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Multidrug-resistance Pattern of Antibiogram of *Escherichia coli* and *Staphylococcus aureus* Isolated from Mastitis Affected Buffaloes in Eastern Uttar Pradesh

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ABSTRACT

Background: Mastitis is a problem of dairy animals including buffaloes. Treatment failure has become a common problem and the most cited reason is antibiotic resistance. The paper presents the scenario of antibiotic resistance with special reference to multi drug resistance pattern.

Methods: Microbiological assay of mastitis affected 48 milk samples was carried out using standard protocols to establish the prevalence of mastitis caused by *E. coli* and *S. aureus*. Antibiotic sensitivity test was carried out against these organisms and the whole milk culture for commonly used antibiotics. The drug resistance pattern was established.

Result: The overall prevalence of mastitis was 20%. The prevalence of *E. coli* and *S. aureus* was *29*.17% and 54.17% respectively. The antibiotic sensitivity revealed that *E.coli* isolates were 100% susceptible to tetracycline, gentamicin, enrofloxacin and streptopenicillin followed by Ceftriaxone and sulbactum combination and streptomycin (92.86%) and ceftriaxone and methicillin (85.71%). The isolates of *S. aureus* were 100% sensitive to only two antibiotics gentamicin and streptopenicillin. The antibiogram of whole milk culture revealed maximum susceptibility to enrofloxacin, gentamicin and streptopeniciliin (95.83% each). Multiple drug resistance has been observed in this study.

Key words: Antibiogram, Antibiotic sensitivity, Buffalo, Drug resistance pattern, Mastitis.

INTRODUCTION

Mastitis is an inflammatory disease of udder, which is an outcome of interaction of various associated factors *viz*. the host, pathogen(s), environment and poor managemental conditions. Large numbers of micro-organisms are known to cause inflammation of udder parenchyma and because of this diverse array of etiological factors like bacteria, virus, fungus, physical, chemical, toxins and other environmental factors *etc*., the treatment of the disease is always a challenge to the veterinarian. This has prompted scientists from all over the world to identify different aspects related to the health of the mammary gland and milk products of buffaloes as in Brazil (Medeiros *et al.,* 2011), India (Yadav *et al., 2020*), Pakistan (Hussain *et al.,* 2013), Nepal (Dhakal *et al*., 2007), Italy (Fagiolo and Lai 2007) and Germany (Braun and Preuss, 2007). This is because of the menace of the antibiotic resistance as reported by several workers (Chavan *et al*., 2007; Roychoudhury and Dutta, 2009; Sharma *et al*., 2010; Tanzin *et al.,* 2016, Yadav *et al*., 2020). Selection of appropriate antibiotic against the common mastitis causing pathogens namely *S. aureus* and *E. coli* is crucial for proper treatment of mastitis in cattle and buffaloes. Time to time evaluation of area specific antibiogram can be a step promoting judicious use of antibiotics and can improve treatment percentage as well as must for welfare of animal.

Therefore, the objective of this study was to isolate and study the prevalence of common mastitogens from buffalo in Eastern Plain Zone of Eastern Uttar Pradesh and to evaluate the antibiotic sensitivity pattern against different isolates from milk.

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MATERIALS AND METHODS

A total of 240 milk samples were collected from the buffaloes of the Sultanpur and Faizabad district. Two blocks from each district were selected and from each block three villages were chosen. From each village 20 samples were collected. In this way a total of 240 milk samples were collected from the buffaloes.

Clinical inspection of the udder

Udders of the buffaloes were examined by visual inspection and palpation for the presence of any lesion, pain, heat and swelling. In addition, milk from each quarter was withdrawn and checked for any change in colour and consistency.

Collection of milk sample

Milk samples were collected following all aseptic norms. The affected quarter was washed with tap water, dried properly and teat and udder was sterilized with cotton soaked with 70% ethyl alcohol. Approximately 10 ml of milk was then collected aseptically from a mastitic quarter into sterile bottle after discarding the first 3 to 4 milking streams. Sample from each quarter were transported on ice to the bacteriology laboratory of department of veterinary microbiology and were immediately cultured or stored at 4°C for a maximum of 24 hrs until cultured on standard bacteriological media.

Microbiological assay of milk sample

Sample processing was carried out in the laboratory of bacteriology in the department of veterinary microbiology. The method used for isolation of bacteria was described by Cruickshank *et al.* (1975) and isolates were identified on the basis of method described by Edwards and Ewing (1972).The collected sample were inoculated into sterile nutrient broth and incubated at 37°C for 24 hrs to see the growth of bacteria. The grown bacteria was streaked onto sterile Mac Conkey lactose agar plate and incubated at 37°C for 24 hrs. Single colony of bacteria was picked and a gram staining was performed. In the same way, one loopful of broth culture was streak upon mannitol salt agar (MSA) plates and plates were incubated at 37°C for 24 hrs. The single colony from MSA plates transferred to nutrient agar slant and incubated aerobically at 37°C for 24 hrs. Bacterial identification was carried out by studying morphology, culture characteristics and Gram's staining (Quinn *et al*., 2004). Lastly antibiotic sensitivity test (ABST) of all isolates was performed.

Isolation of *staphylococcus aureus*

Primary culture was done in nutrient broth for enrichment of bacteria. Pure culture of *S. aureus* was obtained based on Gram staining, morphological study, biochemical characteristics, catalase test, coagulase test, as per the procedures mentioned by Cheesbrough (1985) and Jahan *et al.* (2015).

Isolation of *E. coli*

MC agar media were streaked aseptically with 200 µL of milk sample and incubated at 37°C for overnight. Appearance of bright red or pink color colony was used to culture in EMB agar. Appearance of the colony of pink/red and greenish black with metallic sheen was considered positive for *E. coli* in EMB agar. The positive colonies were sub cultured into EMB agar to obtain pure colony. The pure colonies were subjected for sugar fermentation, biochemical tests, as per the procedure described by Cheesbrough (1985) and Nazir *et al.* (2005).

Antimicrobial susceptibility procedure

The disc diffusion sensitivity test also known as Kirby Bauer disk method is a simple and practical test which uses antibiotic-impregnated discs to test whether particular bacteria is susceptible to specific antibiotic. The bacterial inoculums were uniformly spread using sterile cotton swab on a sterile Petri dish. The antibiotic discs were placed on top of the previously inoculated nutrient agar medium surface with the help of sterile forceps. Each disc was pressed down to ensure complete contact with the agar surface. The plates were incubated for 18-24 hrs at 37°C temperature in bacteriological incubator before interpretation of the result. A zone of inhibition was measured in millimeters by either measuring: (a) Radius: Measure half the distance of the zone and then multiply by two. This method was used when part of the zone is not clear or has grown into another zone. (b) Diameter: Measure the entire length of the zone and subtract the disc diameter (Standard disc size 5 mm). The result of the test can be interpreted by using the criteria published by Clinical and Laboratory Standard Institute. If the organisms were inhibited by the concentration of the antibiotic, there will be no growth in the immediate area around the disks represented as zone of growth inhibition.

Table 1: Codes and concentration of selected antibiotic discs.

Name of antibiotic disc	Code of disc	Concentration of disc
Amoxicillin	AMX	10 mcg/disc
Ceftriaxone	CTR	10 mcg/disc
Enrofloxacin	EX	10 mcg/disc
Ceftriaxone + Sulbactum	CIS	30/15 mcg/ disc
Gentamicin	GEN	10 mcg/disc
Methicillin	MET	10 mcg/disc
Strepto penicillin	DCR	50 mcg/disc
Tetracycline	TE	10 mcg/disc
Streptomycin	S	10 mcg/disc
Vancomycin	VA	10 mcg/disc
Penicillin	P	10 mcg/disc
Oxacillin	Ox	5mcg/disc

The diameter of the zone of inhibition is considered directly proportional to the sensitivity of the isolate and to the diffusion rate of antibiotics through the agar medium. The antibiotics used in this study are given in (Table 1).

Ethical approval

The research work was duly permitted by the Institutional Animal Ethics Committee (IAEC) *viz*. Approval No: IAEC/CVSc/2/P-28/2020/22, dated- 23.01.2020. All samples were collected as per standard procedure without harming or giving stress to the animals.

RESULTS AND DISCUSSION

The present investigation was planned to study the prevalence of mastitis pathogens namely *E. coli* and *S. aureus* and the antibiogram pattern against *E. coli, S. aureus* and whole milk culture with special reference to drug resistance pattern scenario in buffaloes of middle gangetic plains of eastern Uttar Pradesh. Out of the total 240 buffalo milk samples screened 48 samples were tested positive for mastitis. These 48 samples from mastitis affected buffaloes were collected and screened microbiologically for the presence of and *S. aureus* (Fig 1,3 and 5) and *E. coli* (Fig 2, 4 and 6). The overall prevalence of mastitis was 20%. The prevalence of *E. coli* and *S. aureus* was 29.17% (14/48) and 54.17% (26/48) respectively.

The frequency of *E. coli* observed in present study is in close agreement with the data obtained by El-Razik *et al*. (2010); Khan and Muhammad (2005); Farooq *et al.* (2008); Hameed *et al.* (2008); Ali *et al.* (2011); Bhanot *et al*. (2012); El-Sayed Lamey *et al.* (2013) who observed 23.75%, 18%, 16%, 15.38%, 16.18%, 16.3%, 18.47% frequency of E. coli, respectively in buffalo milk samples but less than 52.333% prevalence reported by Singh *et al.* (2018) .

The prevalence of *S. aureus* observed in the present study is in close association with the reports of Patnaik *et al.* (2014) who reported 53.33% prevalence due to *S. aureus*. Saidi *et al.* (2013) and Srinivasan *et al.* (2013) subjected the milk samples positive for California Mastitis Test to bacteriological culture and revealed that *S. aureus* was the most prevalent organism 40.0% and 46.30% respectively. Antibiotic sensitivity of different antibiotics namely Amoxicillin,

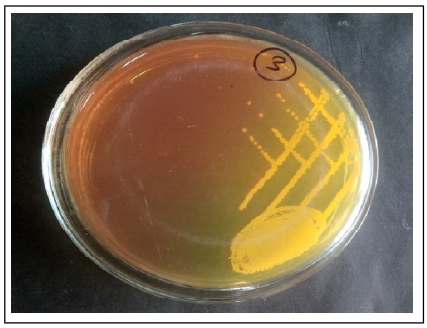


Fig 1: *S. aureus* colony on mannitol salt agar.



Fig 2: *E. coli* colony on EMB agar.

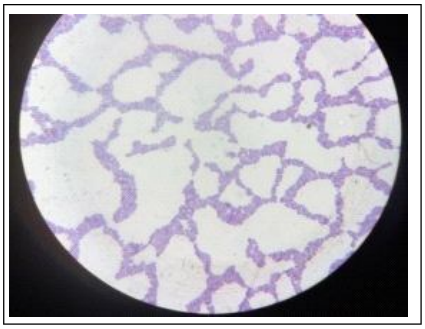


Fig 3: Gram stain of *S. aureus* in characteristic arrangement of "bunch of grapes"

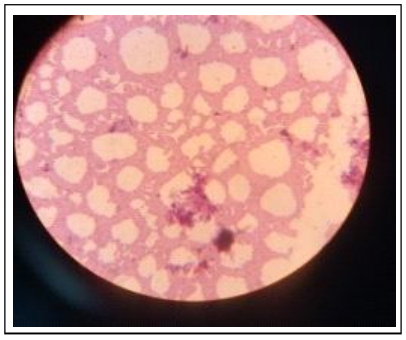


Fig 4: Gram stain of *E.coli* characterised by rod.

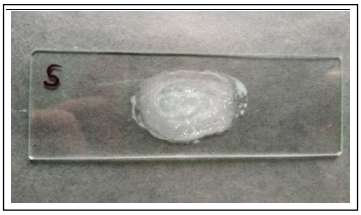


Fig 5: Catalase positive test of *S. aureus.*



Fig 6: Catalase positive E coli.

Ceftriaxone, Ceftriaxone + Sulbactum, Enrofloxacin, Gentamicin, Strepto-penicillin, Methicillin, Tetracycline, Streptomycin, Vancomycin, Penicillin, Oxacillin were studied against *S. aureus*, *E. coli* and milk culture (Table 2.). These 15 antibiotics belonged to the seven groups *viz.* fluoroquinolones, aminoglycosides, tetracycline, cephalosporins, penicillin and beta lactum. The resistance pattern especially the multi drug resistance pattern was established (Table 3).

The antibiotic sensitivity revealed that *E. coli* isolates were 100% susceptible to Tetracycline, Gentamicin, Enrofloxacin and Streptopenicillin followed by Ceftriaxone and sulbactum combination and streptomycin (92.86%) and Ceftriaxone and Methicillin (85.71%) (Fig 7). The isolates were least susceptible to Amoxicillin and vancomycin (71.43%) and oxacillin (78.57%). The isolates of *S. aureus* were 100% sensitive to only two antibiotics Gentamicin and Streptopenicillin (Fig 8). The antibiotics in order of sensitivity are Enrofloxacin (96.15%) > vancomycin (92.31%) > penicillin (88.46%) > streptomycin (84.62%) >tetracycline (80.77%) > Ceftriaxone and sulbactum combination and amoxicillin (76.92% each) > oxacillin (73.07%) > Ceftriaxone and Methicillin (69.23% each). Methicillin resistance was reported in 30.77% *S. aureus* isolates. The antibiogram of whole milk culture (Fig 9) revealed maximum susceptibility to Enrofloxacin, gentamicin and streptopeniciliin (95.83% each) followed in decreasing order by tetracycline, combination of Ceftriaxone and sulbactum (93.75%), penicillin

Table 2: Antibiotic sensitivity pattern of common antibiotics against *S. aureus, E. coli* and whole milk culture of buffalo milk.

Antibiotic	*E. coli*			*S. aureus*			Whole milk culture		
	Susceptible (%)	Range of zone (mm)	Average Zone (mm)	Susceptible	Range of zone (mm)	Average Zone (mm)	Susceptible	Range of zone (mm)	Average Zone (mm)
Ceftriaxone	85.71	10-22	18.75±3.20	69.23	10-14	12.00±1.63	68.75	21-23	22.33±1.25
Cef trioxone+ sulbactum	92.86	20-26	22.5±2.65	76.92	14-18	16.00±2.00	93.75	22-24	23.33±1.54
Tetracycline	100	16-18	16.75±0.96	80.77	16-22	20±3.46	93.75	10-20	15.75±4.34
Gentamicin	100	18-21	19.5±1.29	100	18-23	20.25±2.06	95.83	20-26	23.25±2.75
Enrofloxacin	100	22-29	24.25±3.30	100	17-23	20.25±2.5	95.83	12-28	18.25±6.84
Amoxicillin	71.43	12-16	13.33±2.31	76.92	16-18	17±1.00	87.5	19-28	22.5±3.87
Strepto-penicillin	100	17-20	18.00±1.41	84.62	10-22	17.75±5.31	91.66	18-22	20.5±1.91
Streptomycin	92.86	18-21	19.75±1.25	100	13-22	18.25±3.86	83.33	18-25	21.00±3.6
Vancomycin	71.43	10-11	10.5±0.71	100	10-16	13±2.58	81.25	20-21	19.33±1.15
Oxacillin	78.57	10-12	10.66±0.57	73.07	10-14	12±2	77.08	18-20	20.69±1.154
Penicillin	100	16-20	17.75±1.71	100	16-18	17±0.82	91.66	17-25	20.5±3.69
Methicillin	85.71	10-13	11.33±1.53	69.23	10-17	13.25±2.98	81.25	17-21	19.5±1.73

Table 3: Multi drug resistant pattern against *S. aureus, E. coli* and Whole milk culture of buffalo milk.

Pathogen (Total No. of Isolates)	No. of antibiotics	No. of resistant isolates	Combination of antibiotics
E.coli (14)	2	3 (21.43%)	Amoxicillin + Ceftriaxone
		2 (14.29%)	Methicillin + Vancomycin
	3	2(14.29%)	Amoxicillin + Methicillin +Oxacillin
S. aureus (26)	2	4(15.38%)	Amoxicillin + Methicillin
	4	2(7.69%)	Ceftriaxone + Methicillin + Oxacillin + Tetracyclin
	5	2(7.69%)	Ceftriaxone + Methicillin +Oxacillin+ Tetracycline+ Ceftriaxone and sulbactum
Whole milk culture	2	6 (12.5%)	Streptomycin + Methicillin
		3(6.25%)	Amoxicillin + Tetracycline
		2(4.17%)	Vancomycin + Oxacillin
		1(2.08%)	Methicillin+ Oxacillin
	3	2 (4.17%)	Ceftriaxone + Amoxicillin + Ceftriaxone and sulbactum
	5	3(6.25%)	Ceftriaxone + Ceftriaxone and sulbactum + Tetracycline+ Methicillin+ Oxacillin

(91.66%), amoxicillin (87.5%) and streptomycin (83.33%), vancomycin and Methicillin (81.25% each), oxacillin (77.08%) and Ceftriaxone (68.75%).

Other workers too have reported resistance to wide range of common antibiotics in *E. coli* from animals in India and abroad as in this study (Preethirani *et al.,* 2015, Ranjan *et al.,* 2011, Kisku and Samad, 2013; Verma *et al.,* 2018; Kalmus *et al.*, 2011 and Jeykumar *et al*., 2013). Ranjan *et al.* (2010) has also reported higher resistance of mastitis pathogens to amoxicillin. In *S. aureus* resistance to Ceftriaxone is in concordance with most of the recent reports of drug resistance pattern in investigations conducted on mastitis pathogens (Upadhyay and Kataria, 2009 and Nathawat *et al*., 2013). This high emergence of resistance to cephalosporin's is due to its broad spectrum of action and frequent use by most of the clinicians and all the quacks practicing in the field as the first line of treatment in recent years. Thus, the probable long-term indiscriminate use of these antibiotics in the region has led to the appearance of resistance in mastitogenic bacteria against third generation cephalosporin (Ankita, 2015). Yadav *et al.* (2020) have reported the Methicillin resistance in 19.28% from this region. Studies from North west India and Chennai reported relatively lesser methicillin resistant *Staphylococcus aureus* positive percentages as 13% and 10.44% respectively (Kumar *et al*., 2010 and Chandrasekaran, 2014). Dar *et al.* (2014) from Jammu and Kashmir reported that enrofloxacin, gentamicin, tetracycline and amoxicillin clavulanic acid were effective in descending order of susceptibility against the whole milk cultures positive for SCM.

Multiple drug resistance has been observed in this study (Table 3, Fig 7,8,9). Among the 14 *E. coli* isolates, 05 isolates (35.71%) showed resistance against 2 antibiotics and 02 isolates (14.29%) were found resistant to three antibiotics (Amoxicillin + Methicillin and Oxacillin). Resistance against Amoxicillin and ceftriaxone was observed in 03 isolates (21.43%) and resistance against Methicillin and vancomycin was observed in 02 isolates (14.29%). Out of 26 isolates of *S. aureus*, 8 isolates (30.77 %) were found to be resistant against 02 or more than 02 antibiotics. Maximum *S. aureus* isolates (15.38%) were resistant to 02 antibiotics namely amoxillin +methicillin. In whole milk culture 35.42 % cultures exhibited resistance to 02 or more antibiotics. Maximum isolates (06; 12.5%) were resistant to Streptomycin +

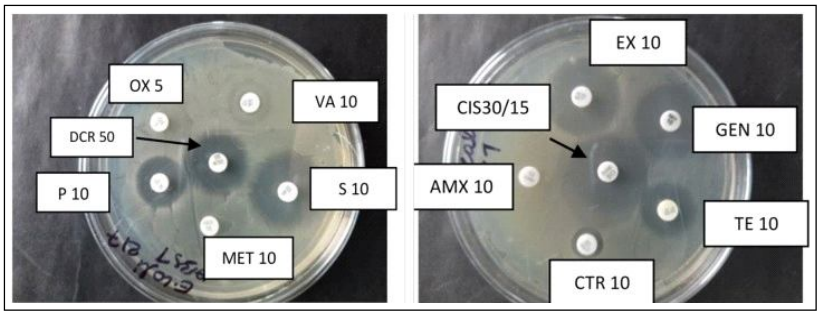


Fig 7: Antibiogram against *E. coli* isolated from buffalo milk.

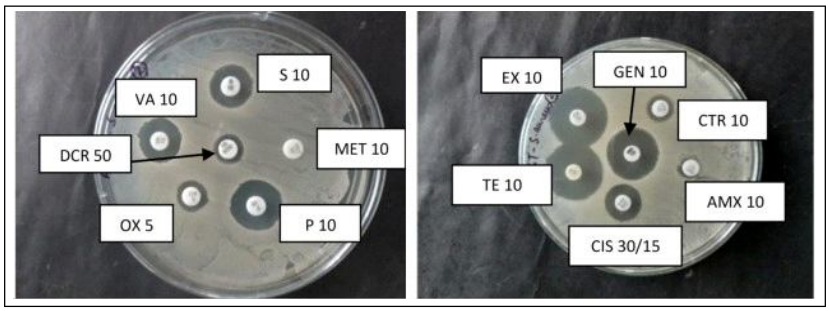


Fig 8: Antibiogram against *S. aureus* isolated from buffalo milk.

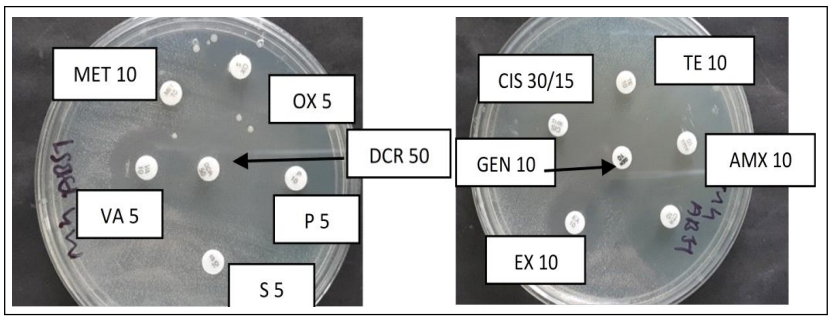


Fig 9: Antibiogram against whole milk culture.

Methicillin. Resistance against 05 antibiotics (Ceftriaxone + Ceftriaxone and sulbactum+Tetracycline + Methicillin+ Oxacillin) were observed in 03 (6.25%) isolates.

There are large numbers of reports from different parts of the world that describe increased trend of developing multiple resistance strains (Shana *et al*., 2009; Rinsky *et al*., 2013, Chavan *et al*., 2007, Sharma *et al*., 2007 Roychodhary and Dutta, 2009). In this region too Ankita (2015) and Yadav *et al.* (2020) observed multidrug resistance in *S. aureus* isolates. The multiple drug resistance in Staphylococcal was also reported by other workers (Ombui *et al*., 2000; Gentilini *et al*., 2002; Rajala-Schultz *et al*., 2004; Adwan *et al*., 2006; Wang *et al*., 2013; Kumar *et al*., 2010). A high level of antimicrobial resistance to many antibiotics and an elevated number of multiresistant strains among mastitis-associated *E. coli* strains have been reported in an earlier studies too (Allen *et al*., 2011; Rangel and Marin 2009). Development of resistance has been attributed to the extensive therapeutic use of antimicrobials (Abo-Shama, 2014). Multidrug resistance in *E. coli* from milk sample was also reported by Zdolec *et al*. (2016). Mahantesh and Basappa (2011) and Jeykumar *et al.,* 2013 were of the view that resistance to multiple antibiotics can be attributed to the injudicious use of antibiotics or selection pressure of antimicrobials on pathogens or colonization of the mammary gland by resistant strains along with the negligence towards bacteriological examination of the mastitis suspected milk.

Susceptibility to gentamicin of the cultures under study was in close agreement with many workers (Sarangi *et al*., 2009; Ranjan *et al*., 2010; Moges *et al*., 2011) . Farooq *et al*. (2008) during their study period have found that norfloxacin, gentamicin and chloramphenicol were the most effective drugs whereas Sumathi *et al*. (2008) reported gentamicin to be most effective among the antimicrobials used in study. The susceptibility to qunilones can be attributed to their lesser use in treatment of mastitis due to its higher dose and twice a day application resulting in higher efficacy of these drugs and corroborates with the quick response to the treatment with Gentamicin.

CONCLUSION

Drug resistance has been established in buffaloes in this region. The overall prevalence of mastitis was 20%. The prevalence of *E. coli* and *S. aureus was* 29.17% and 54.17% respectively. The antibiotic sensitivity revealed that *E. coli* isolates were 100% susceptible to Tetracycline, Gentamicin, Enrofloxacin and Streptopenicillin. The isolates of *S. aureus* were 100% sensitive to only two antibiotics Gentamicin and Streptopenicillin. The antibiogram of whole milk culture revealed maximum susceptibility to Enrofloxacin, Gentamicin and Streptopeniciliin (95.83% each). Multiple drug resistance has been observed in this study. The antibiotics should be used judiciously to curb this menace of antibiotic resistance.

Conflict of interest

All authors declare that they have no conflict of interest.

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