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Phenotypic and Genotypic Analysis of Antibiogram of *Pasteurella multocida* Isolated from Pneumonic Sheep Lungs

K. V. Susmitha[1*], K. Lakshmi Kavitha[1], M. Srivani[1] and V. Ramadevi[2]

[1]Department of Veterinary Microbiology, [2]Department of Veterinary Pathology Gannavaram, ADDL, Labbipet, Vijayawada, India

**Corresponding author*

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A B S T R A C T

Pasteurella multocida is one of the most frequent causes of respiratory infection in sheep. A total of seven *P. multocida* were isolated from 195 sheep lung samples. All the isolates were tested to phenotypic antimicrobial resistance pattern with 14 antimicrobial agents. The organisms were found intermediate resistance 57.14% to Enrofloxacin, 42.85% to tetracycline, amoxicillin, 28.57% to colistin and clindamycin. The isolates were resistant to tetracycline (57.1%), clindamycin (42.86%) and chloramphenicol (28.57%) phenotypically. When the isolates observed for the presence of antibiotic resistance genes it was found100% presence of *catA1*, 71.42% presence of *strB*, 57.14% presence of *strA*, 42.8% presence of *tet B* and no amplification of *sul1*, *sul2* and *tet A*genes. These results indicated the increasing pattern of antibiotic resistance among *P. multocida* isolates.

Introduction

Pasteurella 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 septicaemic 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

Theisolates 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 sensitivity100% 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

S.no	Primer Gene	Primer Sequence (5^1-----3^1)	Amplicon size (bp)	Reference
1	*sul1* F	GTG ACG GTG TTC GGC ATT CT	779	Boerlin *et al.* (2005)
	sul1 R	TCC GAG AAG GTG ATT GCG CT		
2	*sul2* F	CGG CAT CGT CAA CAT AAC CT		
	sul2 R	TGT GCG GAT GAA GTC AGC TC	721	
3	*catA1* F	AGTTGCTCAATGTACCTATAACC	547	Sheryl *et al.* (2008)
	*catA*1 R	TTGTAATTCATTAAGCATTCTGCC		
4	*strA* F	CCTGGTGATAACGGCAATTC	546	Boerlin *et al.* (2005)
	strA R	CCAATCGCAGATAGAAGGC		
5	*strB* F	ATCGTCAAGGGATTGAAACC	509	
	strB R	GGATCGTAGAACATATTGGC		
6	*tetA F*	GGCGGTCTTCTTCATCATGC		Boerlin *et al.* (2005)
	tetA R	CGGCAGGCAGAGCAAGTAGA	500	
7	*tetB F*	CATTAATAGGCGCATCGCTG		
	tetB R	TGAAGGTCATCGATAGCAGG	540	

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

S. No	Gene	Initial Denaturation (°C/min)	Denaturation (°C/sec)	Annealing (°C/sec)	Extension (°C/sec)	Final Extension (°C/min)	No of cycles
1	*sul1*	95/4 min	95/60 sec	68/60 sec	72/60 sec	72/7 min	35
2	*sul2*	95/4 min	95/60 sec	66/60 sec	72/60 sec	72/7 min	35
3	*catA1*	94/4 min	94/30 sec	55/40 sec	72/60 sec	72/10 min	30
4	*strA*	95/4 min	95/60 sec	55/60 sec	72/60 sec	72/10 min	35
5	*strB*	95/4 min	95/60 sec	55/60 sec	72/60 sec	72/ 10min	35
6	*tetA*	95/5 min	94/30 sec	48/30 sec	72/1 min	72/ 10min	30
7	*tetB*	94/5 min	94/30 sec	55/30 sec	72/1 min	72/ 10min	30

Table.3 Distribution of antibiotic resistance genes in *P. multocida* isolates by PCR

Sample no.	*sul1*	*sul2*	*catA1*	*strA*	*strB*	*tetA*	*tetB*
7-1	-	-	+	+	+	-	-
18-1	-	-	+	-	+	-	+
20-1	-	-	+	+	-	-	-
23-4	-	-	+	+	-	-	+
23-5	-	-	+	+	+	-	-
23-6	-	-	+	-	+	-	-
23-7	-	-	+	-	-	-	+

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

Sample no.	SM	*sul1*	*sul2*	C	*catA1*	S	*strA*	*strB*	TE	*tetA*	*tetB*
7-1	S	-	-	S	+	S	+	+	I	-	-
18-1	S	-	-	R	+	S	-	+	R	-	+
20-1	S	-	-	S	+	S	+	-	R	-	-
23-4	S	-	-	S	+	S	+	-	I	-	+
23-5	S	-	-	S	+	S	+	+	R	-	-
23-6	S	-	-	R	+	S	-	+	R	-	-
23-7	S	-	-	S	+	S	-	-	I	-	+

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

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

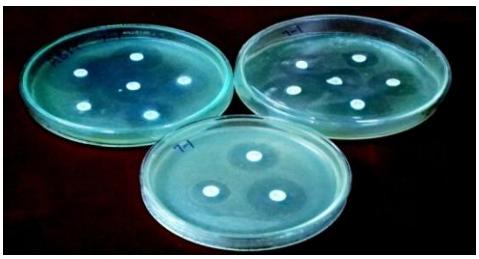


Fig.2 Phenotypic antibiotic sensitivity/resistance pattern of *P. multocida*

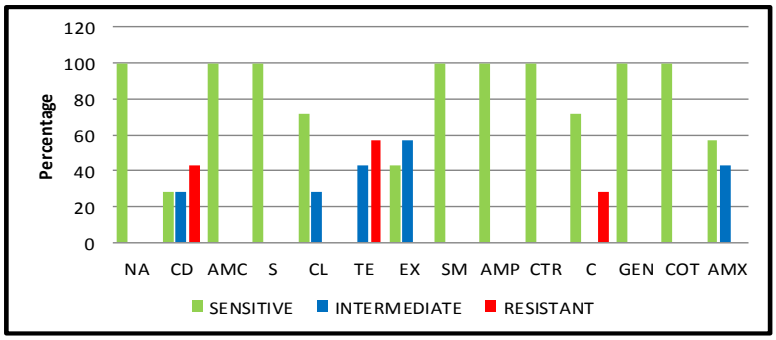
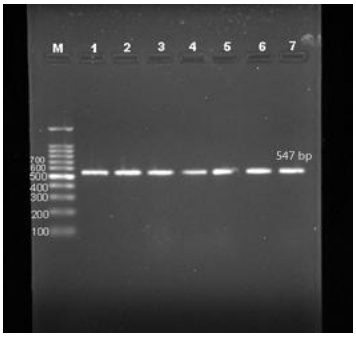
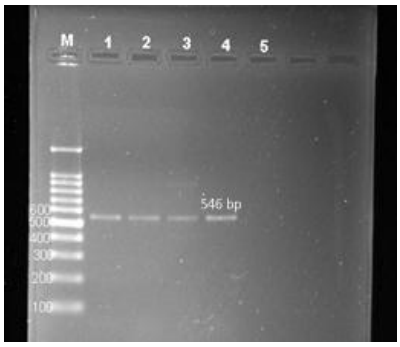


Fig.3 PCR amplification of *catA1* gene



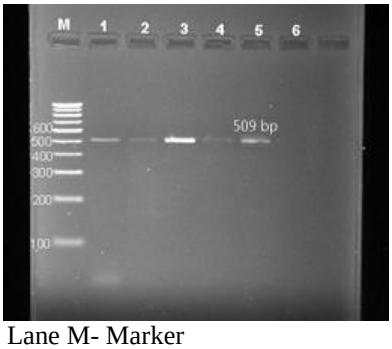
Lane M- Marker
Lane 1, 2, 3, 4, 5, 6, 7- positive

Fig.4 PCR amplification of *strA* gene



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

Fig.5 PCR amplification of *strB* gene



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 7pure 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% enrofloxacin and 28.57% to clindamycin. Though the organisms sensitive to some antibiotics but they showed intermediate resistance 57.14% to Enrofloxacin, 42.85% 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 override 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 suplphonamides, sheep isolates resulted sensitivity to sulphonamides and absence of genes s*ul1*, s*ul2*. 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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