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Isolation, Molecular Identification and Multidrug Resistance Profiling of Bacteria Causing Clinical Mastitis in Cows

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ABSTRACT

Background: Mastitis, the inflammation of parenchyma of mammary gland is frequently considered to be costliest and complex disease prevalent in India. Mastitis is caused by pathogens like *Staphylococcus* spp., *Streptococcus* spp., *Mycoplasma bovis*, *E. coli*, *Klebsiella* spp., *Citrobacter* spp., *Enterobacter* spp. and *Entercoccus*. The treatment of mastitis in animals is carried out using antibiotics. Treatment failure in mastitis is due to increased antibiotic resistance of mastitis pathogens and also due to indiscriminate use of antibiotics without testing *in vitro* antibiotic sensitivity test against causal organisms. In comparison to cultural method, PCR assays takes less time for detection of bacteria from the mastitis milk samples. Present research work was carried out regarding isolation, identification and multiple drug resistance profile of clinical bovine mastitis associated pathogens using conventional as well as molecular approach.

Methods: In the present study, 73 mastitis milk samples were collected from Anand and Panchmahal district of Gujarat. The milk samples were subjected for cultural isolation and DNA extraction for identification of bacteria by cultural and PCR method. Antimicrobial sensitivity pattern of the isolates were carried by disc diffusion method and isolates were categorized in multiple drug resistant.

Result: In the present study, Out of 73 mastitis milk samples collected from cows 48 (65.75%) cows were positive for bacterial isolation and *S. aureus* was the most predominant bacterial species. PCR from the mastitis milk additionally detected bacteria in culturally negative milk samples. Most sensitive drug was gentamicin and most of the isolates (90.19%) showed the multiple drug resistance for the two to nine drugs with 0.1 to 0.6 multiple antibiotic resistance index.

Key words: Antibiotic susceptibility, Cows, Mastitis, Molecular.

INTRODUCTION

The dairy sector in India has shown remarkable development in the past decade. Milk quality is an important aspect of dairy production. The emerging Indian dairy industry is being challenged by the increasing demand to produce high quality dairy products from a milk supply that many times may not be satisfactory in its processing characteristics. The quality control programmes of dairy industry focus on several aspects, including the presence of bacteria and somatic cells in raw milk (Singh and Ramchandran, 2020).

Mastitis, the inflammation of parenchyma of mammary gland is frequently considered to be costliest and complex disease prevalent in India (Sharma *et al*., 2006). Mastitis is generally classified into clinical and subclinical mastitis. Clinical mastitis is characterized by local (*e.g*. swelling of the udder, heat and pain) or systemic (*e.g*. fever, anorexia, depression) symptoms with milk abnormalities (*e.g*. milk clots, flakes, watery secretions, blood) (Gruet *et al*., 2001). Radostitis *et al.* (2007) have classified udder pathogens into 3 categories *viz*. major pathogens, minor pathogens and uncommon mastitis pathogens. The major pathogens are *Staphylococcus* spp., *Streptococcus* spp., *Mycoplasma bovis*, *E. coli*, *Klebsiella* spp., *Citrobacter* spp., *Enterobacter* spp. and *Entercoccus*. The minor pathogens include uncommon species of *Staphylococcus* and *Corynebacteriyum bovis. Streptococcus faecalis*has been also causing clinical mastitis in cows (Zeryehun and Abera, 2017). Major mastitis pathogens such as *S. aureus*, *Str. uberis*, *Str. dysgalactiae* and coliforms are usually considered more virulent and damaging to the udder than minor mastitis pathogens such as *Corynebacterium* spp. and coagulase negative staphylococci (CNS) (Reyher *et al*., 2012).

Intramammary infusion and parenteral route of antibiotics are the main approaches to treatment of mastitis on many dairy farms. Because of increased antibiotic resistance of mastitis pathogens, reduced responses for antibiotic therapy have become very common in veterinary practice (Wang *et al.,* 2006). One important reason for treatment failure is assumed to indiscriminate use of antibiotics without testing *in vitro* antibiotic sensitivity test against causal organisms (Saxena *et al*., 1993). This practice at one hand increases economic losses and on other results

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in development of resistance to commonly used antimicrobials (Owens *et al*., 1997). For effective suitable antibiotic therapy, bacterial isolation followed by antibiotic sensitivity test are always essential.

Efficient control against mastitis requires sensitive, rapid and specific tests to detect and identify the Major bacteria that cause heavy losses to the dairy industry. Molecular detection of pathogenic microorganisms is based on DNA amplification of the target pathogen. Therefore, efficient extraction of DNA from mastitic milk of bacteria for identification by PCR is a major step (Cremonesi *et al*., 2006). Comparison with the cultural method, the PCR assays are less time consuming. It takes less than 24 hours to be complete, while identification of bacteria to the species levels by conventional microbiological and biochemical methods required more than 72 hours (Amin *et al*., 2011). Due to limitations of culture methods, approaches using PCR have been used to identify mastitis pathogens. PCR-based identification provides a promising option for the rapid identification of bacteria. Species-specific DNA sequences such as the highly conserved rRNA genes or the 16S-23S rRNA intergenic spacer of the ribosomal RNA operon can be used for the identification of bacterial species in hours, rather than days. Moreover, the sensitivity of PCR based assays tends to be superior to bacterial cultures (Forsman *et al*., 1997) allowing the detection of small numbers of microorganisms. Current study was undertaken for isolation, identification of clinical bovine mastitis associated pathogens with conventional as well as molecular approach to assess the prevalence of pathogen along with its multiple drug resistance profile.

MATERIALS AND METHODS

The study was conducted for the period from November 2017 to April 2018 at Department of Veterinary Microbiology, College of Veterinary Science and Animal Husbandry, Anand Agricultural University, Anand, Gujarat, India.

Samples

Milk sample aseptically collected from total of 73 clinical mastitis affected cows from Anand (50) district and Panchmahal (23) district of Gujarat. All the cows were showing signs of clinical mastitis like flakes in milk, blood in milk, swelling of teats. Clinical mastitis affected udder and teats were first properly washed with 0.001 per cent (1:1000) potassium permanganate solution. After washing, the udder and teats were wiped with tissue paper and subsequently teat and teat orifices were scrubbed with 70 per cent ethanol. After discarding first 2-3 streams of milk, approximately 15ml foremilk samples were collected from affected quarter in a separate sterile screw-capped vial for conducting bacteriological culture examination.

Isolation and identification of bacteria from the affected quarters

A heavy inoculum of thoroughly mixed milk sample was streaked with the help of sterile platinum loop on 5% sheep blood agar for primary bacterial isolation. The plates were incubated at 37°C for 24-48 h. Following the incubation, the plates were examined for bacterial growth and the morphological characteristics of bacterial colonies were recorded. Identification and biochemical characterization of the bacterial isolates was carried out as per methods described by Cowan and Steel (1974).

Isolation of genomic DNA from bacterial culture

Isolation of genomic DNA from bacterial cultures was done as per Antony *et al.* (2007). A pure colony of bacteria was inoculated into five ml of brain Heart Infusion broth and incubated at 37°C for 18 h. One and half ml of this broth culture was transferred to an Eppendorf tube and centrifuged at 3000 × g for 10 min. The pellet was washed twice in PBS and the final pellet was suspended in 100 µl of triple distilled water. The mixture was boiled for 10 min and immediately chilled on ice for 30 min. The sample was then thawed and centrifuged at 3000 × g for 5 min. The supernatant was stored at -20°C for further use as template DNA.

PCR based identification of the major pathogens

The standard strain of *Staphylococcus aureus* (MTCC 1144) and *Escherichia coli* (MTCC 723) were used for standardization of PCR. The primer details and cycling conditions for each uniplex PCR were as per Table 1 and 2 respectively.

To confirm the targeted PCR amplification, 5 ml of the PCR products from each tube was mixed with one ml of 6X gel loading buffer and electrophoresed along with 100bp DNA molecular weight marker (Gene Ruler, MBI Fermentas) on 2.0% agarose gel containing ethidium bromide (at the rate of 0.5 mg/ml) at constant 80 V for 30 min in 0.5X TBE buffer. The amplified product was visualized as a single compact band of expected size under UV light and documented by gel documentation system (SynGene, Gene Genius Bio Imaging System, UK).

DNA extraction from the mastitic milk

Before milk samples used for DNA extraction they were subjected to process of centrifugation (10000 RPM for three minutes) and remaining milk was discarded and pallet was used for the DNA isolation. Isolation of bacterial genomic DNA directly from mastitic milk samples of cows using Nucleo-poreg DNA Fungal/Bacterial Mini Kit (cat. No. NP-7006D) were carried out following instruction manual of the kit.

Antimicrobial sensitivity pattern

In vitro antibiotic sensitivity patterns of the isolates were conducted as per the method of Bauer *et al.* (1966). Antibiotics disc (Hi Media Ltd., Mumbai, India) used in the present study were Amikacin (30 mcg), Amoxyclav (30 mcg), Ampicillin (10 mcg), Ciprofloxacin (5 mcg), Colistin (10 mcg), Ceftriaxone (30 mcg), Erythromycin (15 mcg), Enrofloxacin (10 mcg), Gentamicin (10mcg), Neomycin (30 mcg), Penicillin-G (10IU), Streptomycin (10 mcg), Sulphadiazine (300 mcg) and Tetracycline (30 mcg). Diameters of the clear

Table 1: Details of primers used.

Primer	Product size	Reference
Staph F 5′- TCAACGATATTCTTCACGACTAA -3′	179bp	Gillespie and Oliver (2005)
Staph R 5′- CCAGCTTCGGTACTACTAAAG -3′		
Strep F 5′- CGTTGGTAGGAGTGGAAAAT -3′	586bp	Riffon *et al.* (2001)
Strep R 5′- CTGCTCCGAAGAGAAAGCCT -3′		
E. coli F 5′- ATCAACCGAGATTCCCCCAGT -3′	232bp	
E coli R 5′- TCACTATCGGTCAGTCAGGAG -3′		

Table 2: Steps and conditions of thermal cycling for different primers in PCR.

Primers	Cycling conditions				
(forward and reverse)	Initial denaturation	Denaturation	Annealing	Extension	Final extension
		Repeated for 30 cycles			
Sau F	95°C	95°C	57°C	72°C	72°C
Sau R	3 min	15 sec	45 sec	30 sec	10 min
(*S. aureus*)					
		Repeated for 35 cycles			
Sag 432	95°C	94°C	52°C	72°C	72°C
Sag 1018	5 min	45 sec	45 sec	90 sec	10 min
(*Str. agalactiae*)					
		Repeated for 35 cycles			
Eco 223	95°C	94°C	64°C	72°C	72°C
Eco 455	5 min	45 sec	45 sec	90 sec	10 min
(*E. coli*)					

zone of inhibition were measured and the interpretation of the results was made in accordance with the instructions supplied by the manufacturer (Hi Media Ltd., Mumbai, India). Multiple Antibiotic resistance index (MARI) were also determined for each isolates by dividing the number of antibiotics to which the isolate is resistant to by the total numbers of antibiotics tested (Adenaike, 2016).

RESULTS AND DISCUSSION

Isolation and identification of bacteria from clinical mastitis milk

Out of 73 mastitis milk samples collected from cows (n=73), 48 (65.75%) cows were positive for bacterial isolation and 25 (34.25%) cows were negative for bacterial isolation. Detection of culturally negative milk samples may be due to antibiotic therapy given to cows during the collection or it may have missed in the loopful milk samples taken for cultural isolation or may be non-cultivable (on sheep blood agar) etiological agents involved. Out of 48 cows positive for bacterial isolation, a total of 51 isolates of three different bacterial genera *viz*. *Staphylococcus*, *Streptococcus* and *Escherichia* were recovered including 3 cows having mixed bacterial infections by morphological, cultural examination and biochemical test. Amongst the 51 isolates, *S. aureus* was the most predominant bacterial species accounting for 39.22 per cent (20/51) of all the isolates, followed by *Str. agalactiae* 31.37 per cent (16/51) and *E. coli* 29.41 per cent (15/51). A total 39 isolates recovered from 50 cows of Anand district and 12 isolates recovered from 23 cows of Panchmahal district. Amongst the 39 isolates of Anand district, *S. aureus* was the most predominant bacterial species accounting for the 41.02% (16/39) of the isolates, followed by the 30.76% (12/39) *E. coli* and 28.20% (11/39) *Str. agalactiae.* Among the 12 isolates from Panchmahal district, predominant bacterial species was *Str. agalactiae* 41.67% (5/12) followed by *S. aureus* 33.33% (4/12) and *E. coli* 25.00% (3/12). The present finding indicated the predominant bacteria in Anand and Panchahal districts were *S. aureus* and *Str. agalactiae* respectively. Present study showed the most predominant bacterial species was *S. aureus* followed by the *Str. agalactiae* and *E. coli*. Higher per cent of Staphylococci reported by Wadhwa *et al.*(1996) isolated *Staphylococcus* spp. (68.83%), *Streptococcus* spp. (16.88%), *E. coli* (7.8%), Kerrodego and Tareke (2003) isolated *Staphylococcus* (62.9%), *Streptococcus* (23.6%) and *Coliforms* (14.1%), Sharma and Prasad (2003) isolated *Staphylococcus* spp. (54.05%), *Streptococcus* spp. (14.41%) and *E. coli* (11.71%), Sidar (2013) isolated *Staphylococcus* spp. (44.44%), *Streptococcus* spp. (11.11%), *E. coli* (33.33%) and Mia *et al.* (2017) isolated *Staphylococcus* spp. (35.42%), *Streptococcus* spp. (18.75%) and *E. coli* (14.58%). Very low percent of isolation was reported by Schukken *et al.* (2009) they isolated higher percentage of *E. coli* (16.2%) followed by the *S. aureus* (9.6 %) and *Str. agalactiae* (0.17%) which showed higher prevalence of gram negative organisms and same was reported recently Manasa *et al.* (2019).

PCR based identification of the isolates

The culturally positive *S. aureus* (20), *Str. agalactiae* (16) and *E. coli* (15) were subjected for PCR based amplification of specific DNA sequence coding for the 23S rRNA. All the *S. aureus* (20), *Str. agalactiae* (16) and *E. coli* (15) isolates were also positive by PCR as they yielded an expected amplification product of 179bp (Fig 1), 586bp (Fig 2) and 232bp (Fig 3) respectively.

PCR based identification of bacteria from mastitis milk

Using DNA of 73 mastitis milk samples, 59 (80.52%) mastitis milk samples were positive by PCR. Out of 59 cows mastitis milk samples, 61 bacterial species with mixed infection comprised of 24/73 (32.87%) mastitis milk samples positive for *S. aureus*, 16/73 (21.93%) positive for *Str. agalactiae* and 21/73(28.76%) positive for *E. coli* by PCR method with amplification of 179bp (Fig 1) for *S. aureus*, 586bp (Fig 2) for *Str. agalactiae* and 232bp (Fig 3) for *E. coli.* Gangwal (2016) detected 36% *E. coli*, 28% *S. aureus* and 4% *Str. agalactiae* and Kalin *et al.* (2017) detected 26.5%, 12% and 6% for *S. aureus*, *Str. agalactiae* and *E. coli* respectively by PCR method. In comparison between cultural and PCR methods, 48 cows (65.75%) milk samples positive for *S. aureus, Str. agalactiae* and *E. coli* by cultural methods were also found positive in PCR but 11 samples (15.07%) mastitis milk samples negative by cultural method were additionally found to be positive for *S. aureus* (4) and *E. coli* (7) by PCR. Overall PCR could additional detected 15.07% more samples positive in comparison with cultural methods. 16S RNA genes are commonly present in multiple copies and have highly conserved and variable sequence segments in all microorganisms (Bentley and Leigh, 1995). PCR based identification from mastitis milk were also carried out previously by Riffon *et al.* (2001), Ramesh *et al.* (2002), Cremonesi *et al.* (2006), Kumar (2009), Nimavat (2015), Gangawal (2016), Bhanderi and Jhala (2016) and Kalin *et al.* (2017).

Antimicrobial susceptibility test

In this study, 51 bacterial isolates subjected for antimicrobial susceptibility test and result revealed that highly sensitive drug was gentamicin (94.11%) followed by the amikacin (86.27%), enrofloxacin (80.39%), streptomycin (80.39%), tetracycline (80.39%), ciprofloxacin (66.66%), ceftriaxone (66.66%), amoxyclav (60.78%) and colistin (52.94%). Whereas low sensitive drugs were sulphadiazine (45.09%), erythromycin (37.25%) and ampicillin (25.49%) and very low sensitive drugs were neomycin (21.56%) and penicillin-G (13.72%) (Fig 4).

In this study, 20 *S. aureus* isolates were subjected to antimicrobial susceptibility testing by disc diffusion method. Results showed that the most sensitive drugs were amikacin (90.00%), gentamicin (90.00%), tetracycline (90.00%) followed by enrofloxacin (80.00%), streptomycin (80.00%), amoxyclav (75.00%), ciprofloxacin (75.00%), colistin (65.00%), erythromycin (60.00%), ceftriaxone (55.00%) and sulphadiazine (50.00%). Least sensitive drugs were neomycin (15.00%) and ampicillin (10.00%). All the isolates were resistance to penicillin-G. Results of 16 *Str. agalactiae* showed that the most sensitive drugs were ceftriaxone (93.75%), enrofloxacin (93.75%) and gentamicin (93.75%) which were followed by the amikacin (81.25%), amoxyclav (81.25%), streptomycin (81.25%), tetracycline (62.50%),

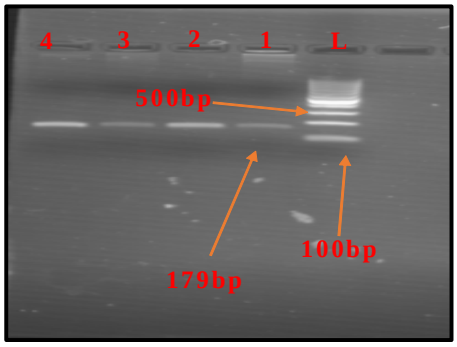


Fig 1: Agarose gel electrophoresis of colony PCR and from mastitic milk PCR amplified *S. aureus* PCR product of 179 bp. L: Ladder, 1 to 4: Representative samples from colony PCR and PCR from mastitic milk sample.

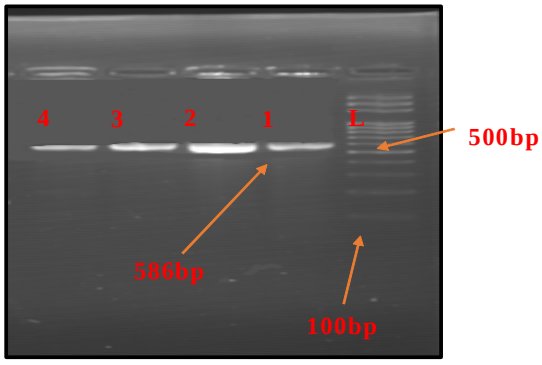


Fig 2: Agarose gel electrophoresis of colony PCR and from mastitic milk PCR amplified *Str. Agalactiae* PCR product of 586bp. L: Ladder, 1 to 4: Representative samples from colony PCR and PCR from mastitic milk sample.

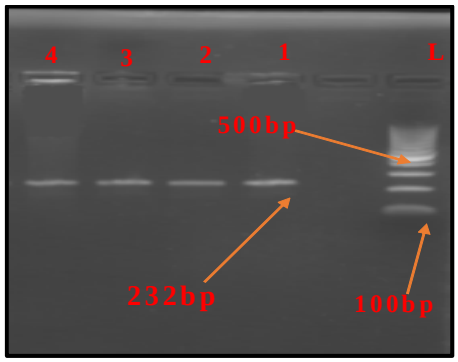


Fig 3: Agarose gel electrophoresis of colony PCR and from mastitic milk PCR amplified *E. coli* PCR product of 232 bp. L: Ladder, 1 to 4: Representative samples from colony PCR and PCR from mastitic milk sample.

ciprofloxacin (56.25%) and penicillin-G (43.75%). Low sensitive drugs were ampicillin (37.50%), erythromycin (37.50%), sulphadiazine (25.00%) and neomycin (12.50%). All the isolates were resistance to colistin. All the *E. coli* (15) isolates were sensitive to gentamicin (100%) followed by the colistin (93.33%), amikacin (86.67%), tetracycline (86.67%), streptomycin (80.00%), ciprofloxacin (66.67%), enrofloxacin (66.67%), sulphadiazine (60.00%), ceftriaxone (53.33%), neomycin (40.00%) and ampicillin (33.33%). Very low sensitive drugs were amoxyclav (20.00%) and erythromycin (6.67%). All the isolates showed the resistance to penicillin-G.

Detection of multiple drug resistance

In the present study, 51 isolates were subjected for antimicrobial susceptibility test against 14 antibiotics. Out of 51 isolates, 90.19% (46/51) were showed multiple drug resistant and 17.64% (9/51) isolates were resistant to two antibiotics, 21.56% (11/51) were resistant to three antibiotics, 11.76% (6/51) isolates were resistant to four antibiotics, 15.68% (8/51) were resistant to five antibiotics, 5.88% (3/51) isolates were resistant to six antibiotics, 7.84% (4/51) were resistant to seven antibiotics, 3.92% (2/51) isolates were resistant to eight antibiotics and 05.88% (3/51) isolates were resistant to nine antibiotics (Table 3). Multiple antibiotic resistance index was also analyzed for each isolate which revealed 27.45% and 9.8% isolates had MARI 0.3 and 0.6, respectively shows higher degree of multiple drug resistance. (Fig 5) which is in agreement with previous study (Adenaike, 2016). Present study showed that there were many isolates from mastitis cases showed the multiple drug resistant, indicated judicial use of antibiotics and veterinarians are advising for antibiotic sensitivity test for appropriate therapy.

Multiple drug resistance of 20 *S. aureus* isolates revealed that 95.00% (19/20) showed the multiple drug resistance and among them 10.00% (2/20) isolates were resistant to two antibiotics, 45.00% (9/20) isolates were resistant to three antibiotics, 10.00% (2/20) isolates were resistant to four antibiotics, 5.00% (1/20) isolates were resistant to five antibiotics, 5.00% (1/20) isolates were resistant to six antibiotics, 15.00% (3/20) isolates were resistant to seven antibiotics, 05.00% (1/20) isolates were resistant eight antibiotics and none of *S. aureus* isolate were resistant to nine antibiotics (Table 3). Elemo *et al.* (2017) estimate the prevalence of multi drug resistance *S. aureus* from bovine mastitis. Among all multi antimicrobial resistance phenotypes of *S. aureus* isolates, 52.05% were resistant tothree or four antibiotics and 41.10% were resistant to five or six antimicrobials, 6.85% were resistance to seven or eight

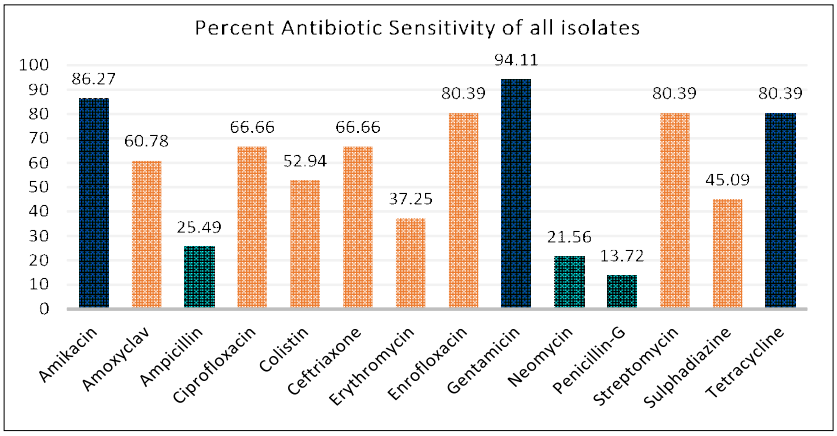


Fig 4: Percentage antibiotic sensitivity of all the isolates.

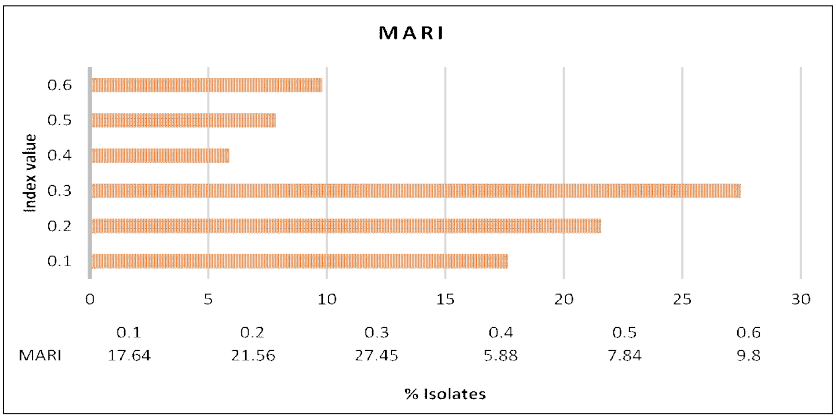


Fig 5: Multiple antibiotic resistance index (MARI) profile of study isolates

Table 3: Detection of multiple drug resistance isolates.

Isolates resistance to	Overall Isolates		Isolates					
	No. of isolates	%	*S. aureus*		*Str. agalactiae*		*E. coli*	
			No.	%	No.	%	No.	%
Two drugs	9/51	17.64	2/20	10.00	4/16	25.00	3/15	20.00
Three drugs	11/51	21.56	9/20	45.00	2/16	12.50	0/15	0.00
Four drugs	6/51	11.76	2/20	10.00	4/16	25.00	0/15	0.00
Five drugs	8/51	15.68	1/20	5.00	4/16	25.00	3/15	20.00
Six drugs	3/51	05.88	1/20	5.00	2/16	12.50	0/15	0.00
Seven drugs	4/51	07.84	3/20	15.00	0/16	0.00	1/15	6.66
Eight drugs	2/51	03.92	1/20	5.00	0/16	0.00	1/15	6.66
Nine drugs	3/51	05.88	0/20	0.00	0/16	0.00	3/15	20.00
Total	46/51	90.19	19/20	95.00	16/16	100	11/15	73.33

antibiotics. Multiple drug resistance of *Str. agalactiae* isolates found that 100% (16/16) showed the multiple drug resistant and 25.00% (4/16) isolates were resistant to two antibiotics, 12.50% (2/16) isolates were resistant to three antibiotics, 25.00% (4/16) isolates were resistant to four antibiotics, 25.00% (4/16) isolates were resistant to five antibiotics, 12.50% (2/16) isolates were resistant to six antibiotics and none of *Str. agalactiae* isolate were resistant to seven, eight and nine antibiotics (Table 3). Ding *et al.* (2016) isolated eighty-one *Streptococcus* isolates from the clinical bovine mastitis cases tested. Of the 81 isolates, 79 (97.5%) were resistant to at least two of the antimicrobial agents tested and greater than or equal to three antimicrobial agents were observed in 72 (88.9%) *Streptococcus* isolates, three isolates (3.7%) were resistant to seven antimicrobials. The observation of multiple drug resistance of *E. coli* were 73.33% (11/15) showed the multiple drug resistant with 20.00% (3/15) isolates were resistant to two antibiotics, 20.00% (3/15) isolates were resistant to five antibiotics, 06.66% (1/15) isolates were resistant to seven antibiotics, 06.66% (1/15) isolates were resistant to eight antibiotics and 20.00% (3/15) isolates were resistant to nine antibiotics (Table 3).

CONCLUSION

In the present study, *S. aureus* was the most predominant bacterial species followed by *Str. agalactiae* and *E. coli.* PCR from the mastitic milk additionally detected bacteria in culturally negative milk samples. Most sensitive drugs were gentamicin and amikacin while penicillin-G and neomycin were most resistance drugs. Among all isolates *Str. agalactiae* isolates showed multiple drug resistance. Most of the isolates (90.19%) showed the multiple drug resistance for the two to nine drugs with Multiple antibiotic resistance index (MARI) range 0.1 to 0.6.

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