Biochemical Characteristics of Bacterial Isolates obtained from Bovine Mastitic Milk Samples

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ABSTRACT

Aim: Bovine mastitis affects the milk production in milking cattle and the milk producer. The major causative organism of mastitis includes the bacteria. The present study is focused on the identification of bacterial isolates obtained from milk samples of bovine mastitis based on their biochemical activities.

Study design: The bacterial isolates obtained from California mastitis test positive milk samples of in and around Bengaluru district, Karnataka were collected from Department of Veterinary Microbiology, Veterinary College, KVAFSU, Bengaluru, Karnataka and subjected to staining for morphology, Gram’s reaction and other biochemical tests. Identification key for both Gram positive and Gram negative bacterial isolates were prepared and based on the activities they were identified up to species level.

Place and Duration of Study: The work was carried out in the Department of Dairy Microbiology, Dairy Science College, KVAFSU, Hebbal, Bengaluru, Karnataka from Feb.2021 to Feb. 2022.

Methodology:Bacterial isolates obtained were subcultured, purified on plate count agar. The bacterial isolates were coded and subjected to simple staining, spore staining, Gram’s staining, oxidase test, catalase test and other biochemical tests like indole, VP, methyl red, citrate tests by preparing specific sterile media. The results obtained for the tests conducted were compared with the standard identification key and declared the species of bacterial genera.

Result:In the present study, 45 isolates of bovine mastitis obtained were phenotyped through staining techniques and biochemical tests. Among 45 isolates, 40 Grampositive isolates and 5 Gram negative, which were identified as *streptococcus agalactiae*(12); *str. uberis* (10); one each of *staphylococcus delphini*, *staph. carnosus* and *staph. gallanarium* while 6 eachof *staph. equorum*; S*taph. epidermis* and 3 *staph. aureus*. Gram negative (5) isolates were identified as *Escherichia coli* (3), *Salmonella enterica* (1) and *Alcaligenes* sp. (1) based on their biochemical characteristics.

Conclusion: The major mastitis causing bacteria isolated from CMT positive bovine milk samples was *Streptococcus agalactiae* followed by*Str. uberis*, S*taphylococcusequorum*, *Staph. epidermis*, *Staph. aureus* and *Escherichia coli*some of which may be harboured in the udder of milch animals while few bacteria may enter through various sources of dairy farm environment. Hence precautions need to be taken about the udder health of the milch animals as well as hygiene of dairy farm to reduce the incidences of mastitis.

***Key words**: Bovine mastitis; Biochemical tests; Bacterial isolates; Gram positive; Gram negative*

1. INTRODUCTION

Bovine mastitis is defined as inflammation of the mammary gland and is most commonly caused by bacterial infection. Bovine mastitis occurs in two different clinical manifestations: subclinical and clinical mastitis that ranges from mild, moderate to severe cases. The California mastitis test (CMT) is one of the most common tests for the diagnosis of subclinical mastitis and an indirect indicator of qualitative measurement of the somatic cell count (SCC) in milk and can be adopted in dairy farm by milkers. The sub clinical mastitis is 15 to 40 times more prevalent than clinical mastitis[1] and the prevalence of subclinical mastitis varied from 10 - 50% in cows and 5-20% in buffaloes when compared to clinical mastitis (1-10%) in Indian condition[2]. In Madhya Pradesh, 74.8% of subclinical mastitis among the cow breeds Jersey Cross, Holstein Friesian, Malvi, Sahiwal and Gir has been reported[3].The coagulase-negative staphylococci were the most predominant (64.8%) bacteria, followed by streptococci (18.1%), *Escherichia coli* (9.8%) and Staphylococcus aureus (7.3%) in 190 buffalo CMT positive milk samples collected from Dharwad and Bengaluru districts[4].

Mastitis directly affects the technical characteristics and hygienic quality of milk, indirectly altering its intrinsic qualities.Mastitis is caused by wide variety of microorganisms including bacteria, mycoplasma, yeasts, algae and among the etiological agents, bacteria play a major role in causing mastitis. Bovine mastitis is classified as: contagious mastitis, generally caused by bacteria presiding on the skin of the teat and inside the udder, transmitted from one cow to another during milking (e.g., *Staphylococcus aureus* or *Streptococcus agalactiae*) and environmental mastitis, caused by pathogens such as *Escherichia coli*, *Streptococcus uberis* and *Klebsiella* sp. normally found in shed surroundings such as bedding, manure, soil, and feed [5].

The herd, cow and quarter level prevalence of mastitis was 73.3, 28.6 and 29.5% respectively, among local breeds and cross bred cows of small-holding dairy farms of Chittagong, Mymensingh and Gazipur of Bangladesh. The subclinical mastitis was the predominant type in all cases. According to bacteriology the overall prevalence of herd, cow and quarter level *Staphylococcus aureus* mastitis was 72.7, 74.0 and 62.0%, respectively, and the pathogen was mostly associated with clinical mastitis [6].

In Shire Dairy Farms, Tigray, Ethiopia, 29.09% positive bovine mastitis cases were observed that showed presence of 32.81% were coagulase-positive *S. aureus*[7]. A total of 125 *Staphylococcus aureus* isolates were identified from 466 mastitis milk samples (clinical and subclinical mastitis) obtained from seven herds of Ningxia, Western China[8].The prevalence of mastitis amongst cattle in Mexico was reported to be up to 35%, depending on the region, the raining season and the system of cattle farming. From 226 milk samples collected from 14 farms in the region of Comarca Lagunera in Mexico having Holstein dairy cows, 33 mastitis‐related *Staphylococcus aureus* isolates were obtained[9]. The bacteria causing bovine mastitis have public health importance causing intoxication or infection in human beings. These bacteria include *Staphylococcus aureus, Streptococcus pyogenes, Escherichia coli and Klebsiella* [10]. *Staphylococcus aureus* was the most common in subclinical mastitis milk samples (34.2%) while *Streptococcus ubersi* (12.2%),other *Streptococcus spp.* (12.2%), *E.coli* (9.8%)andother species of bacteria and yeast candida (31.65) were also observed in 111 subclinical mastitis milk samplesof dairy farms of Bosnia and Herzegovina [11].

Keeping in view all the observations of the above studies, in the present study an attempt has been made to find the types of bacterial species obtained from California Mastitis Test (CMT) positive bovine milk samples through staining and biochemical tests.

2. MATERIALS AND METHODS

2.1Collection of isolates of bovine mastitic pathogens

The pathogens of bovine mastitis from California Mastitis Test (CMT) positive milk samples, selected in and around Bangalore were procured from Veterinary Microbiology Department, Veterinary college, Bangalore in sterile nutrient broth [12] and subjected for purification and characterization.

2.2 Purification and maintenance of isolates of bovine mastitis

The collected pathogens were purified by streaking technique on to poured plates of using the nutrient agar medium sterile inoculation loop and the plates were incubated for 37 ˚C/24 h. After incubation, the obtained single pure colony was selected and transferred to nutrient agar slants and incubated for 37 ˚C/ 24 h. and preserved as stock culture on nutrient agar slant in the refrigerator [12].

The stock culture was subcultured freshly once in a month while working culture was sub cultured whenever required or once a week.

2.3 Characterization of bacterial isolates of bovine mastitis

The **o**btained purified isolates were subjected for Gram staining, Catalase test, Oxidase test. Gram positive through standard procedures [12] and Gram negative isolates were further subjected to specific biochemical tests and based on the identification key placed in genus and species level.

2.3.1 Identification of Gram positive isolates

The isolates were subjected to preliminary test to confirm the genus using Grams staining, catalase test, oxidase test.Gram positive isolates showing coccus shaped, based on chain or bunch arrangement considered as streptococci or staphylococci. Further specific tests [9] like Oxidation-fermentation test, Voges-Proskauer, urease, esculin hydrolysis, arginine hydrolysissugar fermentation like mannitol, arabinose, maltose, sucrose placed the isolates under either streptococci or staphylococci. The identification key for species of *Streptococcus*[13] and *Staphylococcus* [14] were prepared to help in phenotypic identification of the isolates of streptococci and staphylococci.

Chart 1: Identification key for *Streptococcus* species

Tests	**Identity**				
	S. anginous	*S. mitis*	*S. uberis*	*S. agalactiae*	*S. constellatus*
Gram Staining	+				
Catalase	-				
Oxidase					
Oxidative Fermentative	O^+F^+ (oxidative and fermentative)				
Voges Proskauer’s	+	-	-	+	+
Esculin Hydrolysis	+	+	+	-	+
Arginine Utilization	+	-	+	+	+
Sucrose	+	+	+	+	-

Chart 2: Identification key for *Staphylococcus* species

Tests	**Identity**					
	S.delphini	*S.gallinarium*	*S.equorum*	*S.aureus*	*S.carnosus*	*S.epidermidis*
Gram staining	**+**					
Catalase	**+**					
Oxidase	**-**					
Oxidative fermentative	O^+F^+ (oxidative and fermentative)					
Voges Proskauer's	**-**	**-**	**-**	**+**	**+**	**+**
Urease	**+**	**+**	**+**	**+**	**-**	**+**
Mannitol	**+**	**-**	**+**	**+**	**+**	**-**
Arabinose	**-**	**+**	**+**	**-**	**-**	**-**
Maltose	**+**	**+**	**-**	**+**	**-**	**+**

2.3.2.1 Identification of Gram negative isolates

Gram staining, catalase, oxidase, OF test, urease test were followed for Gram negative isolates along with TSI-H_2S test, Indole test, Methyl-red test, Voges Proskauer test and Citrate utilization test. The identification key for Gram negative rods was prepared by referring to Bergey's Manual of Systematic Bacteriology [15].

Chart 3: Identification key for Gram negative isolates

Tests	**Identity**		
	Escherichia coli	*Salmonella enterica*	*Alcaligenes* sp.
Gram staining	-		
Catalase	+		
Oxidase	-		
Oxidative fermentative	O^-F^+ (not oxidative, only fermentative)		O^-F^- (not oxidative and not fermentative)
TSI-H_2S	Acid butt, Acid slant, Gas production	Acid butt, Alkaline slant, H_2S production	Alkaline butt, Alkaline slant, No gas or H_2S production
Indole	+	-	-
Methyl red	+	+	-
Voges-Proskauer	-	–	+
Citrate	-	+	+
Urease	-	-	-

3. RESULTS AND DISCUSSION

Result obtained in the present study are explained below and later the obtained results are discussed with the results of other authors

3.1 Genus level identification of the isolates of bovine mastitis

The cell morphology and cell arrangement of the isolates grouped them as Gram positive streptococci, staphylococci and Gram negative short rods. Few of the preliminary identification tests like oxidase, catalase and oxidative-fermentative tests, TSI H_2S (for Gram negative rods) were conducted to place the isolates under specific genus. Out of 40 Gram positive cocci, 22 were streptococci due to cocci in chains, both oxidase & catalase negative and had fermentative characteristics while another 18 isolates of cocci in bunches were staphylococci as they showed oxidase positive, catalase negative, both oxidative and fermentative properties. Gram negative short rods with oxidase negative, catalase positive, only fermentative with gas production placed them under Genus *Escherichia or Enterobacter*; similarly acid butt, alkaline slant with H_2S production may be genus *Salmonella*. A Gram negative rod, both oxidase & catalase negative as well as no reaction on TSI H_2S was guessed as *Alcaligenes* sp. (Fig. 1).

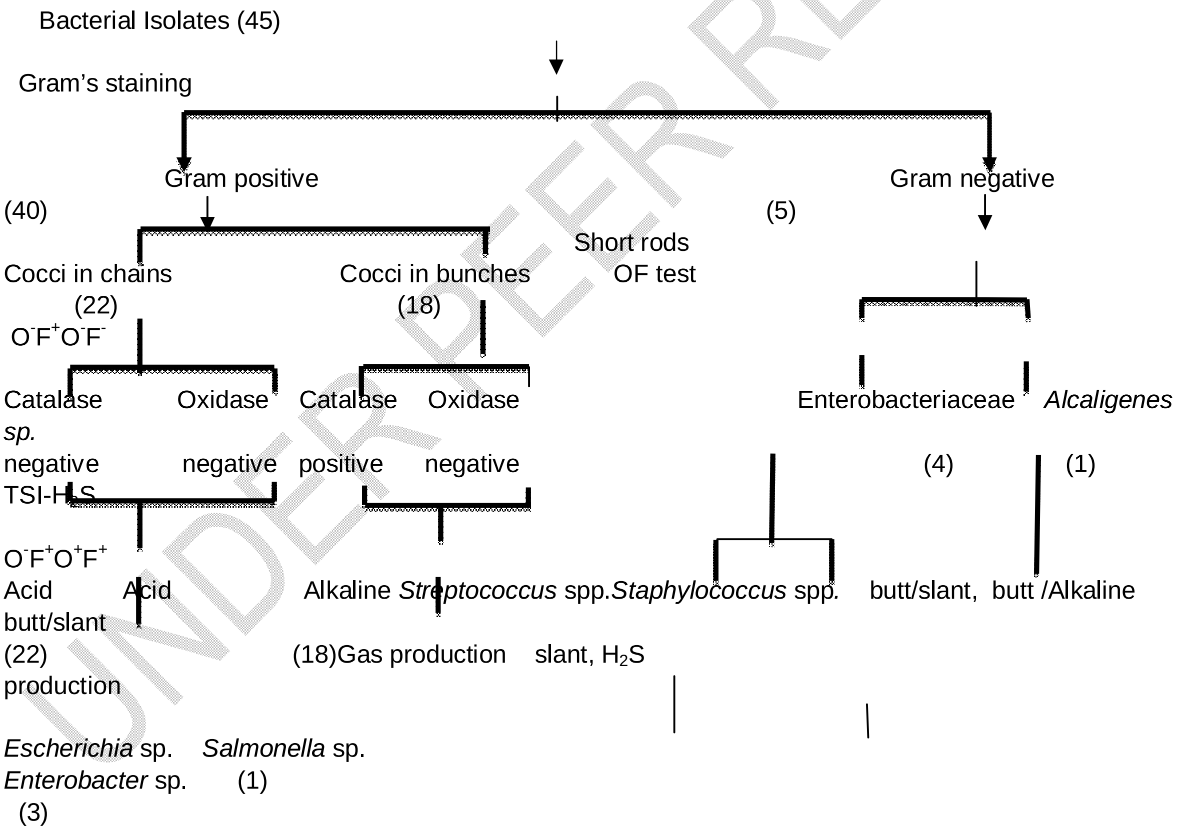

Fig 1: Overview of identity of isolates of bovine mastitis up to genus level

3.2Identification of streptococcal isolates of bovine mastitis

Isolates of streptococci were subjected to Voges Proskauer's, esculin hydrolysis, arginine utilization, sucrose fermentation to identify the species. A total of 10 isolates (M4, M6, M9, M12, M13, M22, M39, M40, M44, M45) were identified based on positive results for Voges-Proskauer’s, esculin hydrolysis, arginine utilization, sucrose fermentation as *Streptococcus uberis* after comparing with identification key. Similarly, another 12 streptococcal isolates matched with identification of *S. agalactiae* (M2, M3, M5, M15, M17, M18, M19, M26, M30, M31, M34, M36) based on specific tests where in the isolates hydrolyzed esculin and fermented sucrose where as VP test arginine tests were negative. Isolates of streptococci of 22 numbers when subjected to specific tests like Voges-Proskauer’s, esculin hydrolysis, arginine utilization, sucrose fermentation, 10 were identified as *Streptococcus agalactiae* while 12 were placed under *Streptococcus uberis* based on identification key (Table 1).

Biochemical tests Conducted	**Isolate codes**	
	M4, M6, M9, M12, M13, M22, M39, M40, M44, M45 (10)	**M2, M3, M5, M15, M17, M18, M19, M26, M30, M31, M34, M36 (12)**
Voges – Proskauer	**-**	**+**
Esculin Hydrolysis	**+**	**-**
Arginine Utilization	**+**	**+**
Sucrose fermentation	**+**	**+**
Identity	***Streptococcus uberis*** **(10)**	***Streptococcus agalactiae*** **(12)**

Table 1: Identification of streptococcal isolates obtained from bovine mastitis up to species level

Note:

- All the 22 isolates were Gram positive cocci in chains with catalase andoxidase negative, non-oxidative but fermentative (O^-F^+)

3.2 Identification of species of *Staphylococcus*obtained from bovine mastitis

All the 18 staphylococcal isolates after conducting specific test for speciation such as Voges-Proskauer’s, urease production, utilization of mannitol, arabinose and maltose, the isolates belonged to *Staphylococcus delphini* (1); *S. gallinarium* (1),*S. carnosus* (1); *S. equorum* (6); *S. aureus* (3) and *S. epidermis* (6). This indicated predominance of both the species *S. equorum* and *S. epidermis* followed by *S. aureus* in causing mastitis among the herd (Table 2).

Table 2: Identification of staphylococcal isolates obtained from bovine mastitis

Biochemical tests conducted	**Isolate codes**					
	M1 (1)	**M16 (1)**	**M7, M10, M23, M24, M37, M31 (6)**	**M35, M28, M21 (3)**	**M20 (1)**	**M8, M11, M25, M29, M32, M43 (6)**
Voges - Proskauer’s	-	-	-	+	+	+
Urease	+	+	+	+	-	+
Mannitol	+	-	+	+	+	-
Arabinose	-	+	+	-	-	-
Maltose	+	+	-	+	-	+
Identity	***Staph. delphini*** **(1)**	***Staph. gallinarum*** **(1)**	***Staph. equorum*** **(6)**	***Staph. aureus*** **(3)**	***Staph. carnosus*** **(1)**	***Staph. epidermis*** **(6)**

up to species level

Note:

- All the 18 isolates were Gram positive cocci in bunches with catalase positive, oxidase negative, both oxidative and fermentative (O^+F^+)

3.3 Identification of Gram negative isolates of bovine mastitis

Gram negativeisolates of 5 numbers were subjected to Indole, methyl red, Voges-Proskauer’s and citrate utilization tests. As per the results obtained for these biochemical tests, the 3 isolates (M14, M33, M38) belonged to *Escherichia* genus with species *E. coli*; while one (M27) was identified as *Salmonella enterica* and last one of Gram-negative isolate (M42) matched with *Alcaligenes* sp. (Table 3). As per the results obtained for the biochemical tests, like IMViC for 5 isolates of Gram negative rods catalase positive and oxidase negative with fermentative nature, the 3 isolates were *E. coli*, while one *Salmonella enterica* and one may be *Alcaligenes* sp.

<table>
<tr><th rowspan="2">Staining and Biochemical tests conducted</th><th colspan="3">Isolate code</th></tr>
<tr><th>M14, M33, M38
(3)</th><th>M27
(1)</th><th>M42
(1)</th></tr>
<tr><td>Gram staining</td><td colspan="3">-</td></tr>
<tr><td>Catalase</td><td colspan="3">+</td></tr>
<tr><td>Oxidase</td><td colspan="3">-</td></tr>
<tr><td>Oxidative Fermentative</td><td colspan="2">$O^- F^+$
(not oxidative , only fermentative)</td><td>$O^- F^-$
(not oxidative and not fermentative)</td></tr>
<tr><td>TSI-H_2S</td><td>Acid butt,
Acid slant,
Gas production</td><td>Acid butt,
Alkaline slant,
H_2S production</td><td>Alkaline butt,
Alkaline slant,
No gas production</td></tr>
<tr><td>Indole</td><td>+</td><td>-</td><td>-</td></tr>
<tr><td>Methyl red</td><td>+</td><td>+</td><td>-</td></tr>
<tr><td>Voges-Proskauer’s</td><td>-</td><td>-</td><td>+</td></tr>
<tr><td>Citrate</td><td>-</td><td>+</td><td>+</td></tr>
<tr><td>Urease</td><td>-</td><td>-</td><td>-</td></tr>
</table>

Table 3: Identification of Gram negative bacterial isolates of bovine mastitis

Identity	***Escherichia coli* (3)**	***Salmonella enterica* (1)**	***Alcaligenes* sp. (1)**

3.4 Per cent occurrence of species of mastitic causing bacteria

Out of 45 isolates (100%) of bovine mastitis, 26.7% (12) *streptococcus agalactiae*,22.2% *Streptococcus uberis* (10); 13.3% *staphylococcus equorum* (6), 13.3% *Staphylococcus epidermis* (6), 6.7% *Staphylococcus aureus* (3) and 2.2% each of *Staphylococcus delphini*(1), *Staphylococcus carnosus*(1)and *Staphylococcus gallinarum*(1) were found.Among Gram negative isolates,6.7% *Escherichia coli* (3), 2.2% *Salmonella enterica* (1) and 2.2% *Alcaligenes* sp. (1) were identified based on their biochemical characteristics.

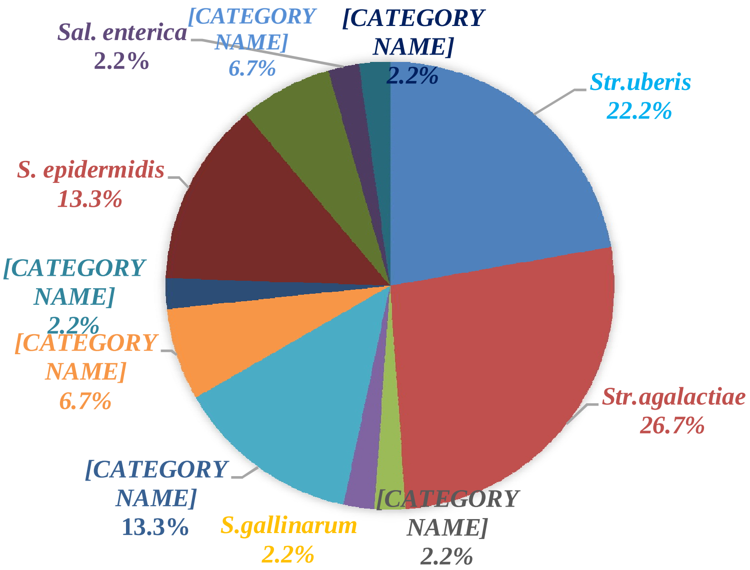

Fig 2: Occurrence of bacterial species obtained from CMT positive bovine milk samples

3.5 Discussion:

In comparison with the present study where predominance of 26.7% *Str. agalactiae followed by*, 22.2% *Str. uberis*,13.3% *Staph. equorum* (6) and *Staph. epidermis* (6), 6.7% *Staph. aureus as well as E. coli* were noticed which may be due to the dairy farm hygenic practices. Species of *Streptococcus* are involved in causing contagious mastitis, while *Staph. aureus* habitat being skin and throat while E.coli enter through contaminated water. On the contrary to the present study, CMT positive milk samples (118) from lactating Murrah buffaloes maintained at Livestock Research Centre farm ICAR-National Dairy Research Institute, Karnal when culturally examined and characterized, samples revealed *Staph. aureus* (35.29%) as the predominant bacteria followed by *Str. agalactiae* with an isolation rate of 25.49% while the incidence of coliforms bacteria was not detected [16].Almost same trend was revealed in 200 CMT positive milk samples of Kashmir valley with *Staphylococcus* spp. showing highest prevalence of 46.4% followed by mixed infection with *Staphylococcus* and *Streptococcus* (20.8%), *Streptococcus* spp. (18.4%) and *Escherichia coli* (14.4%) [17]. In milk samples of dairy cows also showing signs of clinical mastitis in Asella town, Ehiopia with high frequency of *Staphylococcus aureus* (38.6%), *Staphylococcus intermedius*(28.9%)as well as*Escherichia coli*(14.5%) were identified [18].Even major prevalence of

Staphylococcus spp. (42.55 %) and also*E. coli* (21.28%) werereportedin mastitic milk samples collected in and around Meerut[19]. But in case of mastitis-positive cases of cattle and buffaloes of Khyber Pakhtunkhwa, Peshawar the prevalence of wasnot only *Staphylococci* spp. (34%), *Streptococci* spp. (9%), but also *Escherichia coli* (19%) and *Klebsiella* spp. (8%) were identified [20].

The major mastitic bacteria observed in 86 bovine mastitic milk samples from farms of Ludhiana were Gram positive cocci such as 34.02%of S*taphylococcus aureus,Streptococcus agalactiae* of 12.37% and even Gram negative bacteria noticed were 24.74%of *Escherichia coli,*16.49%of *Klebsiella pneumoniae,* 5.15% of *Pseudomonas aeruginosa*[21].*Staphylococcus aureus* was found to be the prime etiological agent involved in the mastitic milk samples of dairy cows of Jammu region, accounting for 379 (34%) out of 1100 bacterial isolates followed by *Escherichia coli* (235 numbers with 21%) and other environmental pathogen*Streptococcus agalactiae* (115 numbers- 10%) [22]. Among 208 clinical milk samples from mastitic cows of four farms in the Jiangsu Province, China, Gram negative bacterial isolates like *Klebsiella pneumoniae* (15.35%) accounted for the highest proportion, followed by *Escherichia coli* (13.70%), *Pseudomonas aeruginosa* (12.33%), while Gram positive *Streptococcus agalactiae* (10.5%) was lower in [23].*Staphylococcus aureus* was the most common in subclinical mastitis milk samples (34.2%) while *Streptococcus ubersi* (12.2%),other *Streptococcus spp.* (12.2%), *E.coli* (9.8%)andother species of bacteria and yeast candida (31.65) were also observed in 111 subclinical mastitis milk samples of dairy farms of Bosnia and Herzegovina [11].

4. CONCLUSION

On the basis of observations made in the present study, *Streptococcus agalactiae followed by Str.uberis*, were found as the major mastitis causing bacteria involved in contagious mastitis in CMT positive bovine milk samples. Other bacterial species included *Staphylococcus equorum; S. epidermis*; *S. aureus* ssp. *aureus* and *Escherichia coli*.*Staph. aureus* as reported by many authors as major mastitic causing organism but in the present study the lower percentage was observed. Mastitis affects the milch animal reducing the milk yield which influences the economy of the milk producer. Health of the milch animals and dairy farm hygiene need to be upgraded to avoid the entry of mastitic causing bacteria.

COMPETING INTERESTS DISCLAIMER:

Authors have declared that they have no known competing financial interests OR non-financial interests OR personal relationships that could have appeared to influence the work reported in this paper.

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Option 1:

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during writing or editing of manuscripts.

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