Indian J Anim Health (2023), 62(2)- Special Issue: 247-253
DOI: https://doi.org/10.36062/ijah.2023.spl.01223

Research Article

Emergence of multidrug resistant strains of *Pasteurella multocida* isolated from cattle and buffaloes in India during the period 2000-2016

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Abstract

The present retrospective study reports the antibiotic resistance pattern among 149 strains of *Pasteurella multocida* isolated from cattle (67) and buffaloes (82) during the period 2000-2016. The isolates of cattle origin were highly resistant to tetracycline (53.7%), followed by sulfatrimethaxazole (41.7%), fluoroquinolone antibiotics pefloxacin (37.3%) and ciprofloxacin (34.3%). The isolates were found to be sensitive to gentamicin and amoxycillin. The isolates of buffalo origin were resistant to sulfatrimethaxazole (50%), followed by tetracycline (35.3%), pefloxacin, ciprofloxacin and streptomycin (26.8%) and the antibiotics gentamicin, erythromycin, cefoperazone and amoxycillin were found to be effective. The time trend analysis of antimicrobial resistance among the *P. multocida* isolates showed a high proportion of resistance against sulfatrimethaxazole, tetracyclines, streptomycin, and ampicillin, a moderate increase in resistance against kanamycin, pefloxacin, ciprofloxacin, gentamicin, enrofloxacin, erythromycin and chloramphenicol. However, the trend was found to be negative against spectinomycin, amoxycillin, cefoperazone and cefepime. The emergence of multidrug resistant strains of *P. multocida* among cattle and buffaloes warrants the judicious use of antimicrobial agents for treating Hemorrhagic septicemia in cattle and buffaloes.

Keywords: Antibiotic resistance, Cattle and buffaloes, India, *Pasteurella multocida*

Highlights

- The resistance to tetracyclines and sulfatrimethaxazole was found to be 53.7% and 41.7% among the cattle isolates and 35.3% and 50% among the buffalo isolates.
- Resistance was observed against sulfatrimethaxazole (50% buffaloes and 41.7% cattle) and sulphadiazine (30.4% buffaloes and 32.8% cattle), the drugs of choice for treating HS in cattle and buffaloes.
- The isolates were found to be sensitive to gentamicin, erythromycin, cefoperazone and amoxycillin.
- The time trend analysis during the periods 2000-2007 and 2008-2016 showed increased antimicrobial resistance (AMR) to commonly used antibiotics against pasteurellosis in animals.
- Emergence of multidrug resistance among the isolates warrants the judicious use of antimicrobial agents for treating HS in animals.

INTRODUCTION

Hemorrhagic septicemia (HS), caused by *Pasteurella multocida*, a Gram-negative coccobacillus bacterium, is the leading cause of mortality in cattle and buffaloes and was also the second most commonly encountered disease from 1991 to 2010 in India (Singh *et al*., 2014). About 74.4% of the total infection rate in the world was distributed among cattle, followed by buffaloes (13.1%). The mortality of HS among cattle and buffaloes increased in 2017–2019 compared to the period between 2014 and 2016 (Almoheer *et al*., 2022). The economic loss per animal due to HS in India was estimated as Rs.11,904, Rs. 13,044 and Rs.20,296 in case of indigenous and crossbred cattle and buffaloes, respectively. The share of buffaloes in total economic loss was highest (55%), followed by indigenous (28%) and crossbred (16.5%) cattle. Thus, the overall economic losses due to HS in bovines in India were found to the tune of Rs. 126.28–127.58 billion (Bardhan *et al*., 2020).

Five serogroups A, B, D, E, and F have been identified in *P. multocida* (Carter, 1967; Rimler and Rhoades, 1987; Harper *et al*., 2012), and 16 serotypes (1–16) have been described using the Heddleston scheme (Carter, 1955; Heddleston *et al*., 1972; Harper *et al*., 2006). The serotype B: 2 is associated with HS in Asia, while E: 2 is associated with Africa (De Alwis, 1999). Prophylactic measures for pasteurellosis in animals include reduction or even elimination of certain predisposing factors like

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stress, exposure to extreme climatic conditions and improved managemental practices supported by the use of vaccines. Lack of timely and proper treatment and insufficient preventive measures lead to high morbidity and mortality among bovines incurring heavy economic losses to farmers. Since the disease has a very short course and rapid onset, successful treatment against HS depends on the early administration of an effective antimicrobial agent before the onset of specific clinical signs (Prescott and Baggot, 1988). Some antibiotics like sulphonamides, tetracycline, penicillin and chloramphenicol are effective in the treatment of HS if administered early (Aiello, 1998). However, the prolonged and indiscriminate use of antibiotics has resulted in the development of resistance among various strains of the organisms (Arora *et al*., 2005; Jamali *et al*., 2014; Furian *et al*., 2016) and even multidrug resistant (MDR) and biofilm forming strains of *P. multocida* have emerged (Shivachandra *et al*., 2004; Furian *et al*., 2016). Nevertheless, for the choice of antibiotics, veterinary surgeons often rely on recommendations based on the resistance data obtained from the national monitoring programmes or on recommendations, which are given in the different textbooks. Albeit a plethora of reports have been published on antimicrobial resistance patterns of isolates of *P. multocida* in livestock and poultry in several countries, the reports for Indian isolates of *P. multocida* are scanty. The lack of antimicrobial resistance surveillance data on *P. multocida* in India is a major limitation in initiating early and effective antibiotic therapy for diseased animals.

Hence, the present study was done to determine the antimicrobial susceptibility of *Pasteurella multocida* isolated from cattle and buffaloes during the period 2000-2016.

MATERIALS AND METHODS

Bacterial strains: A total of one hundred and forty nine (149) freeze dried cultures of *P. multocida* isolated from cattle (62) and buffaloes (87) from different parts of India over a period of 16 years (2000-2016) and maintained in culture repository of Haemorrhagic Septicaemia (HS) laboratory, Division of Bacteriology and Mycology, Indian Veterinary Research Institute, Izatnagar were used in the study (Table 1).

Revival and confirmation of *P. multocida* isolate: The freeze-dried ampoules were broken, and the cultures were revived in brain heart infusion (BHI) broth. The revived broth cultures were then inoculated on 5% sheep blood agar. The genomic DNA of the isolates was extracted by CTAB method. Confirmation of the isolates was done by *P. multocida* specific PCR (PM-PCR) as per the method described (Townsend *et al*., 2001), and capsular typing was done using capsular PCR (Townsend *et al*., 1998) in Thermal cycler (PeqSTAR, USA). The primers used for PM-PCR and multiplex capsular typing are listed in Table 2. The PCR was set up in 25 µL reaction

Table 1. Number of isolates of *P. multocida* obtained from different avian species

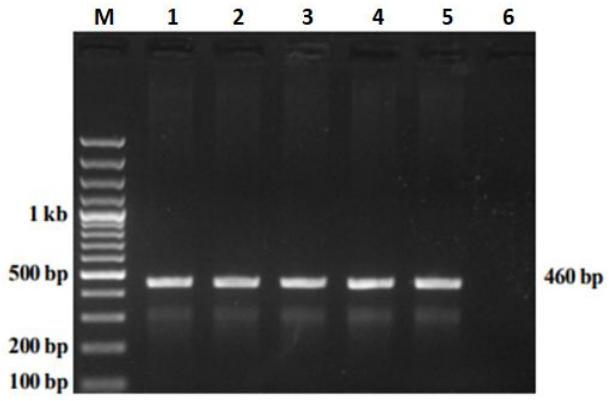
Species	No. of isolates	Serogroup				
		A	B	D	E	F
Cattle	67	17	50	None	None	None
Buffaloes	82	14	61	6	None	1

Table 2. List of primers used for PM PCR and Capsular PCR assays

Gene	Primer	Primer sequence (5′ -3′)	Amplified product size (bp)
KMT1	PM PCR	F- ATCCCGCTATTTACCCAGTGC R- GCTGTAAACGAACTCGCCAC	460
hyaD-hyaC	capA	F- GATGCCAAAATCGCAGTCAG R- TGTTGCCATCATTGTCAGTG	1044
bcbD	capB	F- CATTTATCCAAGCTCCACC R- GCCCGAGAGTTTCAATCC	760
dcbF	capD	F- TTACAAAAGAAAGACTAGGAGCCC R- CATCTACCCACTCAACCATATCAG	657
ecbJ	capE	F- TCCGCAGAAAATTATTGACTC R- GCTTGCTGCTTGATTTTGTC	511
fcbD	capF	F- AATCGGAGAACGCAGAAATCAG R- TTCCGCCGTCAATTACTCTG	851

tube with 2.5 µL of 10X Dream taq buffer (Thermoscientific, USA), 0.5 µL of 10 mM dNTPs (Thermoscientific, USA), 0.5 µL each of 10 pmol/µL forward and reverse primers (M/s. Eurofins, Bangalore), 2 µL of 100 ng/µL of template DNA, 0.25 µL of Dream taq DNA polymerase (5U/µL) (Thermoscientific, USA) and the final volume made to 25 µL with Nuclease free water. The PCR cycling conditions were standardized with Initial denaturation at 94°C for 5 min, followed by 34 cycles of denaturation at 94°C for 45 sec, annealing at 55°C for 45 sec, extension at 72°C for 45 sec and final elongation at 72°C for 10 min. The reaction conditions were same for both PM-PCR and Multiplex PCR. The PCR products were subjected to agarose gel electrophoresis using 1.5% agarose (Sigma, USA) and then visualized by a UV gel documentation system (Alpha Imager, Germany).

Antibiotic sensitivity test: Antibiotic susceptibility testing was performed using 16 antibiotics as per the method described by Bauer *et al*. (1966). On the basis of the results, the isolates were classified as sensitive, intermediate, and resistant according to Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2016). The antibiotics (Hi-Media laboratories, Mumbai, India) used are ampicillin (10 µg), amoxycillin (30 µg), chloramphenicol (30 µg), ciprofloxacin (5 µg), cefepime (5 µg), cefoperazone (75 µg), ceftriaxone (30 µg), erythromycin (15 µg), enrofloxacin (10 µg), gentamicin (10 µg), kanamycin (30 µg), pefloxacin (5 µg), streptomycin (10 µg), sulfamethoxazole trimethoprim (SXT), tetracycline (30 µg) and sulphadiazine (100 µg). Briefly, a single colony of each isolate is inoculated into BHI broth and incubated for a period of 10-12 h. The inoculum was adjusted to 0.5 Mc Farland and spread on a Muller-Hinton agar medium, and allowed to adsorb for 10 min. The antibiotic discs were placed onto the plate at an appropriate distance from each other, and the plates were incubated aerobically at 37°C for 24 h. The diameters of the zone of inhibition surrounding the antibiotic discs were measured and subsequently matched with the standard zone of inhibition diameters of respective antibiotic discs. The isolates were interpreted as sensitive, intermediately sensitive and resistant based on the interpretative criteria.



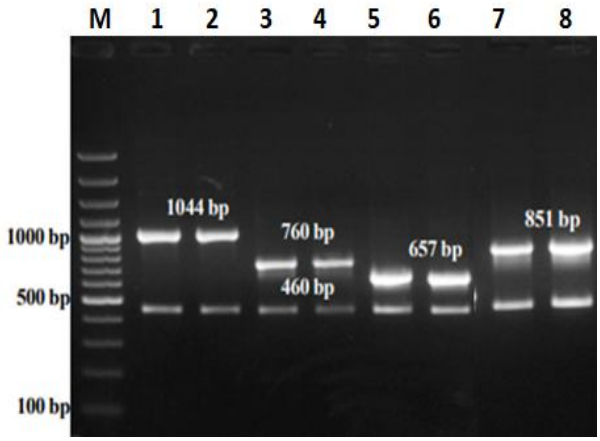
[Lane M: 100 bp DNA marker; Lanes 1-5: Agarose gel showing amplicon of 460 bp in PM-PCR; Lane 6: No Template control]

Fig. 1. PM-PCR of the isolates

RESULTS

Revival and confirmation of *P. multocida* isolates: All the cultures of *P. multocida* were successfully revived in BHI broth in pure cultures. On 5% sheep blood agar, the cultures were found to be pure, and the colonies were small, non-hemolytic and dewdrop-like in appearance. On Gram's staining of the single colony of cultures, small Gram negative coccobacillary organisms were observed suggestive of *P. multocida*. The genomic DNA isolated by CTAB method was found to be pure with O.D value of 1.8 at 260/280 nm. All the isolates gave an amplicon of ~460 bp in agarose gel electrophoresis in PM-PCR assay (Fig. 1). By capsular PCR assay, the isolates were re-confirmed. An amplicon of ~1044 bp, ~ 760 bp, ~657 bp, and ~851 bp were considered as confirmatory for capsular types A, B, D and F, respectively (Fig. 2).

Antibiogram of isolates: The strains of *P. multocida* isolated from cattle and buffaloes showed resistance to



[Lane M: 1 kbp DNA marker; Lanes 1-2: Amplicon showing 1044 bp (capsular type A) and 460 bp (*P. multocida*); Lanes 3-4: Amplicon showing 760 bp (capsular type B) and 460 bp (*P. multocida*); Lanes 5-6: Amplicon showing 657 bp (capsular type D) and 460 bp (*P. multocida*); Lanes 7-8: Amplicon showing 851 bp (capsular type F) and 460 bp (*P. multocida*)]

Fig. 2. Multiplex capsular PCR

Table 3. List of antimicrobial agents and resistance patterns observed among cattle and buffalo isolates

Antimicrobial agent	Host species resistance (%)	
	Buffalo	Cattle
Ampicillin (10 µg)	22	13.4
Amoxycillin (30 µg)	6	6
Chloramphenicol (30 µg)	29.2	28.3
Ciprofloxacin (5 µg)	26.8	34.3
Cefepime (5 µg)	15.8	8.9
Cefoperazone (75 µg)	2.4	0
Ceftriaxone (30 µg)	9.7	5.9
Erythromycin (15 µg)	3.6	5.9
Enrofloxacin (10 µg)	2.4	2.9
Gentamicin (10 µg)	1.2	1.4
Kanamycin (30 µg)	25.6	22.3
Pefloxacin (5 µg)	26.8	37.3
Streptomycin (10 µg)	26.8	20.8
Sulfatrimethaxazole (25 µg)	50	41.7
Sulphadiazine (100 µg)	30.4	32.8
Tetracycline (30 µg)	35.3	53.7

the majority of the antibiotics tested in the study (Table 3).

Tetracyclines and sulfatrimethaxazole, the most commonly used antibiotics for treating pasteurellosis in animals, showed the highest resistance to the isolates from cattle (53.7%, 41.7%) and buffaloes (35.3%, 50%). The resistance of the isolates from cattle for other antibiotics pefloxacin, ciprofloxacin, sulphadiazine, chloramphenicol, kanamycin, streptomycin, ampicillin, cefepime were in the order 37.3%, 34.3%, 32.8%, 28.3%, 22.3%, 20.8%, 13.4% and 8.9%, respectively (Fig. 3). Nevertheless, least resistance was observed for the antibiotics amoxycillin, ceftriaxone, erythromycin, enrofloxacin, gentamicin in the order 6%, 5.9%, 5.9%, 2.9% and 1.4%, respectively. The isolates were found to be 100% sensitive to cefoperazone drug. Whereas, the isolates from buffaloes showed resistance to sulphadiazine (30.4%), chloramphenicol (29.2%) and ciprofloxacin (26.8%). The resistance to other antibiotics, pefloxacin, streptomycin, kanamycin, ampicillin, and cefepime, was in the order 26.8%, 26.8%, 25.6%, 22% and 15.8%, respectively. The resistance pattern was found to be increased in the isolates over a period of time (Table 4). The isolates during the period 2008-2016 showed increased resistance to the majority of the commonly used antibiotics sulfatrimethaxazole, tetracyclines, streptomycin and ampicillin, a moderate increase in resistance against kanamycin, pefloxacin, ciprofloxacin, gentamicin, enrofloxacin, erythromycin and

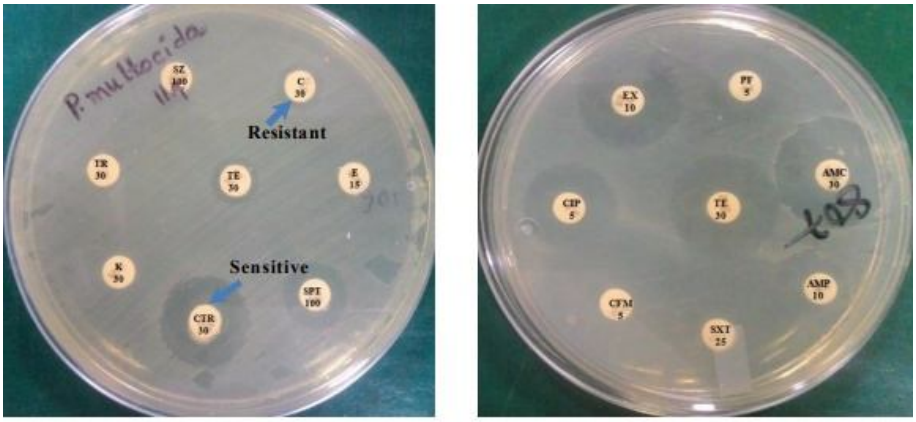


Fig. 3. Antibiogram of *P. multocida* isolate using different antibiotics on Mueller Hinton Agar

Table 4. Time trend analysis of antibiotic resistance of *P. multocida* isolates from cattle and buffaloes to various antibiotics during two different periods (2000 - 2007 and 2008 - 2016)

Sl. No.	Antibiotics	2000-2007		2008-2016	
		(n=82)	(%)	(n=67)	(%)
1	Ampicillin	8	9.76	19	28.36
2	Amoxycillin	5	6.10	4	5.97
3	Chloramphenicol	23	28.05	20	29.85
4	Ciprofloxacin	24	29.27	22	32.84
5	Cefepime	11	13.41	8	11.94
6	Cefoperazone	2	2.44	0	0.00
7	Ceftriaxone	7	8.54	5	7.46
8	Erythromycin	2	2.44	5	7.46

Cont. Table 4.

Table 4., Cont. ...

Sl. No.	Antibiotics	2000-2007 (n=82)	(%)	2008-2016 (n=67)	(%)
9	Enrofloxacin	2	2.44	2	2.99
10	Gentamicin	1	1.22	1	1.49
11	Kanamycin	16	19.51	20	29.85
12	Pefloxacin	26	31.71	21	31.34
13	Streptomycin	15	18.29	21	31.34
14	Sulfatrimethaxazole	23	28.05	46	68.66
15	Spectinomycin	33	40.24	19	28.36
16	Tetracycline	28	34.15	37	55.22

n = total number of isolates tested against each antibiotic

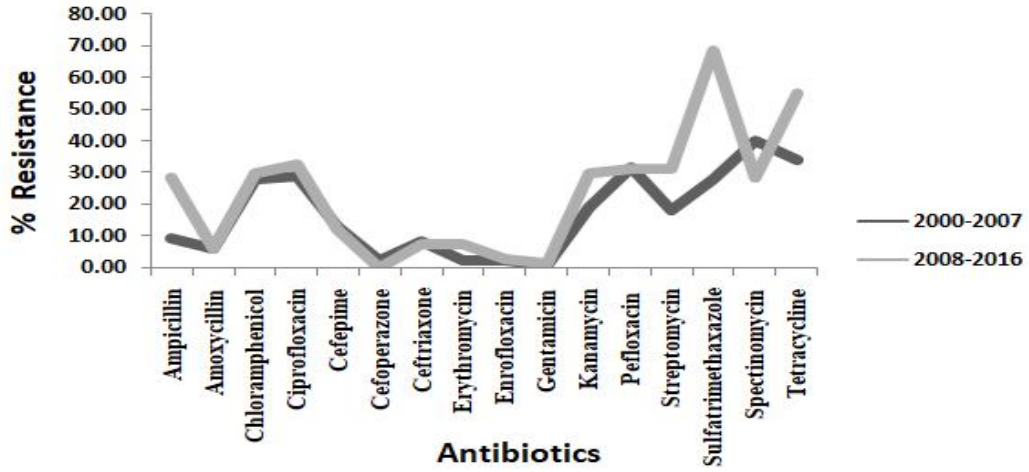


Fig. 4. Time trend analysis of antibiotic resistance pattern observed among the 149 isolates of *P. multocida* during the periods 2000-2007 and 2008-2016

chloramphenicol than those isolated during the period 2000-2016. However, for the antibiotics spectinomycin, amoxycillin, cefoperazone and cefepime, the trend was found to be negative (Fig. 4).

DISCUSSION

All the revived cultures of *P. multocida* were found to be pure on 5% sheep blood agar, with small, non-hemolytic and dewdrop-like colonies in appearance. On Gram's staining, small Gram negative coccobacillary organisms were observed suggestive of *P. multocida***.** The genomic DNA isolated by CTAB method was found to be pure with O.D value of 1.8 at 260/280 nm. All the isolates gave an amplicon of ~460 bp in agarose gel electrophoresis in PM-PCR assay, which is consistent with other studies (Kumar *et al*., 2009). By capsular PCR assay, the isolates were re-confirmed as capsular type A (~1044 bp), capsular type B (~760 bp), type D (~657 bp) and type F showing an amplicon of ~851 bp. The results of the capsular PCR assay were in accordance with Townsend *et al*. (2001), who reported that amplification of 1044 bp, 760 bp, 657 bp, 511 bp and 851 bp amplicon corresponding to capsular types A, B, D, E and F, respectively of *P. multocida*.

In India, the antibiotics like penicillins (ampicillin, amoxycillin), cephalosporins (ceftriaxone, cefepime), fluoroquinolones (ciprofloxacin, enrofloxacin), aminoglycosides (gentamycin, streptomycin), tetracyclines, sulphadiazine and trimethoprim are commonly used for the treatment of various infectious diseases of livestock and poultry (Selleyi *et al*., 2009). Generally, *P. multocida* are susceptible to most of the presently available antibacterial drugs. However, strains with different antimicrobial resistance patterns are always present in animal populations and may vary according to the host origin, geographical location and the previously applied antimicrobial therapy in the given animal population (Selleyi *et al*., 2009).

In the present study, 53.7% of the *P. multocida* isolates from cattle and 35.3% of the isolates from buffaloes were resistant to tetracyclines. Anwar *et al*. (2000) reported that 37.5% and 25% of isolates from cattle and buffaloes in Pakistan were resistant to

oxytetracycline and doxycycline, respectively. Similar findings were recorded by Qureshi and Saxena (2014) in India, Katsuda *et al*. (2013) 21.7% in Japan, and Jamali *et al*. (2014) 19.9% in Iran. The resistance to sulfatrimethaxazole was found to be 41.7% in isolates from cattle and 50% in buffalo isolates. The present findings are similar to earlier reports where majority of the isolates from cattle and buffaloes were found to be resistant to sulphonamides and sulphonamide-trimethoprim combinations (Anwar *et al*., 2000; Biswas *et al*., 2004; Kumar *et al*., 2009; Naz *et al*., 2012; Sugun *et al*., 2013; Elshemey and Abd-Elrahman, 2013; Qureshi and Saxena, 2014). Since tetracyclines and sulphonamides are commonly used for the treatment of pasteurellosis in animals, the selective pressure could have resulted in the emergence of resistance in the isolates in the present study. The resistance to fluoroquinolone antibiotics in isolates from cattle was found to be pefloxacin (37.3%) and ciprofloxacin (34.3%), and in isolates from buffaloes was found to be pefloxacin, ciprofloxacin and streptomycin (26.8%). Ciprofloxacin resistant isolates from cattle have been documented by several workers (Durrani *et al*., 2013; Elshemey and Abd-Elrahman, 2013; Sugun *et al*., 2013; Qureshi and Saxena, 2014). In the present study, the antibiotics gentamicin, erythromycin and amoxycillin were found to be sensitive, and no resistance was observed against cefoperazone in cattle and buffalo isolates. The resistance to ampicillin was observed among 18% of the isolates. Similar to our findings, penicillins were found to be sensitive against *P. multocida* isolates from cattle in Turkey (Onat *et al*., 2010). Anwar *et al*. (2000) reported 81.3% of resistant isolates to ampicillin from cattle in Pakistan, Qureshi and Saxena (2014) and Elshemey and Abd-Elrahaman (2013) in Egypt, Sugun *et al*. (2013), Khamesipour *et al*. (2014) and Jamali *et al*. (2014) in Iran observed resistance to penicillins in cattle isolates. Similar to our results, Biswas *et al*. (2004) reported that the isolates from cattle in India were sensitive to erythromycin. The results of the present study are similar to the findings of Biswas *et al*. (2004), Elshemey and Abd-Elrahman (2013), Sugun *et al*. (2013) and Jamali *et al*. (2014), who also observed gentamicin as a highly effective antimicrobial agent for *P. multocida* isolates from cattle. Moreover, the time trend analysis of the resistance pattern among the isolates showed that the resistance to the majority of the antibiotics was increased in the isolates during the period 2008-2016 than those isolated during 2000-2007. Even though, the number of isolates is limited and being a retrospective study, cannot infer to the large extent antibiotic resistance, increased antimicrobial resistance pattern (AMR) over a period of time suggests that the organisms are gaining resistance by selective pressure with increased usage of antibiotics. Moreover, the emergence of MDR isolates is a matter of concern as the number of effective antimicrobials will be greatly reduced, resulting in frequent treatment failures and huge economic loss to animal owners as a result of high morbidity and mortality. Hence, judicious use of antibiotics will significantly reduce the cost of treatment and also prevent treatment failures. Wherever possible, antimicrobial susceptibility testing should be carried out before initiating antimicrobial therapy to animals.

Conflict of interest: The authors declare that they have no conflict of interest.

Author's contributions: VKS: Revived the cultures, carried out the antimicrobial sensitivity part and drafted the manuscript; VKN, RKA: Conceived the study and participated in designing the research work.

ACKNOWLEDGEMENTS

The authors thank Indian Council of Agricultural Research (ICAR), New Delhi, for providing financial support under the 'All India Network Programme on Haemorrhagic Septicaemia' and the Director, Indian Veterinary Research Institute (Izatnagar), for providing facilities for carrying out this study.

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Received- 03.09.2023, Accepted- 08.11.2023, Published- 01.12.2023 (Print)
Section Editor: Dr. I. Samanta, Associate Editor