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Chandravathi T
Assistant Professor,
Department of Veterinary
Pathology, College of
Veterinary Science,
PVNRTVU, Rajendranagar,
Hyderabad, Telangana, India

V Ramadevi
Professor and Head,
Department of Veterinary
Pathology, NTR College of
Veterinary Science, SVVU,
Gannavaram, Andhra Pradesh,
India

K Sathish
Professor, Department of
Veterinary Pathology, NTR
College of Veterinary Science,
SVVU, Gannavaram, Andhra
Pradesh, India

Corresponding Author:
Chandravathi T
Assistant Professor,
Department of Veterinary
Pathology, College of
Veterinary Science,
PVNRTVU, Rajendranagar,
Hyderabad, Telangana, India

Screening of virulence genes in *Pasteurella multocida* associated with poultry respiratory diseases

Chandravathi T, V Ramadevi and K Sathish

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Abstract

Pasteurella multocida is a crucial pathogen causing fowl cholera, a highly contagious disease affecting a wide range of poultry worldwide, resulting in significant economic losses due to high morbidity and mortality. This study aimed to isolate, identify, and characterize field strains of *P. multocida* associated with fowl cholera in avian species in Andhra Pradesh. Samples (n = 289) were collected from various districts experiencing sporadic outbreaks, from which 40 isolates of *P. multocida* were recovered. The isolates were recovered from liver, heart, and lung cultured under anaerobic conditions on brain heart infusion (BHI) agar and blood agar, and stained by Gram's stain, Leishman's stain, and biochemical tests were conducted. Molecular confirmation was performed using species-specific PCR with KMT1T7 and KMT1SP6 primers, amplifying a 460 bp fragment. Capsular typing using capA primers identified that all isolates are capsular type A (product size 1044 bp). Virulence gene profiling through multiplex PCR indicated that all isolates possessed the hgbA, ptfA, and ompH genes, while hgbB and pfhA genes were present in 75% of the isolates. The study highlights the prevalence of capsular type A *P. multocida* strains harbouring key virulence genes in poultry from Andhra Pradesh, providing valuable insights for disease diagnosis and control strategies.

Keywords: *Pasteurella multocida*, virulence genes, poultry, respiratory-disease

Introduction

Pasteurella multocida is a Gram-negative, facultative anaerobic bacterium that plays a critical role in respiratory infections among a wide range of avian species. As the causative agent of avian pasteurellosis, this pathogen is responsible for significant morbidity and mortality in poultry flocks worldwide, leading to severe economic losses in the poultry industry. The disease presents in both acute and chronic forms, varying in clinical severity and often complicating diagnosis and management.

Traditionally, *P. multocida* classification has relied on serological methods based on capsular and somatic antigens, but these methods are complicated and have been increasingly supplemented by molecular typing techniques that identify 5 (A, B, D, E, and F) distinct capsular (Townsend *et al.*, 2001) [31] and 8(L1-L8) LPS genotypes (Harper *et al.*, 2015) [17]. Molecular studies reveal considerable genetic diversity among avian isolates, indicating differences in virulence and pathogenic potential across strains from different poultry species. This variation poses challenges for diagnosis, treatment, and vaccine development, highlighting the need for comprehensive characterization of *P. multocida* strains to better understand their role in avian diseases and improve control strategies.

The pathogenic potential of *P. multocida* is largely determined by a complex array of virulence factors, including its polysaccharide capsule, lipopolysaccharides (LPS), outer membrane proteins (OMPs), adhesins, fimbriae, and iron acquisition systems. These components not only facilitate colonization and immune evasion but also play a crucial role in tissue damage and systemic dissemination. Molecular and histopathological investigations have highlighted the strain-specific nature of these factors, particularly in avian hosts, where variations in host immunity, environmental exposure, and geographical area can influence infection dynamics [19, 26, 27].

This study aims to isolate *P. multocida* from pulmonary lesions in chickens and analyze the virulence markers responsible for pathogenesis. Understanding these factors is essential to improve diagnostic approaches and inform prevention strategies against this highly adaptable and pathogenic organism.

Materials and Methods

Samples were collected from both clinically ill and deceased poultry birds from farms located in the different districts of Andhra Pradesh. These cases were examined at the Department of Veterinary Pathology, NTR College of Veterinary Science, Gannavaram. During necropsy, tissue samples exhibiting lesions were aseptically collected in sterile phosphate-buffered saline (PBS) for further analysis. The tissue swabs were then cultured in brain heart infusion (BHI) broth and incubated anaerobically at 37 °C for 12-18 hours using a candle jar method. Post-incubation, the samples were streaked on BHI agar, sheep blood agar (5%), and MacConkey agar to observe bacterial growth and colony characteristics. Gram's staining was performed to determine cell wall characteristics. Additionally, blood and tissue impression smears prepared at necropsy were stained with Leishman stain to identify the bipolar-staining characteristic of *P. multocida* organisms.

Bacterial motility was assessed using the hanging drop

technique, and biochemical assays were performed for catalase, oxidase, nitrate, methyl red, Voges-Proskauer, sugar fermentation, indole, citrate, gelatin liquefaction, and urease by standard methods [5, 22]. For molecular confirmation and strain typing, bacterial DNA was extracted from cultured isolates using a rapid heat lysis method. Culture was suspended in phosphate-buffered saline (PBS), followed by washing, thermal lysis at 95 °C, and centrifugation to collect the DNA-containing supernatant. This DNA was used as a template for PCR-based identification. A multiplex PCR assay targeting species-specific and capsular-type genes was performed. Virulence typing of *P. multocida* was done with the detection of virulence-associated genes *pfhA*, *ptfA*, *ompH*, *hgbA*, and *hgbB*. The primers used in this study are given in Table 1, and the reaction conditions are given in Table 2. The DNA amplified by PCR was subjected to agarose gel electrophoresis. The bands were visualized under UV illumination using a Gel documentation system.

Table 1: Details of the primers used for molecular detection of *P. multocida* and its virulence genes

S. No	Genes	Primer sequence (5'---3')	Size	Reference
1	KMT1T7 KMT1SP6	ATCCGCTATTTACCCAGTGG GCTGTAAACGAACTCGCCAC	460 bp	Townsend <i>et al.</i> (1998) [32]
2	Cap A F Cap A R	TGCCAAAATCGCTGTCAG TTGCCATCATTGTCAGTG	1044 bp	Ewers <i>et al.</i> (2006) [12]
3	ompH F ompH R	CGCGTATGAAGGTTTAGGT TTTAGATTGTGCGTAGTCAAC	438	
4	ptfA F ptfA R	TGTGGAATTCAGCATTCTTAGTGTG TC TCATGAATTCTTATGCGCAAAA TCCTGCTGG	488	
5	pfhA F pfhA R	AGCTGATCAAGTGGTGAAC TGGTACATTGGTGAATGCTG	275	
6	hgbA F hgbA R	TGGCGGATAGTCATCAAG CCAAAGAACCACTACCCA	419	
7	hgbB F hgbB R	ACCGCGTTGGAATTATGATTG CATTGAGTACGGCTTGACAT	788	

Table 2: Thermal cycling conditions

S. No	Gene	Initial denaturation	Denaturation	Annealing	Extension	Final Extension	No. Cycles
1	KMT1	94/3 min	94/30 sec	57/40 sec	72/60 sec	72/10 min	30
2	Cap A	94/3 min	94/30 sec	57/40 sec	72/60 sec	72/10 min	30
3	ompH	94/3 min	94/30 sec	54/30 sec	72/1 min	72/10 min	30
4	ptfA	94/5 min	94/30 sec	55/30 sec	72/60 sec	72/5 min	25
5	pfhA	94/5 min	94/30 sec	55/30 sec	72/60 sec	72/5 min	25
6	hgbA	94/3 min	94/30 sec	53/30 sec	72/30 sec	72/10 min	30
7	hgbB	94/3 min	94/30 sec	54/30 sec	72/45 sec	72/10 min	30

Results, Discussion and Conclusion

In this study, samples were collected from birds exhibiting characteristic lesions during post-mortem examinations. From a total of 289 samples obtained from multiple poultry farms experiencing acute mortality, 30(10.38%) isolates of *P. multocida* were successfully recovered. The identification of *P. multocida* in these 30 isolates was confirmed through cultural, biochemical, and molecular techniques, aligning with findings from previous studies. Notably, incidence varied across studies, as 2.6% by Dogra *et al.* (2015) [9], 9.6% by Aboob *et al.* (2021) [2], 16% by El-Demerdash *et al.* (2023) [10], and 12.4% by El-Tarabili *et al.* (2025) [11].

The bacterial cultures on Brain Heart Infusion (BHI) agar showed small, whitish, discrete, circular, and translucent colonies, which later became opaque. On blood agar, *P. multocida* formed small, whitish, circular colonies (Fig.1) that were initially translucent and gradually turned opaque, with no hemolysis observed. No growth was detected on

MacConkey agar plates, consistent with reports from Dogra *et al.* (2015) [9] and Aboob *et al.* (2021) [2].

Microscopic evaluation of the isolates using Gram staining revealed small, Gram-negative coccobacilli (Fig.2) exhibiting a characteristic bipolar appearance, suggesting *P. multocida*. Impression smears prepared from the liver and heart of deceased birds, stained with Leishman's stain (Fig.3), demonstrated bipolar organisms displaying a capsule. Leishman's stain proved to be a simple, rapid, and effective method for identifying the distinctive bipolar bacteria in impression smears. These staining characters are in accordance with the Markam *et al.* (2009) [21], while some researchers [3, 8] have utilized Giemsa and Methylene blue stains to detect *P. multocida* in hemorrhagic septicaemia. Initially, isolates exhibiting characteristic growth on brain heart infusion (BHI) agar and blood agar, no growth on MacConkey agar, and positive results for indole, catalase, oxidase, and nitrate reduction tests were presumptively

identified as *P. multocida*. These isolates were further tested on triple sugar iron (TSI) agar to assess hydrogen sulfide (H₂S) production, with all isolates demonstrating typical reactions consistent with *P. multocida*. Subsequent sugar fermentation tests showed that all isolates fermented glucose (100%) without gas production; 24 (80%) isolates fermented sucrose, and 30 (62.5%) fermented maltose, both also without gas production. The biochemical test results align with the observations of Charitha Devi *et al.* (2012) [6] and Penz *et al.* (2019) [23].

Molecular identification of *P. multocida* was conducted using species-specific primers KMT1T7 and KMT1SP6, which generated an amplified product of 460 base pairs (bp) in the isolates, as compared to the 100 bp molecular weight marker. Among the 289 samples analyzed, 30 (10.38%) were confirmed positive for *P. multocida* by PCR. For capsular typing, multiplex PCR was performed employing capsule-specific primers alongside the species-specific primers for confirmation. All isolates (100%) produced an amplified DNA fragment of 1044 bp corresponding to the *capA* gene, indicating that they belong to capsular group A (Fig.4). This predominance of capsular type A among poultry *P. multocida* isolates aligns with previous findings [2, 26, 30]. Therefore, the geographical region investigated in this study primarily harbors capsular type A strains. Combining molecular identification and capsular typing into a single multiplex PCR reaction proves advantageous by saving time and reagents.

The pathogenic potential of *P. multocida* is largely determined by a complex array of virulence factors. The major virulence factors identified in *P. multocida* are capsular proteins, lipopolysaccharide, fimbriae, bacterial adhesins, and outer membrane proteins (OMPs). In the present study, the occurrence of virulence genes, namely, *hgbA* and *hgbB* (iron uptake proteins), *ptfA* (Type IV fimbrial protein), *pfhA* (haemagglutinin protein), and *ompH* (porin) were screened with all the isolates.

The five virulence-associated genes, namely *ompH*, *ptfA*, *pfhA*, *hgbA*, and *hgbB* were detected using PCR in the presence of specific primers, which yielded PCR products of 438 bp, 488 bp, 275 bp, 419 bp, and 778 bp, respectively. The virulence-associated genes, namely *ompH*, *ptfA*, and *hgbA* were present in all isolates (100%).

The *ompH* gene encodes an outer membrane porin protein that functions as a general transporter facilitating the diffusion of nutrients and molecules, crucial for bacterial survival and adaptation within the host. Additionally, *OmpH* is involved in nutrient acquisition and possibly immune evasion, supporting bacterial colonization and persistence during infection [18]. The *ptfA* encodes a type IV fimbrial protein, an adhesion factor that enables the bacterium to attach to host mucosal surfaces. Adhesion is a critical first step for colonization and establishment of infection, allowing the bacteria to resist clearance by host defenses [24]. The *hgbA* codes for a hemoglobin binding protein involved in iron acquisition from the host. Iron uptake is critical for bacterial growth and virulence because iron is a limited but essential nutrient within the host environment. The presence of *hgbA* ensures that *P. multocida* can efficiently scavenge iron from host hemoglobin, promoting survival and multiplication [13]. Filamentous hemagglutinins encoded by the *pfhA* gene have an important role in the colonization of bacteria in the upper respiratory tract initially and this gene in several avian species varies frequently [16, 20]. Together,

these genes facilitate key steps of infection-adherence to host cells (*ptfA*), nutrient acquisition including iron uptake (*hgbA*), and maintenance of bacterial integrity and nutrient exchange via outer membrane functions (*ompH*) essential for bacterial pathogenesis and successful infection.

The virulence-associated genes *hgbB* and *pfhA* were 75% in Cap A isolates. The *hgbB* gene encodes a hemoglobin-binding outer membrane protein that helps in acquiring iron from host hemoglobin, a critical factor for bacterial survival and virulence within the host [7]. The *pfhA* is commonly, though not universally, found in *P. multocida* isolates, and its presence is linked with enhanced ability to colonize and initiate infection, especially in respiratory and oral environments [28].

Among all the 40 isolates, 30 isolates possessed all the virulent genes, and the other 10 isolates lacked some virulent genes (*hgbB* and *ptfH*), suggesting that those two genes might play a less critical role in disease causation compared to others. Similar findings were reported by Ewers *et al.*, (2006) [12] while working with virulence genes of *P. multocida*, reporting 100% of *ompH* and *ptfA* genes, 90% of *hgbA*, 85% of *hgbB*, and 45% *pfhA* from poultry. Previous studies showed *ptfA* is present in 100% of isolates irrespective of their capsular type [15, 29, 33]. In a study by El-Demerdash *et al.* (2023) [10], *ptfA* is present in 86.2% of isolates. Aski and Tabatabaei (2016) [4] reported the 100% occurrence of *ptfA*, *hgbA*, and *ompH* genes, 93% occurrence of *hgbB*, and 46% of *pfhA* genes and various degrees of other virulence genes. In contrast to the present study, Furian *et al.* (2013) [14] observed 100% occurrence of the *hgbB* virulence gene among all the isolates, whereas 92% occurrence of the *ptfA* gene. These results are also in agreement with the present study. Sarangi *et al.* (2015) [29] reported the presence of multiple virulence-associated genes *ptfA*, *hgbA*, *hgbB*, and *ompH*, which were frequently detected among Indian strains.

It is noteworthy that the presence of virulence-associated genes in *P. multocida* isolates varies widely across different geographical areas and study populations [25, 29]. Differences in virulence profiles between investigations revealed that various *P. multocida* isolates may have different pathogenic mechanisms [1, 20].

This study concludes that Complete presence (100%) of *ptfA*, *hgbA*, and *ompH* virulence genes, coupled with variable presence of other virulence genes such as *hgbB* and *pfhA* highlights the regional genetic diversity of *P. multocida* in Andrapradesh. These genes collectively contribute to the pathogen's ability to cause disease and represent crucial molecular determinants for epidemiological and intervention strategies, suggesting the need for ongoing surveillance of gene patterns to understand pathogenesis and inform control measures like vaccines or therapeutics.

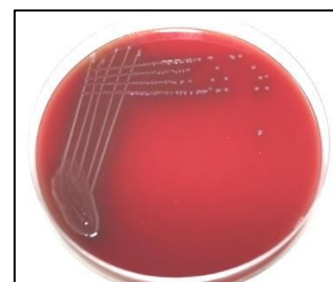


Fig 1: *P. multocida* colonies on blood agar

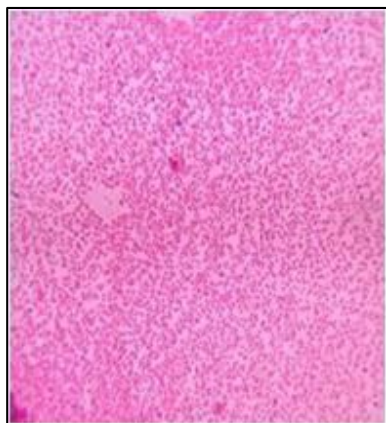


Fig 2: Grams staining of *P. multocida* organisms 100x

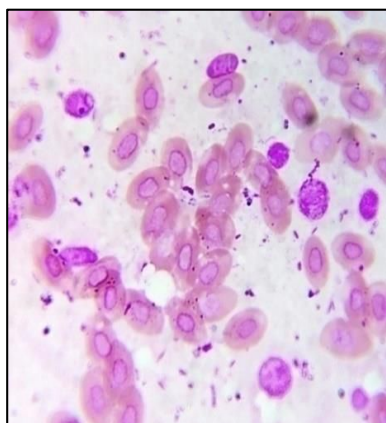


Fig 3: Heart impression smear showing *P. multocida* organisms 1000x

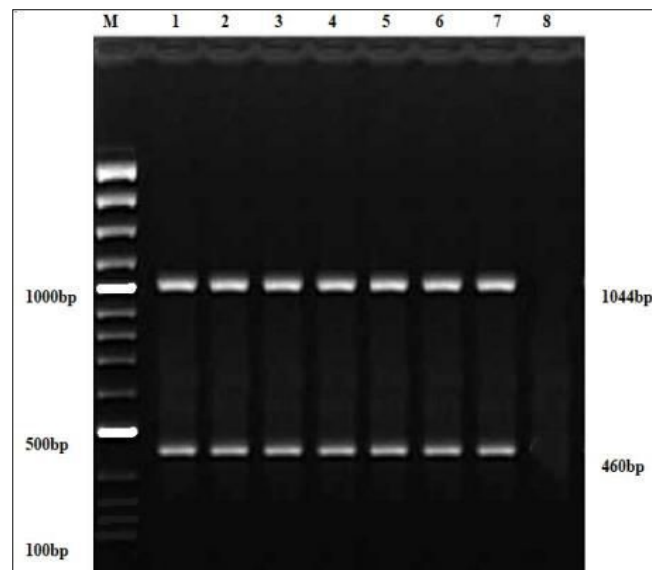


Fig 4: Gel electrophoresis of amplified PCR products of species specific primers (460 bp) and capsular A primers (1044 bp) of *P. multocida*
Lane M-Ladder 100 bp
Lane 1 to 7-DNA samples
Lane 8-Negative control

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