Journal Homepage: - www.journalijar.com
INTERNATIONAL JOURNAL OF ADVANCED RESEARCH (IJAR)
Article DOI: 10.21474/IJAR01/4818
DOI URL: http://dx.doi.org/10.21474/IJAR01/4818

RESEARCH ARTICLE

ISOLATION AND MOLECULAR IDENTIFICATION OF *STAPHYLOCOCCUS* SPP. FROM BOVINE MASTITIS MILK SAMPLES.

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Manuscript Info

Manuscript History

Received: 11 May 2017
Final Accepted: 13 June 2017
Published: July 2017

Key words:-
Staphylococcus; MRSA, Mastitis, Somatic Cell Count, 23S

Abstract

Mastitis is an inflammation of mammary gland caused by major etiological agent *Staphylococcus* sp. mainly antibiotic resistant strains. In this study, antibiotic resistant *Staphylococcus* spp. were isolated, characterized and confirmed by molecular techniques from a total of 120 lactating cows including 30 buffaloes (480 quarter milk samples). They were screened for mastitis using Somatic Cell Count (SCC) and California Mastitis Test (CMT). Isolation and identification of *Staphylococcus* spp. was done using standard microbiological methods by species specific media as well as various biochemical tests. Antibiotic susceptibility tests (ABST) were performed to screen resistant isolates. Genotypic identification was confirmed using *23S rRNA* amplification and *16S rDNA* sequencing. A total of 193 non-duplicated *Staphylococcus* spp. were isolated having higher than 500 somatic cells/µl and CMT score was 1 or 2. Methicillin resistance in 31 and other multidrug resistivity in 17 *Staphylococcus* spp. were observed. Multidrug resistant strains were identified using Sanger sequencing and assigned accession number KX821621 to KX821638 for 11 *Staphylococcus aureus* and 7 coagulase negative *Staphylococcus* spp. These findings suggested that milk represents a potential infection risk threat of multidrug-resistant *S. aureus* in India due to neglected hygienic practices during lactation and maintenance of herd.

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Introduction:-

Mastitis infection in bovines is a critical and a stubborn intramammary infection (IMI), producing economic losses; drop in milk production, increased cost of treatment and culling process (Abd-Elrahman 2013). In dairy animals, *Staphylococcus* spp. are most ubiquitous pathogen specially *Staphylococcus aureus* is the one causing mastitis infections (Mullen et al. 2013).

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Natural immunity to fight against environment of *S. aureus* has been detected in clinical and subclinical bovine mastitis cases (Davis MP and Gamier P. 2013). *Staphylococcus aureus* infection causes main concern regarding treatment using experimental antimicrobials and leads challengeable tasks for veterinarians. Intramammary infections in animal caused by methicillin-resistant *staphylococci* (MRS) are indeed challenging task which include health risk of animal as well as milkers (Gómez et al. 2015).

Coagulase negative *Staphylococcus* spp. (CNS), which are in acquisition of interest for researchers because of its augmented exposure as an infection causing agents including human (Embden and Barkema 2009). At present, CNS are generally deliberated to be opportunistic pathogens in the research field. Epidemiology of CNS is not clear, which is sole reason for not having treatment against this pathogen. CNS group consists of about 40 different *Staphylococcus* species (Shittu and Lin 2006).

Majority of mastitis causing pathogens are normally found intermediate resistant to the antimicrobials used for treatment, but in past two decades few studies have signposted an augmented resistance of *S. aureus* and CNS to β-lactam antibiotics (Trojan et al. 2016). In a view of this, the emergence of methicillin resistance among *Staphylococcus* spp. isolated from bovine milk has been reported in different countries and continents (Bergonier et al. 2014).

The surveillance of methicillin-resistant *Staphylococci* should be prioritized because the accurate diagnosis of these bacteria is paramount for appropriate mastitis treatment. The purpose of this study was to identify and characterize multidrug resistant including methicillin in *Staphylococcal* isolates from bovine mastitis milk samples.

Methodology:-

Sample collection and processing:-

A total of 480 quarters milk samples from 120 lactating cows and buffaloes were collected from the different regions of Anand and Kheda district in Gujarat. Sample collection was done by maintaining proper hygiene conditions as described (Patel et al. 2017), At beginning, few drops of milk were discarded and samples were collected in autoclaved 50mL falcon tube (Tarson). California Mastitis Test was executed to check infections level simultaneously (Bhutto et al. 2012). Samples were brought to the laboratory in desired cooling condition and subjected to further microbiology evaluation. Somatic cells present in milk sample were analyzed on Fossomatic™ Cell Minor (Foss, Denmark) (Bhutto et al. 2012).

Isolation of *Staphylococci* and culture Conditions:-

For isolation of Staphylococci, milk samples were diluted and streaked on Baird-Parker agar base (Himedia, Catalog No. M043, India) plate with added 3.5 % potassium tellurite and incubated at 37°C for 24 to 36 h. *S. aureus* colonies were identified on the basis of colony morphology as mentioned in Himedia reference (Oliveira et al. 2011). Such colonies were further transferred to mannitol salt agar (Himedia, Catalog No. MH118, India) for differentiation and selection of pathogenic *Staphylococcus aureus* and *Staphylococci sp*. (Kateete et al. 2010). Confirmed isolates were cultured in Luria Broth (Himedia M575, India) at 37°C with aeration and 25% glycerol stocks were prepared for storage purpose. Isolates were further confirmed as *S. aureus* by a coagulase test using coagulase rabbit plasma (HiMedia, FD248) and species specific biochemical tests. Gram's morphology was also observed by preparing smear from the isolated culture on clean grease free microscopic glass slide and stained with gram's method of staining (Carles et al. 2004).

Biochemical examination:-

Various biochemical tests specific for confirmatory *Staphylococcus* sp. identification named methyl red test, Voges-Proskauer test, hemolytic test, catalase test, urea hydrolysis, indole and ammonia production were performed for characterization of isolates (Makki 2011).

Antibiotic susceptibility test:-

According to the National Committee of Clinical Laboratory Standards (NCCLS), an *in vitro* antibiotic susceptibility for isolated *Staphylococci* were checked against 20 different antibiotics by agar disc diffusion method (Carey et al. 2010). Antibiotic disc were used according to manufacturer's instructions, Himedia, India. Antibiotics used in this study were, amoxycillin (10 mcg/disc), ampicillin (10 mcg/disc), cfataxime (30 mcg/disc), chloramphenicol (30 mcg/disc), ciprofloxacin (5 mcg/disc), cloxacillin (10 mcg/disc), enrofloxacin (10 mcg/disc), erythromycin (15 mcg/disc), gentamicin (10 mcg/disc), kanamycin (30 mcg/disc), meropenem (10 mcg/disc),

methicillin (5 mcg/disc), nalidixic acid (30 mcg/disc), ofloxacin (5 mcg/disc), oxacillin (1 mcg/disc), penicillin-G (10 mcg/disc), rifampicin (5 mcg/disc), streptomycin (10 mcg/disc), tetracycline (30 mcg/disc), vancomycin (30 mcg/disc).

Extraction of genomic DNA:-
DNA was extracted from 24 hr grown *Staphylococcal* cultures using Proteinase-K-SDS method mentioned with slight modification(Prosperi et al. 2013). Concisely, 400 µl of culture was taken, centrifuged at 8000 rpm, supernatant was removed and dissolved in 500 µl TE buffer (10 mm of Tris HCl, 1 mM of EDTA, pH 8.0). Treatment of lysozyme (Sigma-Aldrich), 10% w/v freshly prepared SDS and 10 µl proteinase-k (10 mg/ml) (Sigma-Aldrich) was given t culture and incubated at 37°C for 1 hr. Subsequently, addition of 10% w/v CTAB and 200 µl NaCl (5M) in the suspension and was re-incubated for 10 min at 56 ^{o}C in boiling water bath. The undesirable contaminants that are chiefly made of proteins were removed by Phenol: Chloroform: isoamyl alcohol (25:24:1) and Chloroform: isoamyl alcohol (24:1). 100 µl of 5M sodium acetate is added to the contents and is mixed gently. The salts and alcohol remnants are removed by washing with 80% alcohol. After removal of alcohol by centrifugation, desiccated the tubes and elution was done in 50 µl warm TE buffer. Quantity of extracted DNA was checked using Nanodrop 1000 (ThermoScientific, USA) and quality on 0.8 % agarose gel in 0.5X TBE (Tris 45 mM, Boric acid 45 mM, EDTA 1 mM) buffer (pH 8.0). DNA was stored at -20 ^{o}C.

Species specific identification through *23S rRNA* gene amplification:-
Amplicons of *23S rRNA* gene were produced by species specific forward primer: 5'-ACG GAG TTA CAA AGG ACG AC-3' and Reverse Primer: 5'-AGC TCA GCC TTA ACG AGT AC-3' (Coelho et al. 2011). Each PCR mixture (25 µl) consisted of PCR Taq Buffer with MgCl2 (2.5 µl), 2.5mMdNTP mix (2.5 µl), 0.2 U of Taq DNA polymerase (Genei, Bangalore, India), 1 µl of each forward and reverse primer (10 pM/ µl) each, and 16.7 µl molecular biology grade water by using PCR program slightly modified by initial denaturation at 94°C for 3 min followed by 28 cycles of denaturation, annealing, extension (94°C for 60 seconds, 57°C for 60 seconds, 72°C for 60 seconds), and final extension at 72°C for 5 min followed by hold at 4°C (Khichar et al. 2014).

***16S rDNA* amplification:-**
16S rDNA amplification was carried out using universal primer 8-F (5′AGAGTTTGATCCTGGCTCAG3′) and 926R (5′CCGTCAATTCCTTTGAGTTT3′). PCR amplification cycle consisted of initial denaturation step of 3 min at 94 °C, followed by 25 cycles of 40 seconds at 94 °C, 45 seconds at 54 °C, and 75 seconds at 72 °C. Following amplification, final extension was performed at 72 °C for 3 min and cooled at 4 °C (Patel et al. 2016). Amplified PCR product was confirmed using 1.5 % agarose gel electrophoresis (Türkyilmaz et al. 2010). A DNA marker of known DNA fragment sizes (500 bp ladder) was run alongside the specimens to aid in identification of the products. Desired amplified product was cut from the gel and purified using QIAGEN gel purification kit (QIAGEN, Germany) (Bergonier et al. 2014).

Nucleotide sequencing:-
Purified 16S products of isolates which includes, multidrug resistivity against conventional antibiotics (ABST assay) and positive *23S rRNA* gene amplification were sent for Sanger deodoxy sequencing (SciGenome Pvt. Ltd., Kerala). Obtained Sequences were blasted with the GenBank NCBI nr database (http://www.ncbi.nlm.nih.gov/). The highest identity was selected as the identified and according to the results, sequences were submitted for species assignment (Hijazin et al. 2011).

Results:-
Out of total 120 animals harboring 480 quarter milk samples, 128 (26.66%) and 52 (10.83%) were positive for CMT score 1 and 2 respectively. SCC score more than 500 cells/µl was found in 140 (29.17) quarters (Table 1). Milk samples from these contagious quarters were proceeded further for isolation of *Staphylococcus* spp.

Isolation and characterization of *Staphylococcus* spp:-
193 non-duplicate *Staphylococcus* spp. were identified on the basis of colony morphology on Baird-Parker agar (Figure 1). On mannitol salt agar a total of 139 (72.02%) (Pink to yellow positive pigmentation) and 40 (20.73%) (Pink pigmentation) were differentiated (Figure 2). Gram positive cocci were observed in Gram's staining. Positive results for different specific biochemical tests viz. Methyl Red test, Voges- Proskauer test, Hemolytic test, Catalase test, Urea hydrolysis, Indole and Ammonia production test were obtained. Positive coagulase test in 121 isolates and negative in 28 isolates were found.

Screening of methicillin-resistant *Staphylococcus* spp. (MRS):-
On the basis of antibiotic resistivity pattern, multidrug resistivity of isolates were observed mainly against methicillin, oxacillin, tetracyclin and vancomycin. A total of 31 (22.30%) MRSA (Methicillin Resistant *Staphylococcus aureus*) out of 139 *S. aureus* and methicillin resistant in 17 (42.5%) *Staphylococcus* spp. were observed (Table 2). Fascinatingly, *S.aureus* and *Staphylococcus* spp. showed sensitivity to gentamicin (15.11%, 17.50%), ampicillin (15.83%, 10%) and least enrofloxacin (10.79%, 52.50%) respectively. Interestingly, all the rest organisms exhibited intermediate response to antibiotics. Some of which are rampantly used in the treatment of various disorders of bovines including mastitis.

Genomic DNA Isolation, *23S rRNA* and *16S rDNA* gene amplification:-
Electrophoresis of genomic DNA having concentration above 60 ng/µl of bacteria under UV transilluminator is shown in figure 3. An Amplicon of 1250 bp size of *23S rRNA* gene and 850 bp of *16S rDNA* gene was observed as shown in figure 4 and 5.

Identification of isolates using *16S rDNA* sequencing:-
Output sequences of sanger dideoxy sequencing were submitted to GenBank under the accession number KX821628 to KX821638 for *Staphylococcus aureus* (11 strains) and KX821621 to KX821627 for coagulase negative *Staphylococcus* spp (07 strains).

Table 1:- Results of CMT and SCC of collected Milk samples

No. of Cow	Quarters	CMT (Score)			SCC	
		Negative	1	2	0-500 cells/µl	>500 cells/µl
120	480	300	128	52	340	140
		62.50 %	26.67 %	10.83 %	70.83 %	29.17%

(CMT: California Mastitis Test, SCC: Somatic Cell Count)

Table 2:- Antibiotic Susceptibility test results

Antibiotics	% Resistant		% Sensitive	
	S. aureus (139)	*Staphylococcus* spp. (40)	*S. aureus* (139)	*Staphylococcus* spp. (40)
Amoxycillin (10 µg)	0	0	0	0
Ampicillin (10 µg)	13 (9.35 %)	8 (20 %)	22 (15.83 %)	4 (10 %)
Cfataxime (30 µg)	0	0	0	0
Chloramphenicol (30 µg)	0	0	0	0
Ciprofloxacin (5 µg)	41 (29.49 %)	22 (55 %)	0	0
Enrofloxacin (10 µg)	0	0	15 (10.79 %)	21 (52.5 %)
Erythromycin (15 µg)	0	0	0	0
Gentamicin (10 µg)	35 (25.18 %)	10 (25 %)	21 (15.11 %)	7 (17.5 %)
Kanamycin (30 µg)	18 (12.95 %)	13 (32.5 %)	0	0
Meropenem (10 µg)	0	0	0	0
Methicillin (5 µg)	31 (22.30 %)	17 (42.5 %)	0	0
Nalidixic acid (30 µg)	21 (15.11 %)	7 (17.5 %)	0	0
Cloxacilin (10 µg)	38 (27.34 %)	11 (27.5 %)	0	0
Ofloxacin (5 µg)	27 (19.42 %)	17 (42.5 %)	0	0
Oxacillin (1 µg)	23 (16.55 %)	9 (22.5 %)	0	0
Rifampicin (5 µg)	45 (32.37 %)	21 (52.5 %)	0	0
Streptomycin (10 µg)	33 (23.74 %)	16 (40 %)	0	0
Tetracycline (30 µg)	17 (12.23 %)	6 (15 %)	0	0
Penicillin-G (10 µg)	22 (15.83 %)	12 (30 %)	0	0
Vancomycin (30 µg)	20 (14.39 %)	14 (35 %)	0	0

(Sensitivity was observed against Ampicillin, Enrofloxacin and Gentamicin Only)

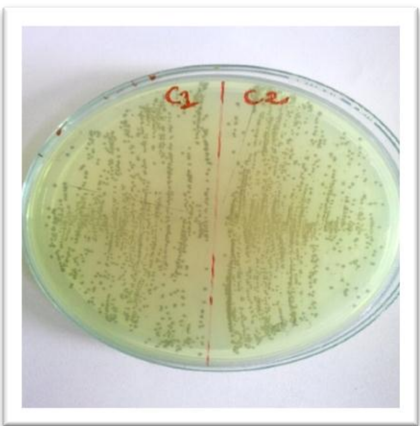

Figure 1:- Representative image of baird-parker agar plate showing black colored *Staphylococcus* spp.

Figure 2:- Representative image of mannitol salt agar plate showing positive fermentation by color change from pink to yellow; indicate *S. aureus*

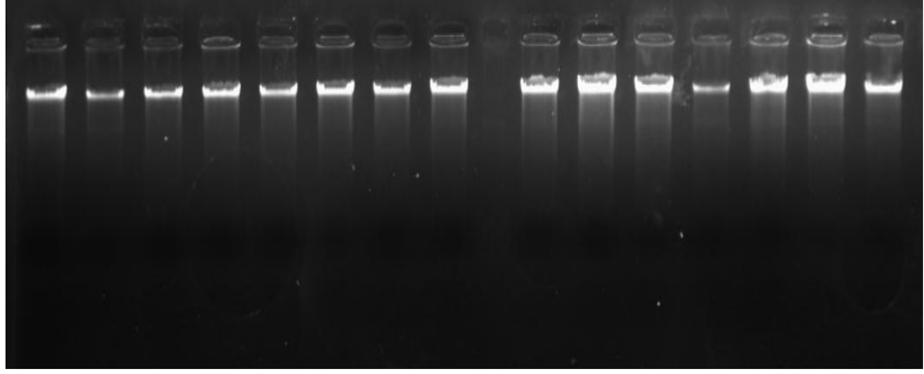

Figure 3:- Agarose gel (0.8%) showing isolated genomic DNA in UV transilluminator

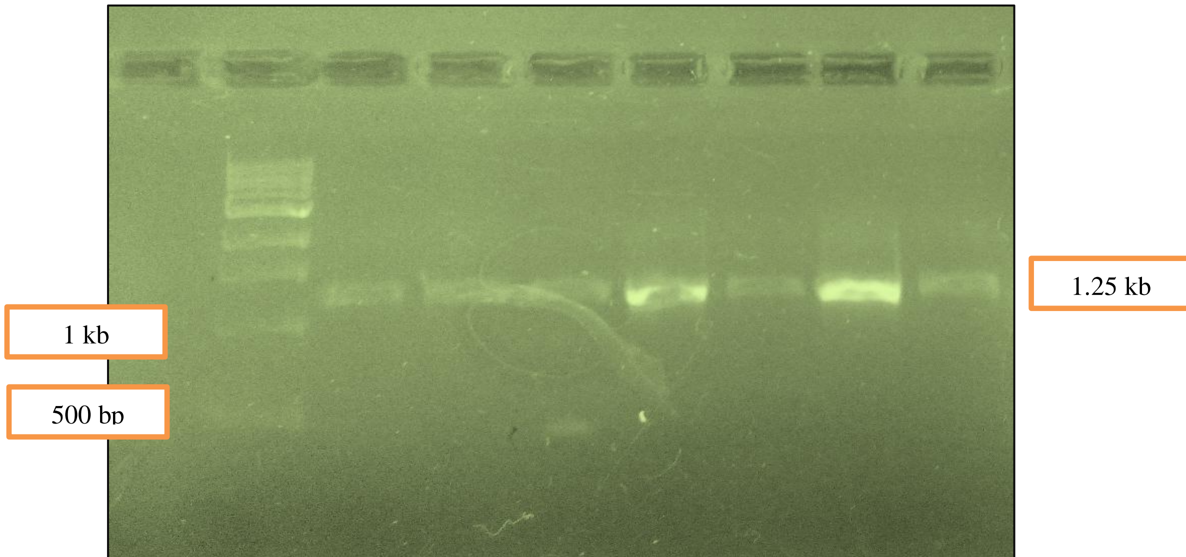

Figure 4:- Agarose gel (1.5%) showing amplified product *23S rRNA* gene under UV transilluminator with 1 kb ladder, Product size seems to be approx. 1250 bp

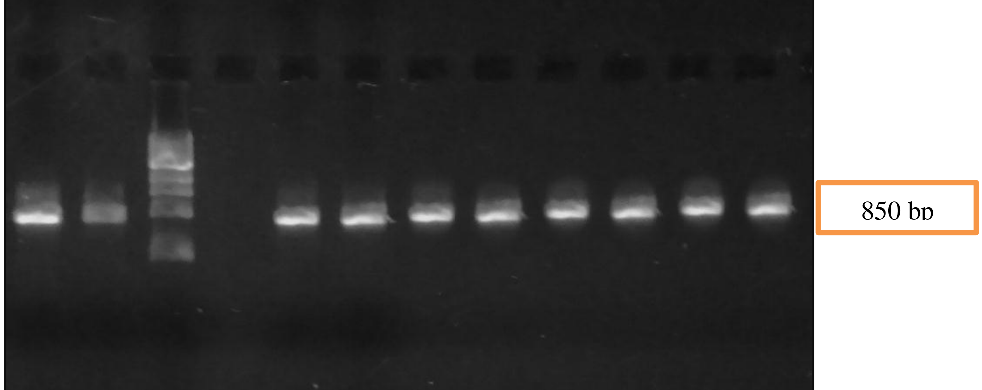

Figure 5:- Agarose gel (1.5%) showing amplified product *16S rDNA* gene under UV transilluminator with 1 kb ladder, Product size seems to be approx. 850 bp.

Discussion:-

In this study, we have isolated multidrug resistant *Staphylococcus* spp. from bovine mastitis milk. *Staphylococcus aureus* remains one of the most significant micro-organisms associated with bovine mastitis worldwide. The isolation of MRSA from animals was first reported in 1972 (Devriese et al. 1972). For identification of bacteria, majority of the researchers tend to use isolation on selective media and molecular identification. In this study, biochemical characterization of each isolate was used to eliminate sequencing cost for molecular identification at later stage.

Since the use of antibiotic has been boundless in recent years, presence of MRSA in dairy farm has been recognized (Haran et al. 2012a). Many studies on prevalence of MRSA investigation have been reported in cattles in India; however, limited information with respect to the characterization of MRSA in cattle milk is available (Sumathi et al. 2008).

For veterinarians, one of the paramount microbial threats of the twenty-first century is developing antimicrobial resistance in pathogens. One of the pathogen, *S. aureus* has always been a stumbling block for antimicrobial chemotherapy and the introduction of new classes of antimicrobial agents is usually followed by the emergence of resistant forms of this pathogen (Shittu and Lin 2006; Harrison et al. 2013). Therefore, surveillance on the antimicrobial susceptibility patterns of *S. aureus* mainly methicillin resistant is of utmost importance in understanding new and emerging resistance trends and in the management.

Our results suggest that there is a high prevalence of MRSA that is 22.30%. Consistent with our findings, other countries have reported numerous rates of prevalence of MRSA in bovine milk. A study of *S. aureus* from mastitic cattle in India reported a high prevalence of MRSA, i.e., 13% of *S. aureus* isolates (14 of 107 samples) which is quite differ from our result in which it shows prevalence of MRSA is 22.30 % (31 of 139). Such high prevalence has not been reported in studies from the United States and Europe (Türkyilmaz et al. 2010; Bardiau et al. 2013). Such resistance against antibiotics is only the excessive use of β-lactam antibiotics that are used as primary choice of treatment provoking strong β- lactamase producers *S. aureus* isolates (Saei 2012).

In a recent study of MRSA isolated from milk samples from mastitic cows, 100% of isolates were resistant to clindamycin, chloramphenicol, erythromycin, and gentamicin (Lee et al. 2015). In another study in Switzerland, two MRSA isolates from 142 milk samples from from mastitic cows were found resistant to all beta-lactam antibiotics (Haran et al. 2012b; Lange et al. 2015), while Vanderhaeghen et al. reported resistance to tetracycline, with frequent resistance to macrolides, lincosamide, and aminoglycosides, in MRSA from milk from mastitic cows (Bardiau et al. 2013).

The CoNS species in our data, *S. hominis and S. pasteuri* has been reported most frequently in numerous studies on bovine intramammary infection (IMI) or mastitis. *S. chromogenes* has been isolated most commonly in almost all studies but we couldn't isolate it in our study (Lange et al. 2015).

The presence of MRSA isolates in milk may surge a potential public health risk, and MRSA may spread between animals and humans due to many reasons like milk practice and improper hygiene maintenance. Spread of such microbes may be depending upon environmental condition in the herd. Virulence factors associated to this pathogen helps to protest against antimicrobial agents. The indiscriminate use of antibiotics/antimicrobials agents for therapeutic purpose could be the reasons for increased antimicrobial resistance of *S. aureus*. Different strains other than *Staphylococcus* spp. are also resistant to the antibiotics commonly used to treat all the microbes in therapeutic protocols of many human and animal infections. Further study on virulence factors and antimicrobial resistance genes may highlight the strain's response and strength in diverse condition during treatment with antimicrobials.

Conclusion:-

In this study eleven Coagulase positive methicillin resistant *Staphylococcus aureus*, six coagulase negative *Staphylococcus* spp. (two *S. hominis*, four *S. pasteuri*) and one *Staphylococcus* sp. having multidrug resistivity including methicillin were found from mastitis cases. Stimulatingly, obtained coagulase negative *Staphylococcus* sp. in this study are usually found in human origins, which leads us to understand that the mobile pathway of such pathogen into milk udders may be because of human activities during milking process. Increase in population of MRSA is affected by persistent use of antibiotics for treatment. Further study on virulence factors might help to understand the pathogenicity and treatment. Careful monitoring of the resistance status of *S. aureus* in dairy environments is needed, as *Staphylococcus* spp. transmission is dynamic and involves human approach, animal health, and likely the farm production environment.

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