrich [21], emu [15, 22]. Chickens and water fowl act as natural host for Pasteurellosis. It has been reported that occurrence of *P.multocida* infection in other avian species point out its role as reservoir and they could spread the disease to other susceptible flocks [18]. *P.multocida* cause major disease on duck farms results in severe economic losses. Hence, it is essential to choose a rapid and reliable detection method in order to contain the disease.

In the present study, *P. multocida* was isolated and identified from ducks by conventional bacteriological method. On blood agar, the isolated bacteria produced non hemolytic, mucoid, small, round, whitish colonies and did not grow in MacConkey agar

as reported by various authors [23, 24]. Gram's staining of the organism revealed Gram negative small rod shaped or coccobacillary. Biochemical tests exposed that the isolates were catalase, oxidase and indole positive. The results of morphological, cultural and biochemical tests are in agreement with past studies [23, 25, 26].

In addition to conventional methods, *P. multocida* was also isolated and identified by PM-PCR from migratory ducks in Cauvery delta region of Tamil Nadu by amplifying the KMT1 gene. Our results obtained agree with *P.multocida* stains which were isolated from avian origin [15, 27].

It is apparent from present and past studies, *P. multocida* specific PCR provides rapid identification of avian pasteurellosis irrespective of serotypes and can be used as rapid, robust and highly specific diagnostic method for confirmatory detection of *P. multocida* infection [4, 28, 29]. Molecular detection was found to be highly sensitive and rapid method compared to conventional cultural and biochemical tests which are less sensitive and time consuming [30, 31].

Further, the isolate also subjected to sequencing and phylogentically characterized as *P.multocida*. The partial sequences of KMT1 gene of the duck isolate of the present study showed 97 -100 similarity with the available sequences on comparison. The KMT1 sequences of the present study showed 100 per cent similarity with the sequence of the duck isolate from Kerala (Accession No. CP023304.1) and goose isolate of China (Accession No.KP036619.1) [32]. It showed 99 per cent similarity with the KMT1 partial sequences of sheep isolate from Rajasthan (Accession No. KY825088.1) and goat isolate from Uttar Pradesh (Accession No. KX348143.1) [32]. It is concluded that, nucleic acid based detection methods was found to be sensitive, specific for rapid diagnosis than conventional methods.

5. Conclusion

P. multocida was isolated from the migratory ducks in the Cauvery delta region of Tamil Nadu. In addition to conventional methods, *P. multocida* was also confirmed by PM-PCR. Further, the isolate was also subjected to phylogenetic analysis and showed 100% similarity with duck isolate of Kerala. The present study can serve as baseline for development of control strategies in future.

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