The Pharma Innovation Journal 2023; SP-12(9): 1391-1395

www.ThePharmaJournal.com

The Pharma Innovation

ISSN (E): 2277-7695
ISSN (P): 2349-8242
NAAS Rating: 5.23
TPI 2023; SP-12(9): 1391-1395

www.thepharmajournal.com
Received: 03-06-2023
Accepted: 09-07-2023

Chandravathi T
Department of Veterinary Pathology, College of Veterinary Science, Mamnoor Telangana, India

Rama Devi V
Department of Veterinary Pathology, NTR College of Veterinary Science, Gannavaram, Andhra Pradesh, India

Satheesh K
A.H. Polytechnic, Banavasi, Kurnool, Andhra Pradesh, India

Ravi Kumar P
Department of Veterinary Pharmacology and Toxicology, NTR College of Veterinary Science, Gannavaram, Andhra Pradesh, India

Sudhakar K
Department of Animal Genetics and Breeding, College of Veterinary Science, Garividi, Andhra Pradesh, India

Corresponding Author:
Chandravathi T
Department of Veterinary Pathology, College of Veterinary Science, Mamnoor Telangana, India

Pathology, molecular identification and capsular typing of *Pasteurella multocida* isolates in chicken

Chandravathi T, Rama Devi V, Satheesh K, Ravi Kumar P and Sudhakar K

Abstract
Fowl cholera was caused by gram negative bacteria *Pasteurella multocida*. Thirty birds from private poultry farm suffering with pyrexia were brought to the Department of Veterinary Pathology, NTR College of Veterinary science, Gannavaram, Andhra Pradesh. Birds were dehydrated and emaciated and showing pyrexia, pale combs, mucus discharge from nostrils and greenish white coloured diarrhoea. On necropsy examination haemorrhages were observed on abdominal fat, epicardium, caecal tonsils, and intestinal mucosa. Lungs were congested. Liver was swollen, congested and containing multiple small focal areas of necrosis. The impression smears from liver and blood smears from heart in on Leishman's staining revealed characteristic bipolar organisms. Histopathological studies of heart revealed congestion, haemorrhages, myocardial degeneration and liver showed congestion of central vein and sinusoids and mild degree of degenerative changes and focal areas of necrosis with heterophils and mononuclear cell infiltration. The disease was confirmed by amplifying species specific KMT gene through PCR which yielded 460bp size amplicon and cap A capsule-specific primers for capsular genotyping yielded 1044bp size amplicon. Based on bacterial isolation, blood smear examination and pathological lesions, disease was diagnosed and confirmed by molecular study.

Keywords: Fowl cholera, *Pastuerella multocida*, pathology, lesions, PCR

1. Introduction
Pasteurella multocida, a gram -negative organism causes Pasteurellosis/ Fowl cholera in domestic and wild birds. The disease was highly contagious, septicemic and fatal disease. High morbidity and mortality were recorded in poultry flocks affected with septicemic form of disease and it leads to great economic impact in poultry production [1, 2, 3]. Organisms spreads within flocks through mucus discharges from beak, nose, and eyes which contaminate poultry environment and it also survives in contaminated crates, feed bags and equipment. These bacteria first they colonize in the upper respiratory tract and lung then spreads through blood vessels to different internal organs. Severely affected birds died due to endotoxic shock and bacteraemia [4].
Although the disease was old, now also observing outbreaks as a consequence to primary immunosuppressive diseases. The severity of disease depends on Virulence genes, toxins, strain, route of infection and host immunity. It has been frequently reported from the different states of India and trough-out the world [1, 5, 6, 7].
P. multocida consist of 5 serogroups (A, B, D, E and F) based on capsular composition and 16 somatic serovars (1-16) based on lipopolysaccharide antigens [1, 8]. *P. multocida* type A:1, A:3 and type D in Asian countries were responsible for fowl cholera infection [9]. The infectious capacity of *P. multocida* was related to its capsular polysaccharide. Serogroup A bacteria produces a hyaluronic acid capsule, which similar to mammalian and avian hyaluronic acid chemically and evade the host's immune system [10].
This disease can be diagnosed based on isolation of bacteria and its biochemical, morphological and cultural characteristics, but it is time consuming, hence this research was attempted to study the pathological changes and to identify the organisms and its capsule type through molecular techniques.

2. Materials and Methods
Post mortem examination was conducted for thirty birds presented to the department of Pathology. The tissue samples of trachea, lungs, caecal tonsil, liver, heart and kidneys were collected and fixed in 10% neutral buffer formalin for histopathology.

The samples were processed, sectioned (5 µm) and stained with H&E stain for histopathological examination as per the standard procedure.

The suspected tissue swabs were inoculated into BHI broth and incubated at 37 °C under anaerobic conditions in an anaerobic jar with a candle lighted for 12-18 hrs. After incubation, the cultures were streaked on BHI agar, 5% sheep blood agar and MacConkey agar and observed for growth.

On BHI agar, small white dewdrop like, smooth and glistening colonies and on blood agar greyish, non-haemolytic colonies were observed. Smears were prepared from the colonies and Gram's staining was done and pink colored organisms were interpreted as gram negative bacteria. At the time of post mortem examination, blood smears from heart and impression smears lung, heart and liver were prepared and stained with Leishman's staining and observed for the bipolar organisms.

In the present study, for the rapid identification and characterization of bacterial isolates by PCR, the bacterial DNA template was prepared from cultures as per the method described by Markam *et al.* (2009) [11]. Briefly, a loop full of bacterial culture was taken from parent colonies with the help of sterile inoculation loop and was suspended in 1000 µl of sterile PBS and centrifuged at 10,000 rpm for 1 min at room temperature. The pellet was resuspended in 1000 µl of sterile Milli-Q water and centrifuged at 10,000 rpm for 1 min at room temperature. The supernatant was discarded and 300 µl of sterile Milli-Q water was added to the pellet and mixed well. The 300 µl suspension taken in eppendorf tube was heated for 10 min in a boiling water bath at 95 °C and transferred immediately onto ice and kept for 5 min. The tube was centrifuged at 10,000 rpm for 1 min at 4 °C and the supernatant was used as a template for PCR assay for detection of *P. multocida*.

Multiplex PCR was carried out by using species specific primers namely KMT17 and KMT1SP6 and for serotyping/capsular typing by capsular specific primers as described by Townsend *et al.* (2001) [12].

Table 1: Primers for the detection of *P. multocida* isolates and serotypes

Primer gene	Primer Sequence (5' ----3')
KMT1T7	F: ATCCGCTATTTACCCAGTGG
KMT1SP6	R: GCTGTAAACGAACTCGCCAC
Cap A F	F: TGCCAAAATCGCTGTCAG
Cap A R	R: TTGCCATCATTGTCAGTG

The PCR reaction mix was initially denatured by heating to 94 °C for 3 min in a thermal cycler. The samples were amplified for 30 cycles and each cycle consisted of a denaturation step at 94 °C for 30 sec, followed by annealing at 57 °C for 40 sec, extension at 72 °C for 60 sec and final extension at 72 °C for 10 min. The PCR products were kept at 4 °C until they were removed from the thermal cycler. The amplified PCR products were detected by electrophoresis.

3. Results and Discussion

Thirty birds were brought for necropsy examination from the affected poultry flock. Affected birds showed respiratory distress, mucous discharges from the mouth, nose and ears, cyanosis of comb and wattles, facial edema, ataxia, back ward retraction of head, rise in temperature, generalised depression, ruffled feathers, off feed and diarrhoea. These results were in accordance with the results of previous authors [1, 5, 13].

P. multocida infects through the tissues of the respiratory tract and adhere to the mucosa and then enter the blood stream resulting in septicemia. The release of large amounts of endotoxins are responsible for the pathogenesis. The recruitment of heterophils into the lungs and other organs leads to tissue damage and invasion.

The affected birds showed congestion throughout the carcass and petechial haemorrhages on the epicardium of heart (Fig.1), and abdominal fat. Lungs, liver, spleen, kidneys, ovaries and intestines were congested. Liver was swollen, friable and contained multiple small focal areas of necrosis (Fig.3). These lesions are in accordance with the findings of previous studies [5, 14]. Swapnil *et al.* (2011) [14] described lesions like congestion throughout the carcass, petechial haemorrhages in the abdominal and coronary fat, congestion of lungs, liver, spleen, kidney and heart, fibrinous pleuropneumonia later on and swollen liver with multiple small focal areas of necrosis in *P. multocida* infected broilers.

Fig 1: Heart showing petechial haemorrhages

Heart blood smear and culture smears on Leishman's staining showed bipolar organisms (Fig. 3).

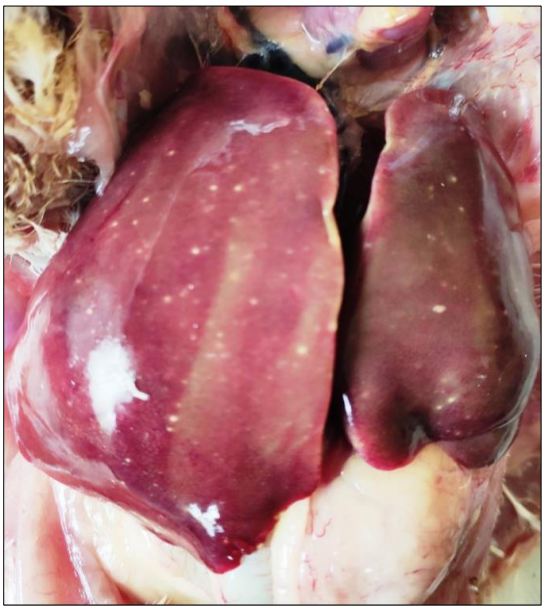

Fig 2: Liver showing multiple necrotic spots

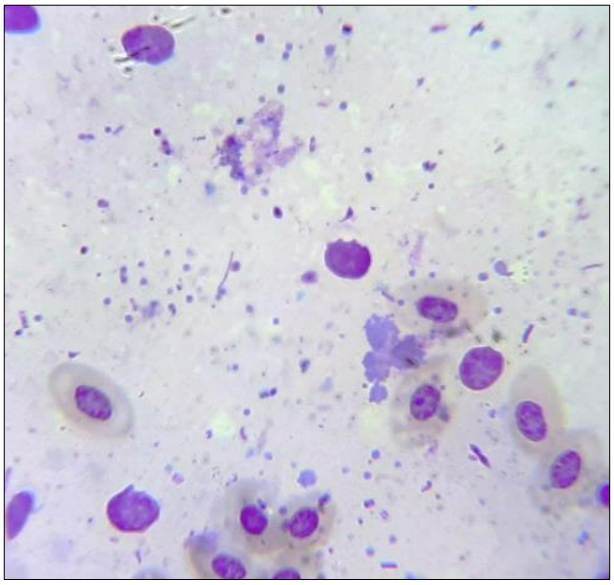

Fig 3: *P. multocida organisms*

Histopathology of lungs revealed congestion of parabronchial blood vessels and alveolar capillaries and fibrinous exudates in the parabronchi along with infiltration of heterophils and mononuclear cells in the alveoli and fibrinous thickening of parabronchial septa (Fig.4). Secondary bronchus showed severe congestion and haemorrhages along with heterophils and mononuclear cell infiltration in the mucosa (Fig. 5). In few cases desquamation of epithelial cells of bronchi was observed. Heart revealed congestion, haemorrhages and myocardial degeneration (Fig.6). The lesions found in the lungs and heart are in accordance with the earlier findings of Swapnil *et al.* (2011) [14] and Kurkure *et al.* (2011) [11] in *P. multocida* infected birds. Liver revealed congestion, necrotic foci (Fig.7) with mononuclear cell and heterophilic infiltration (Fig.8) as found by Kurkure *et al.* (2011) [11].

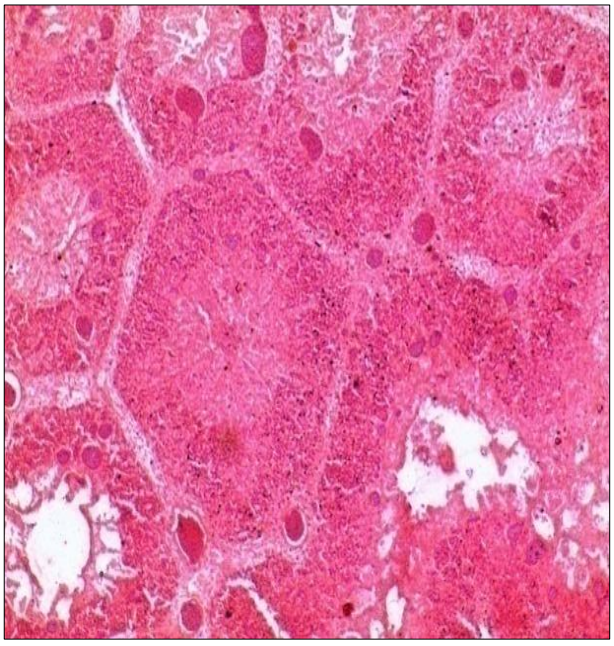

Fig 4: Parabronchi showing fibrinous exudates and thickening of parabronchial septa H&E x40

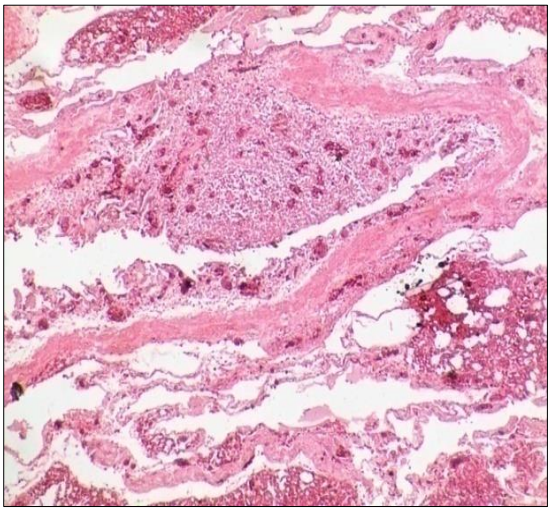

Fig 5: Secondary bronchus showing congestion and haemorrhages along with heterophils and mononuclear cell infiltration in lumen H&E x40

Microscopically, spleen revealed congestion, haemorrhages and depletion of lymphoid cells that are in accordance with the findings of Samia (2009) in *P. multocida* infected birds. Intestines showed mild desquamation of epithelium, dilated crypts with abundant number of inflammatory cells and lymphoid cell depletion in the submucosa as reported by Swapnil *et al.* (2011) [14].

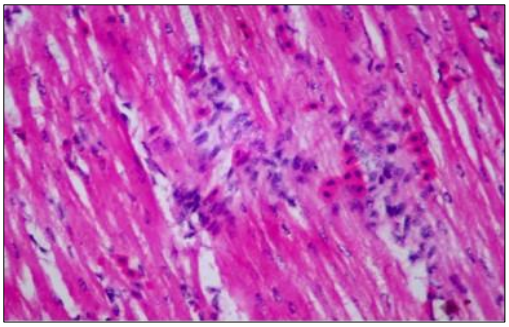

Fig 6: Heart showing congestion, haemorrhages, infiltration and myocardial degeneration H&E x100

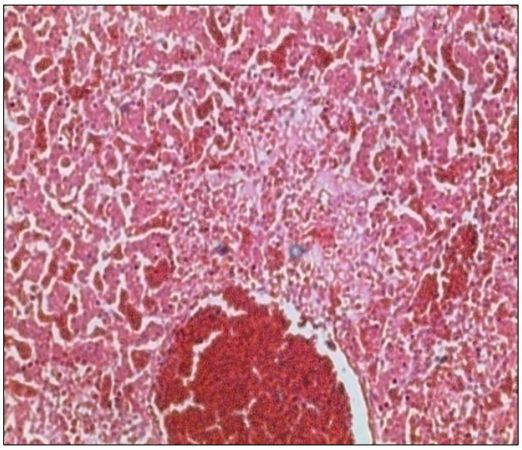

Fig 7: Liver showing congestion of central vein and sinusoids and coagulative necrosis with infiltration of heterophils and mononuclear cells H&E x100

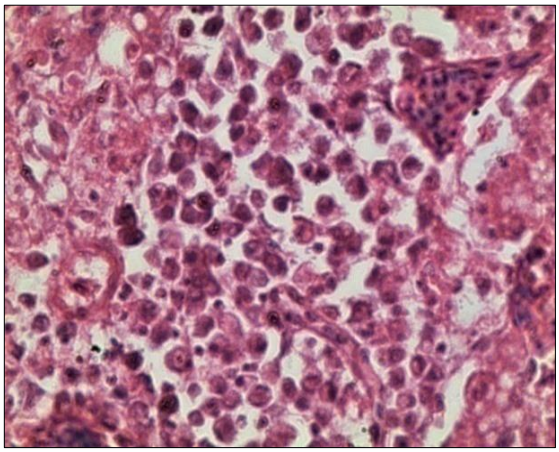

Fig 8: Liver showing focal necrosis with infiltration of heterophils and mononuclear cells H&E x400

In kidney, degenerative changes in renal tubular epithelial cells and focal interstitial nephritis with infiltration of heterophils and mononuclear cells were found. These results are in agreement with that of Samia (2009) who also noticed degenerative changes and focal interstitial nephritis in the kidneys [15]. The changes observed in the ovaries such as congestion and degeneration of ovarian follicles and mononuclear cell infiltration and congestion, necrosis and degeneration of Purkinji cells in the brain were also found by Kurkure *et al*. (2011) in layer birds in *P. multocida* infection.

The DNA extracted from *P. multocida* isolates was used in the PCR assay for amplification. Detection of *P. multocida* and its capsular genotyping was carried out by multiplex PCR in the presence of species and capsule-specific primers. PCR primers targeting KMT1 gene of *P. multocida* amplified an expected size of 466 bp amplicon that confirmed the identity of *P. multocida***.** All the positive isolates that were typed for the capsule were found to be amplified with cap A primers and a product of 1044 bp was obtained (Fig.9). All the isolates in the present study had *P. multocida* type A capsule.

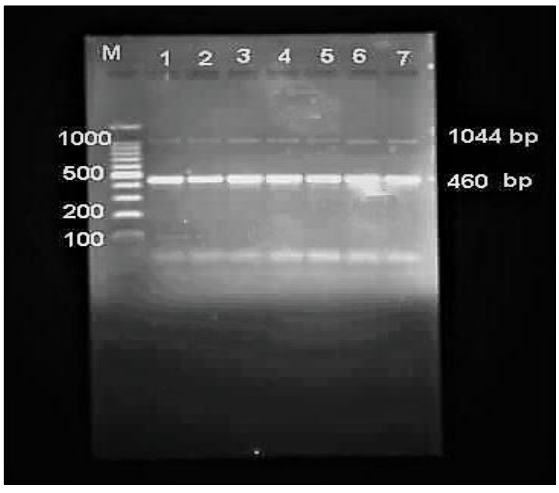

Fig 9: Agar gel electrophoresis of pcr products of KMT gene(460bp) and capsular A specific gene (1044bp). M-ladder,1-6: DNA samples,7-Positive control

These results are in similar to the previous studies [16, 17]. Townsend *et al*. (2001) [12] developed a multiplex PCR assay for rapid detection of *P. multocida* and its capsular serotyping system, using specific primers KMT1T7 and KMT1SP6 that amplified KMT1 gene of *P. multocida* and yielded 466 bp product and the serogroup specific primers used in this assay for serotypes A, B, D, E and F yielded an amplicons of 1044, 760, 657, 511 and 851 bp respectively. Shivachandra *et al.* (2006) [16] and Bhimani *et al.* (2014) [17] suggested that a multiplex capsular PCR assay might be suitable for the rapid initial identification of the serotypes of *P. multocida* during an epidemic and the authors also used species-specific primers and cap A primers described by Townsend *et al.* (2001) [12].

4. Conclusions

The PCR assay with rapidity and accuracy, irrespective of sample type gives the confirmatory diagnosis of *P. multocida* isolates and its capsular typing in a single reaction which saves time.

5. References

1. Rhodes KR, Rimler RB. Pasteurellosis: In Diseases of Poultry. Hofstad MS, Barner HJ, Calnek BW, Reid Wand Yoder HW (Ed.) London Academic Press Limited; c1991. p. 154-162.
2. Serbessa TA, Geleta YG, Terfa IO. Review on diseases and health management of poultry and swine Int J Avian & Wildlife Biol. 2023;7(1):27-38.
3. Roshdy S, Soliman R, Aly M, Omar L, Sameer A, Abo-Elyazeed H, *et al*. (Preparation of a Newly Developed Trivalent *Pasteurella multocida, Avibacterium paragallinarum*, and *Ornithobacterium rhinotracheale* Vaccine with an Evaluation of its Protective Efficacy in Chickens. Journal of Advanced Veterinary Research. 2023;13(5):737-742.
4. OIE-Office International Des Epizootics, Fowl cholera in Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. 2008;2.3.9:524-530.
5. Mohamed MA, Mohamed MW, Ahmed AI. *P. multocida* in backyard chickens in Upper Egypt: incidence with polymerase chain reaction analysis for capsule type, virulence in chicken embryos and antimicrobial resistance. Veterinaria Italiana. 2012;48(1):77-86.
6. El-demerdash GO, Elsebaey HS, Mohamed SR, Mahmoud AE. *Pasteurella multocida* Infection in Duck, Detection of Virulence Genes and Antimicrobial Susceptibility of the Isolates. Alexandria Journal for Veterinary Sciences. 2023 Jan 1;76(1).
7. Kurkure NV, Hedau M, Raut SS, Kalorey DR, Bhandarkar AG. Genotypic characterization of *Pasturella multocida* isolated from an outbreak of fowl cholera. Indian Journal of Veterinary Pathology. 2011;35(1):48-51.
8. Carter GR. Proposed modification of the serological classification of *Pasteurella multocida*. Veterinary Record. 1963;75:1264.
9. Ranjan R, Panda SK, Acharya AP, Singh AP, Gupt A. Molecular diagnosis of Hemorrhagic septicemia. Veterinary World. 2011;4:189-192.
10. Harper M, Boyce JD, Adler B, *Pasteurella multocida* pathogenesis: 125 years after Pasteur. FEMS Microbiology Letters. 2006;265:1-10.
11. Markam SK, Khokhar RS, Kapoor S, Kadian SK, Molecular characterization of field isolates of *Pasteurella multocida* by Polymerase chain reaction in the state of Haryana. Indian Journal of Comparative Microbiology, Immunology and Infectious Diseases. 2009;30(1):31-34.
12. Townsend KM, Boyce JD, Chung JY, Frost AJ and Adler B Genetic organization of *Pasteurella multocida* cap loci

and development of a multiplex capsular PCR typing system. Journal of Clinical Microbiology. 2001;39(3):924-929.
13. Shah AH, Kamboh AA, Rajput N, Korejo NA. Optimization of physico chemical conditions for the growth of *Pasteurella multocida* under *in vitro*. Journal of Agricultural Society Sciences. 2008;4:176-179.
14. Swapnil S, Swamy M, Verma Y. Ameliorative effect of mannan oligosaccharide on pathology of fowl cholera in broiler birds. Asian Journal of Poultry Science. 2011;5(2):86-98.
15. Samia Mohamed M. Hematological, biochemical, immunological and pathological studies on pasteurellosis in chicken. Egyptain Journal of Comparative Pathology and Clinical Pathology. 2009;22(2):232-241.
16. Shivachandra SB, Kumar AA, Gautam R, Singh VP, Saxena MK, Srivastava SK. Identification of avian strains of *Pasteurella multocida* in India by conventional and PCR assays. The Veterinary Journal. 2006;172(3):561-564.
17. Bhimani MP, Roy A, Bhanderi BB, Mathakiya RA. Isolation, identification and molecular characterization of *Pasteurella multocida* isolates obtained from emu in Gujarat state, India. Veterinarski Arhiv. 2014;84(4):411-419.