Introduction

*Ph*asurella multocida is a gram negative pathogen responsible for wide range of diseases in domestic animals. Pasteurellosis caused by *Pasteurella multocida* manifest often as respiratory infection in farmed small ruminants and has major economic and welfare implications worldwide (Odugbo *et al.*, 2006). This organism mainly takes the form of pneumonia and septicæmic pasteurellosis which has been reported in lambs (Watson and Davis, 2002). It has a zoonotic impact in human; it can cause soft tissue infection after animal bites or via inhalation causing respiratory tract infection. Use of antibiotics has widely been reported for the treatment of various diseases caused by *P. multocida*.

However, the prolonged and indiscriminate use of antibiotics has resulted in the development of resistance among various strains of the organism and even multi drug resistant (MDR) forms of *P. multocida* have emerged (Vu-Khac *et al.*, 2020). The present study focused on the phenotypic and genotypic antibiotic resistance pattern of *P. multocida* isolated from sheep lung samples.
Materials and Methods

Sample Collection

A total of 195 pneumonic lung samples were collected from sheep slaughter house Gannavaram, Krishna district, Andhra Pradesh.

Bacterial isolation

From 195 samples, seven P. multocida were isolated as pure culture by following standard bacteriological procedures with specific modifications as described by Sujatha et al., 2018.

Molecular confirmation of the isolates

Template nucleic acid was isolated using boiling method as described by Sujatha et al 2018. P. multocida specific PCR (PM-PCR) was done using a set of primers as reported by Townsend et al. (1998). A 10µl reaction mixture was used as described by Sujatha et al., 2018 with PCR conditions initial denaturation 94°C for 3 min followed by 30 cycles of denaturation 94°C for 30 sec, annealing for 57°C for 40 sec, extension 72°C for 60 sec and final extension 72°C for 10 min. The amplified gene products were subjected to agarose gel electrophoresis using 1.5% agarose (Invitrogen, UK) and then visualised by UV gel documentation system ((Bio-Rad)).

Phenotypic antibiotic resistance/ sensitivity

All the isolates were tested for antibiotic resistance/ sensitivity as per Bauer- Kirby et al. (1966) with 14 antibiotics namely Ampicillin-AMP (10µg), Amoxicillin-AMX (10µg), Amoxicillin-Clavulanic acid-AMC (30µg), Clindamycin- CD (2µg), Chloramphenicol - C (30µg), Colistin - CL (10µg), Ceftriaxone – CTR (30µg), Enrofloxacin - EX (10µg), Gentamicin - GEN (10µg), Nalidixic acid- NA (30µg), Streptomycin - S (30µg), Cotrimoxazole- COT (30µg), Sulfamethoxazole- SM(300µg) and Tetracycline – TE(30µg).

Twenty ml of Muller Hinton Agar (MHA) medium was poured in 90 mm diameter sterile petridishes to a depth of 4 mm and the plates were incubated at 37°C/ 24 h for sterility check. Test cultures of P. multocida enriched in BHI broth (using overnight cultures incubation at 37°C for 16 to 18 h.) were used as inoculums. The inoculum was spread to get lawn culture with the help of sterile cotton swab evenly over the entire surface of the MHA plates by swabbing back and forth across the agar. Antibiotic discs were deposited aseptically by using sterile forceps. The plates were incubated at 37°C for 16-24 h. The zones of growth inhibition around the antibiotic discs were measured to the nearest millimetre. The zone diameters of each drug were interpreted using the criteria chart provided by CLSI, (2007). Based on the disc diffusion diameters, results were interpreted qualitatively as susceptible, intermediate, and resistant.

Genotypic antibiotic resistance / sensitivity

The isolates were tested for presence of antibiotic resistance genes genotypically using sul1 and sul2 (sulphonamides), catA1 (chloramphenicol), strA and strB (streptomycins), tetA and tetB (tetracyclines) primers. The PCR reaction mixture and conditions for each primer has been listed in table 1 and 2, respectively.

Results and Discussion

Phenotypic antibiogram pattern of P. multocida

In the present study all the 7 P. multocida isolates were tested for their antibiotic
resistance/sensitivity pattern by disc diffusion method against commonly used antibiotics. The organisms revealed sensitivity 100% to gentamicin, cotrimoxazole, ceftriaxone, ampicillin, sulfamethoxazole, streptomycin, amoxicillin-clavulanic acid, nalidixic acid, 71.42% to chloramphenicol and colistin, 57.1% to amoxicillin, 42.85% enrofloxacin and 28.57% to clindamycin.

The organisms were intermediate resistance 57.14% to Enrofloxacin, 42.85% Tetracycline, amoxicillin, 28.57% to colistin and clindamycin.

The organisms were resistant to tetracycline (57.1%), clindamycin (42.86%) and chloramphenicol (28.57%). The results were presented in Fig. 1 and 2.

### Table 1. Primers used for detection of antibiotic resistance genes

<table>
<thead>
<tr>
<th>S.no</th>
<th>Primer Gene</th>
<th>Primer Sequence (5'-----3')</th>
<th>Amplicon size (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>sul1 F</td>
<td>GTG ACG GTG TTC GGC ATT CT</td>
<td>779</td>
<td>Boerlin et al. (2005)</td>
</tr>
<tr>
<td></td>
<td>sul1 R</td>
<td>TCC GAG AAG GTG ATT GCG CT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>sul2 F</td>
<td>CGG CAT CCGT CAA CAT AAC CT</td>
<td>721</td>
<td></td>
</tr>
<tr>
<td></td>
<td>sul2 R</td>
<td>TGT GCG GAT GAA GTC AGC TC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>catA1 F</td>
<td>AGTTGCTCAATGTACCTATAACC</td>
<td>547</td>
<td>Sheryl et al. (2008)</td>
</tr>
<tr>
<td></td>
<td>catA1 R</td>
<td>TTGTAATTCAAGGATTGCATTCTGCC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>strA F</td>
<td>CCTGGTGATAACCGCAATTC</td>
<td>546</td>
<td>Boerlin et al. (2005)</td>
</tr>
<tr>
<td></td>
<td>strA R</td>
<td>CCAATCGCAGATAGAAGGC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>strB F</td>
<td>ATCGTCAAGGGATTGAAACC</td>
<td>509</td>
<td></td>
</tr>
<tr>
<td></td>
<td>strB R</td>
<td>GGATCGTAGAACATATTGC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>tetA F</td>
<td>GCCGGTCTTCTTCTCATCTG</td>
<td>500</td>
<td>Boerlin et al. (2005)</td>
</tr>
<tr>
<td></td>
<td>tetA R</td>
<td>CGGCGAGCAGAGCAAGTAGA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>tetB F</td>
<td>CATTAATAGCGCATCGCTG</td>
<td>540</td>
<td></td>
</tr>
<tr>
<td></td>
<td>tetB R</td>
<td>TGAAGGTCATCGATAGCAGG</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Table 2. Standardization of PCR protocols for the detection of Antibiotic resistance genes for *P. multocida* and *M. haemolytica*

<table>
<thead>
<tr>
<th>S. No</th>
<th>Gene</th>
<th>Initial Denaturation (°C/min)</th>
<th>Denaturation (°C/sec)</th>
<th>Annealing (°C/sec)</th>
<th>Extension (°C/sec)</th>
<th>Final Extension (°C/min)</th>
<th>No of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>sul1</td>
<td>95/4</td>
<td>95/60</td>
<td>68/60</td>
<td>72/60</td>
<td>72/7 min</td>
<td>35</td>
</tr>
<tr>
<td>2</td>
<td>sul2</td>
<td>95/4</td>
<td>95/60</td>
<td>66/60</td>
<td>72/60</td>
<td>72/7 min</td>
<td>35</td>
</tr>
<tr>
<td>3</td>
<td>catA1</td>
<td>94/4</td>
<td>94/30</td>
<td>55/40</td>
<td>72/60</td>
<td>72/10 min</td>
<td>30</td>
</tr>
<tr>
<td>4</td>
<td>strA</td>
<td>95/4</td>
<td>95/60</td>
<td>55/60</td>
<td>72/60</td>
<td>72/10 min</td>
<td>35</td>
</tr>
<tr>
<td>5</td>
<td>strB</td>
<td>95/4</td>
<td>95/60</td>
<td>55/60</td>
<td>72/60</td>
<td>72/10 min</td>
<td>35</td>
</tr>
<tr>
<td>6</td>
<td>tetA</td>
<td>95/5</td>
<td>94/30</td>
<td>48/30</td>
<td>72/1 min</td>
<td>72/10 min</td>
<td>30</td>
</tr>
<tr>
<td>7</td>
<td>tetB</td>
<td>94/5</td>
<td>94/30</td>
<td>55/30</td>
<td>72/1 min</td>
<td>72/10 min</td>
<td>30</td>
</tr>
</tbody>
</table>
Table 3 Distribution of antibiotic resistance genes in *P. multocida* isolates by PCR

<table>
<thead>
<tr>
<th>Sample no.</th>
<th>sul1</th>
<th>sul2</th>
<th>catA1</th>
<th>strA</th>
<th>strB</th>
<th>tetA</th>
<th>tetB</th>
</tr>
</thead>
<tbody>
<tr>
<td>7-1</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>18-1</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>20-1</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>23-4</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>23-5</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>23-6</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>23-7</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

SM-Sulfamethoxazole (300μg), C-Chloramphenicol (30μg), S-Streptomycin (30μg), TE - Tetracycline (30μg)

Table 4 Comparison of phenotypic and genotypic pattern of antibiotic resistance genes in *P. multocida* isolates

<table>
<thead>
<tr>
<th>Sample no.</th>
<th>SM</th>
<th>sul1</th>
<th>sul2</th>
<th>C</th>
<th>catA1</th>
<th>S</th>
<th>strA</th>
<th>strB</th>
<th>TE</th>
<th>tetA</th>
<th>tetB</th>
</tr>
</thead>
<tbody>
<tr>
<td>7-1</td>
<td>S</td>
<td>-</td>
<td>-</td>
<td>S</td>
<td>+</td>
<td>S</td>
<td>+</td>
<td>+</td>
<td>I</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>18-1</td>
<td>S</td>
<td>-</td>
<td>-</td>
<td>R</td>
<td>+</td>
<td>S</td>
<td>-</td>
<td>+</td>
<td>R</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>20-1</td>
<td>S</td>
<td>-</td>
<td>-</td>
<td>S</td>
<td>+</td>
<td>S</td>
<td>+</td>
<td>-</td>
<td>R</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>23-4</td>
<td>S</td>
<td>-</td>
<td>-</td>
<td>S</td>
<td>+</td>
<td>S</td>
<td>+</td>
<td>-</td>
<td>I</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>23-5</td>
<td>S</td>
<td>-</td>
<td>-</td>
<td>S</td>
<td>+</td>
<td>S</td>
<td>+</td>
<td>+</td>
<td>R</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>23-6</td>
<td>S</td>
<td>-</td>
<td>-</td>
<td>R</td>
<td>+</td>
<td>S</td>
<td>-</td>
<td>+</td>
<td>R</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>23-7</td>
<td>S</td>
<td>-</td>
<td>-</td>
<td>S</td>
<td>+</td>
<td>S</td>
<td>-</td>
<td>-</td>
<td>I</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

**Fig.1** Phenotypic antibiotic sensitivity / resistance pattern of *P. multocida*

**Fig.2** Phenotypic antibiotic sensitivity/resistance pattern of *P. multocida*
**Fig. 3** PCR amplification of *catA1* gene

![Image of PCR amplification of catA1 gene](image)

Lane M- Marker  
Lane 1, 2, 3, 4, 5, 6, 7- positive

**Fig. 4** PCR amplification of *strA* gene

![Image of PCR amplification of strA gene](image)

Lane M- Marker  
Lane 1, 2, 3, 4- positive samples  
Lane 5- negative control

**Fig. 5** PCR amplification of *strB* gene

![Image of PCR amplification of strB gene](image)

Lane M- Marker

---

**Genotypic antibiotic resistance pattern of *P. multocida***

The genotypic antibiotic resistance gene distribution of *P. multocida* was checked with the help of PCR. Primers for *sul1*, *sul2*, *catA1*, *strA*, *strB*, *tetA* and *tetB* were used which resulted in amplified band width of 779 bp, 721 bp, 547 bp, 546 bp, 509 bp and 540bp, respectively. The positive amplification was shown in Fig. 3, 4 and 5.
Of the 7 antimicrobial genes examined, no isolate was found to be positive for sul1 and sul2, all the isolates were positive for catA1 (100%), 4 were found to be positive for strA (57.14%) and 5 for strB (71.42%), tetA was negative in all the isolates and tetB was found in 3 isolates (42.85%). Two isolates (7-1 and 23-4) possessed 3 genes catA1, strA and strB.

Distribution of the antibiotic resistance genes and their comparison with phenotypic resistance were shown in Table 3 and 4.

The isolation and identification of Pasteurella multocida from primary cultures was done by observing standard cultural and biochemical properties of the P. multocida. The conventional procedures were supported by observing an amplified product of 460 bp in PCR that confirmed the occurrence of P. multocida in the isolated sample (Sujatha et al., 2018; Sarangi et al., 2014; Prabhakar et al., 2012; Ewers et al., 2006). Out of 195 samples 7 pure cultures were obtained and were used for studying antibiogram pattern.

The antibiogram pattern of the pathogen is an important aid for veterinarian to select most effective therapeutic agents. Hence, the pure colonies of P. multocida were subjected to antibiotic sensitivity test. Among the 14 antibiotics tested, the organisms revealed sensitive 100% to gentamicin, cotrimoxazole, ceftriaxone, ampicillin, sulfamethoxazole, streptomycin, amoxicillin-clavulanic acid, nalidixic acid, 71.42% to chloramphenicol and colistin, 57.1% to amoxicillin, 42.85% to enrofloxacin and 28.57% to clindamycin.

Though the organisms sensitive to some antibiotics but they showed intermediate resistance 57.14% to Enrofloxacin, 42.85% to Tetracycline, amoxicillin, 28.57% to colistin and clindamycin and resistance 57.14% to tetracycline, 42.86% to clindamycin, 28.57% to chloramphenicol. The multidrug resistance of P. multocida of sheep was also supported by Emikpe et al., (2014), Marru et al., (2013) and Rajkhowa et al., (2012). In the study, sheep isolates revealed 100% sensitive to sulfamethoxazole which is a positive indication. It was also evident that sulfonamides are the drug of choice in the treatment of pasteurellosis (Radostits et al., 2006). However the use of sulfonamides in the treatment of P. multocida was overide by the use of fluoroquinolone and other higher antibiotics in day to day practice. In present study it was observed that the isolates showed increased resistance to Enrofloxacin. Moreover, the organisms also exhibited intermediate resistance pattern to colistin at 28.57% which is an alarming sign indicating that organisms are acquiring higher antibiotic resistance as colistin is being treated as the last resort antibiotic for Gram negative organisms (Cheng et al., 2015). These results necessitate the prudent use of antibiotics during therapy.

The genotypic antibiogram of P. multocida resulted no amplification for sul1 and sul2 genes, 100% amplification for catA1, 71.42% for strB, 57.14% for strA, 42.85% to tetB genes and no amplification for tetA gene. Among these isolates, some possesses three genes (catA1, strA, strB for 7-1 isolate and catA1, strB, tetB for 18-1 isolate). When it was compared with previous study on Pasteurella multocida in bovines (Sujatha, 2016) which showed resistance to sulphonamides, sheep isolates resulted sensitivity to sulphonamides and absence of genes sul1, sul2. None of the bovine isolates harbored catA1, strA and strB (Sujatha, 2016) but ovine isolates harbored the genes related to chloramphenicol (catA1), streptomycin (strA, strB) and tetracycline (tetA, tetB) which is due to the variation in the use of antibiotics in the treatment of pneumonia in bovines and ovines. Furthermore the comparison of phenotypic and genotypic pattern of antibiotic resistance, the correlation was observed with sulphonamides. But there was less correlation
between presence of gene and phenotypic
antibiogram of other antibiotics. Such type of
discrepancies was also observed in P. multocida and other gram negative bacteria
(Vu-Khac et al., 2020, Srivani. 2017, Sujatha.
2016, Davis et al., 2011).

The present study provided useful information
on the distribution of antibiotic resistance in
P. multocida. It was also recommended that
not only the treatment of the disease with
antibiotics but also improvement of
managemental practices by providing optimal
sanitation, air quality in housing, minimizing
transportation stress, providing good quality
hay and water along with proper vaccination
is necessary to reduce the occurrence of
disease (Marru et al., 2013).

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