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Dinesh Kumar Saharan
Teaching Associate at Livestock Research Station, RAJUVAS, Bikaner, Rajasthan, India

Kavita Jaidiya
Ph.D. Scholar at Department of Veterinary Medicine, College of Veterinary and Animal Science, Bikaner, Rajasthan, India

Yogendra Meena
Ph.D. Scholar at Department of Veterinary Medicine, Post Graduate Institute of Veterinary Education and Research, Agra Road, Jaipur, Rajasthan, India

Shivendra Kumar Bhalothia
Ph.D. Scholar at Department of Veterinary Gynaecology and Obstetrics, College of Veterinary and Animal Science, Bikaner, Rajasthan, India

Sunita Choudhary
Assistant Professor at Department of Veterinary Medicine, College of Veterinary and Animal Science, Bikaner, Rajasthan, India

Corresponding Author
Dinesh Kumar Saharan
Teaching Associate at Livestock Research Station, RAJUVAS, Bikaner, Rajasthan, India

Isolation and identification of bacterial flora in milk of cattle affected with clinical mastitis

Dinesh Kumar Saharan, Kavita Jaidiya, Yogendra Meena, Shivendra Kumar Bhalothia and Sunita Choudhary

Abstract
The current study aimed to isolate and identify bacterial flora in milk of cattle affected with clinical mastitis. a total of 16 animals involving 23 quarters exhibiting clinical manifestations of mastitis in were included.Clinical mastitis was observed high in 4th parity, early lactation stage and in hind quarters. On cultural examination of mastitic milk of 23 quarters of 16 cattle, 18 quarters (78.26%) were having single bacterial infection, whereas 5 quarters (21.73%) were having mixed infection. The bacteria isolated in this study were *Staphylococcus aureus*, accounting for 12 of the isolates (42.85%), followed by 6 (21.42%) *E. coli*, 5 (17.85%) *Streptococcus agalactiae*, 3 (10.71%) *Klebsiella pneumoniae* and 2 (7.14%) *Streptococcus dysaglactiae*, respectively. In mixed infection combination of four genera were found. Organisms isolated in mixed infection were *Staphylococcus spp., Streptococcus spp., Klebsiella pneumoniae* and *E. coli.* Major pathogens found were Staphylococcus spp., Streptococcus spp, Klebsiella pneumonia and E.coli on bacteriological culture.

Keywords: Clinical mastitis, bacterial culture, *E. coli, Staphylococcus*

1. Introduction
Mastitis is a multi-etiological complex disease, which is defined as inflammation of parenchyma of mammary gland characterized by physical, chemical changes in milk and pathological changes in glandular tissue (Radostits *et al*., 2009) [24]. Clinical mastitis is a condition characterized by abnormalities of udder (swollen, hard and hot quarters) and milk (flakes, clots and watery appearance), which results in decrease in total milk production as well as changes in milk composition. Mastitis occurs due to various factors such as bacteria, fungi, mycoplasma, yeast along with decreased resistance due to stress, shape of udder, teats, and environment including milking and feeding system (Chahar, 2001) [10].
Clinical mastitis is often classified according to severity as mild (abnormal milk), moderate (abnormal milk and swollen udder), or severe (cattle exhibits systemic signs). Immediate action using systemic treatment is generally recommended for severe cases of clinical mastitis (Neeser *et al*., 2006 and Lago, 2009) [20].
More than 140 various microorganisms can cause mastitis (Nunes *et al*., 2013) [21]. The causative bacteria can be classified as major and minor pathogens (Harmon, 1994) [14]. The major pathogens responsible for bovine mastitis can be further classified as environmental (*Escherichia coli, Streptococcus dysgalactiae* and *Streptococcus uberis*) and contagious (*Staphylococcus aureus* and *Streptococcus agalactiae*) (Bramley *et al*., 1996 and Riffon *et al*., 2001) [9, 27] and *Mycoplasma bovis* depending on their primary reservoir and other pathogens that cause bovine mastitis, such as *Klebsiella pneumoniae (*Bannerman *et al*., 2004) [7] and *Pseudomonas aureginosa* (Hameed *et al*., 2014) [13] and *Listeria monocytogenes* are emerging problems in dairy herds. Bacteriological diagnosis is the most specific technique and still it is golden test for intra mammary infection but it is too expensive for routine use (Chahar, 2001) [10].

2. Materials and Methods
2.1Selection of Animals
The presentstudy entitled "Therapeutic studies on clinical mastitis in cattle" was carried out in the animals presented to VCC, CVAS, Bikaner or from individual holding of owner during June to November 2019. A total of 16 cattle showing signs of clinical mastitis such as inflammatory swelling of udder, pain on palpation and physical composition of milk such as color, presence of clots, flakes, pus and any other abnormalities.

2.2 Collection of milk samples
Milk sample was collected under aseptic conditions. The udder and teats were cleaned with water and dried. The teat orifice and the skin around the teat were wiped with cotton soaked in 70% alcohol. The first few milk stripping were discarded and about 30ml of milk sample from each affected quarter was collected separately into sterilized test tubes with caps. Care was taken to avoid any type of contamination. These were marked as right fore (RF), right hind (RH), left fore (LF) and left hind (LH). The milk samples from a total 16 cattle were collected in the present study.
All the samples of milk were brought to the laboratory and kept in refrigerator until analyzed. The analysis was carried out as soon as possible. Samples were collected on 0^{th} day as pre treatment and on 5^{th} day as post treatment for analysis. Both pre and post treatment samples were analysed for physical changes and further cultural examination was done for isolation and identification of organisms.

2.3 Cultural examination
The Isolation and identification of bacteria in clinical mastitis affected milk samples was done as per Cowan and Steel (1975).

2.4 Isolation of bacteria
The milk samples collected aseptically were shaken thoroughly. With the help of a four mm diameter platinum loop 0.01 ml of the sample was streaked on 5% sheep blood agar, Nutrient agar plate and Mannitol salt agar/Mac-Conkey agar/Edward media plates in primary, secondary and tertiary fashion in order to obtain isolated colonies of bacteria. These petri dishes were incubated for 24 hours at 37ºC and in case colonies did not appear or were found small, the plates were incubated for further 24 hours. Following incubation, the plates were observed for colonial characteristics and haemolytic zones on blood agar plates. If more than one type of colonies appeared on the agar plates, the different colonies were fished out and subcultured separately for obtaining the pure culture of the bacterial isolates.
Mannitol salt agar culture plates were observed for appearance of *Staphylococci* and *Micrococci* colonies as it is selective media for Gram positive bacteria *Staphylococci* and *Micrococci*, as high level of NaCl is inhibitory to most other bacteria. In Mannitol salt agar *Staphylococcus aureus* (Mannitol fermenting) produced yellow colonies with yellow zones, whereas other coagulase-negative *Staphylococci*eg. *S. epidermidis* (Non mannitol fermenting) produce small pink or red colonies with no colour change to the medium.
Mac-Conkey agar culture media plates were also observed for the appearance of colonies. The colonies were further examined for fermentation reaction of lactose. The lactose fermenting colonies were distinguished by their red or pink colour and non lactose fermenting colonies were colour less. Now, pure lactose fermenting culture were streaked on Eosine Methylene Blue (EMB) agar plates and incubated for 24 hours. The cultures that gave metallic sheen were considered as having *E. coli*. This appearance of metallic sheen on culture was due to the formation of eosinate from eosine.
Edwards Medium Base, Modified is a selective medium for the rapid isolation of *Streptococcus agalactiae* and other *streptococci* associated with mastitis. Esculin differentiates esculin- positive group D *Streptococci* (black colonies) from esculin-negative *Streptococcus agalactiae* (blue to colourless colonies).
The smears were prepared from pure colonies of bacteria, fixed by gentle heating and stained by Gram's method. The stained smears were examined under oil immersion objective for determining the type of Gram's reaction, morphological characters and to ascertain homogenicity of organisms. The pure isolates were taken on the nutrient agar slants and preserved in refrigeration at 4ºC after proper sealing and numbering was done for reference. The other bacterial growth slants were subjected to further characterization.

2.5 Identification of bacteria
Identification of mastitis causing bacteria was done as per following procedures:

2.5.1 Morphology
Colonies of bacteria on nutrient agar plates were purified and bacteria were observed for their shape, arrangement, sporulation, capsulation and presence of any other distinctive feature.

2.5.2 Motility
Motility was studied in hanging drop preparation of broth culture of bacteria.

2.5.3 Growth in air
Growth in air was studied to confirm whether the bacterial isolates were able to grow under aerobic or anaerobic condition.

2.5.4 Gram's reaction
Smears of young culture of bacterial isolates were stained by modified Gram's Method of staining described by Hucker and Cohan (1923). The results of staining were noted as Gram's positive (+) staining blue of primary stain and Gram's negative (-ve) staining pink of counter stain.

2.5.5 Spore formation
Spore formation was observed in smear prepared from bacterial colonies.

2.5.6 Identification of Bacterial species on basis of biochemical testing
Identification of the mastitis causing bacteria was done by biochemical identification kit of Himedia. Each kit has a standardized colorimetric identification system based on carbohydrate utilization and other biochemical tests specific for the identification of different bacterial species. These tests are based on the principle of pH change and substrate utilization. On incubation, the organisms undergo metabolic changes which are indicated by a colour change in the media that was either visible spontaneously or after addition of a reagent.

2.5.7 Procedure
The organisms to be identified were first isolated and purified on common media. Then single isolated colony was picked up and inoculated in 5 ml Brain Heart Infusion Broth and then incubated at 35-37 ^{0}C for 6 to 8 hours until inoculums turbidity was 3 0.1 OD at 620nm or 0.5 McFarland standard. Some organisms may require more than 6 hours of incubation. Therefore, the inoculum was incubated till the inoculum turbidity reached 0.1 OD at 620nm. The kit was opened aseptically. The sealing foil was peeled off. Each well was inoculated with 50µl of the above inoculum by surface

inoculation method. Alternately, the kit could also be inoculated by stabbing each individual well with a loopful of inoculum. Then it was incubated at 35-37 ^{0}C for 24-48 hrs. At the end of incubation period the reagents were added in some wells wherever required as per the instructions provided by manufacturer. Lastly, results were interpreted as per the standards given in the identification index. The results were noted in the entry data sheet and were compare colour reaction with the standard chart which was provided along with kits.

The oxidase test was performed on the organism to be tested. The test was performed using oxidase disc provided with the *Enterobacteriaceae* kit. The well isolated colony was picked up and was rubed it on a single oxidase disc. Positive reaction was indicated by development of deep purple colour within 10 seconds. Colour change in 10-60 seconds indicated delayed positive reaction. Colour development after 60 seconds or no change in colour indicated a negative reaction.

Following kits of Himedia were used for identification of bacteria:

1) HiStaph identification kit (KB004):each kit is a combination of 12 tests for identification of *Staphylococci*. i.e. Voges Proskauer's, Alkaline Phosphatase, ONPG, Urease, Arginine utization, Mannitol, Sucrose, Lactose, Arabinose, Raffinose, Trehalase and Maltose utilization.
2) HiStrep identification kit (KB005A):each kit is a combination of 12 tests for identification of *Streptococci*. Each kit contains sterile media for Voges Proskauer's, Esculin hydrolysis, PYR, ONPG, Arginine utization and 7 carbohydrate utilization tests Glucose, Lactose, Arabinose, Sucrose, Sorbitol, Mannitol and Raffinose utilization.
3) HiE. Coli identification kit (KB010): each kit is a combination of 12 tests for identification of *E. coli*. Each kit contains sterile media for Methyl Red, Voges Proskauer's, Citrate utilization, Indole test, Glucouronidase, Nitrate reduction, ONPG, Lysine utilization, Lactose, Glucose, Sucrose and Sorbitol utilization tests.
4) Hi Enterobacteriaceae Identification kit (KB003): kit for identification of *Enterobacteriaceae* (eg. *Enterobacter*, *klebsiella* and *Proteus* etc.). is a combination of 25 tests, contain a set of 2 kits, one kit contains sterile media for ONPG, Lysine utilization, Ornithine utilization, Urease, Phenylalanine, Nitrate reduction, H_2S production, Citrate utilization, Voges proskauer's, Methyl red test, Indole test, Malonate utilization and another kit contains sterile media for Esculin hydrolysis, Arabinose, Xylose, Adonitol, Rhamnose, Cellobiose, Melibiose, Saccharose, Raffinose, Trehalose, Glucose, Lactose and also having oxidase disc for oxidase test.

3. Statistical analysis

The results obtained were subjected to statistical analysis as per the methods described by Snedecor and Cochran (1994) and by using SPSS 20.0.0 version.

4. Results and Discussion

The The study was carried out in the Department of Clinical Veterinary Medicine E&J, College of Veterinary and Animal Science, Bikaner from June to November 2019. Some of the milk samples were also collected from individual holdings.

Clinical mastitis was manifested by change in gross appearance of udder like swelling, pain on palpation, erythema, warmth and hardness. There was gross change in appearance of milk like change in colour (yellow), consistency (viscous and purulent), presence of flakes and clots. Change in milk and udder of cattle were examined and recorded before and after treatment.

4.1 Bacterial culture examination

A total of 23 quarters milk samples from 16 clinical mastitis affected cattle were taken for bacteriological examination and for isolation of bacteria**.**

The cultural isolation of organisms involving 23 clinical mastitic milk samples was carried out. On the basis of Gram staining, 13 quarters (56.52%) showed Gram positive, 5 quarters (21.73%) showed Gram negative organisms and rest 5 quarters (21.73%) showed mixed infection. By culture examination of milk samples a total of 28 isolates were detected.

In the present study the relative frequency of different microorganisms in 28 isolates from 23 clinical affected quarters are presented in Table-1**.** As evident from the Table-1 *Staphylococcus aureus* was the most frequent isolate, accounting for 12 of the isolates (42.85%), followed by *E. coli* 6 (21.42%), *Streptococcus agalactiae* 5 (17.85%), *Klebsiella pneumoniae* 3 (10.71%) and *Streptococcus dysaglactiae* 2 (7.14%), respectively.

Table 1: Total bacteria isolated from clinical mastitic milk of cattle

S. No	Organisms isolated	No. of isolates from affected quarters
1.	*Staphylococcus aureus*	12(42.85%)
2.	*E. coli*	6 (21.42%)
3.	*Streptococcus agalactiae*	5 (17.85%)
4.	*Streptococcus dysgalactiae*	2 (7.14%)
5.	*Klebsiella pneumoniae*	3(10.71%)
Total isolates		28

Amongst different bacteria isolated in present study *Staphylococcus aureus* was found to be the most frequent isolated organism based on isolation on specific media and microscopic identification of organisms.

In a report Radostits *et al.* (2007) [25] cited that *S. aureus* had a wide ecological distribution being present inside the mammary gland and on the skin which was responsible for higher isolation rate of *S. aureus.*

The highest presence of *Staphylococci* spp. in the present study may possibly be due to the fact that these are present in large number in various body sites such as teat surfaces and teat orifices (Prabhakar *et al.*, 1990) [25] and *Staphylococci* can survive better in the environment than other micro organisms like *Streptococci spp.* (Shukla *et al.*, 1998) [32]. The presence of *Streptococci* spp. in milk was found to be lower than *Staphylococci* spp., which possibly is due to the fact that *Streptococci* spp. was unable to survive for long periods in the environment outside the body (Schlam *et al.*, 1971).

Staphylococcus aureus was found to be most common cause of mastitis and it may be due to the invading property of *Staphylococcus aureus* within the mammary gland macrophage and polymorphonuclear cells and capacity of pathogens to produce a polysaccharide capsule to the host factors (Sandholm *et al.*, 1990) [29]. Invasive properties of *Staphylococcus* enables to survive in udder tissue and during a cycling infection, inefficient phagocytosis and killing contribute to the relapse of infection (Michael *et al.*, 1991) [19].

E. coli infects the udder via teat canal from the unhygienic enviromental conditions (Akram *et al.*, 2013) [3]. The low incidence of gram negative rods may be due to their destruction by mammary gland (Wagge *et al.*, 1994 and Saidi *et al.*, 2013) [36, 28].

Almost similar result have been reported by Almobarak and Saeed (2019) [4], Gemechu *et al*. (2019) [12], Saravanajayam *et al.* (2015) [30], Jeykumar *et al*. (2013) [16], Abd-Elrahman (2013) [1], Sumathi *et al.* (2008) [34], Abera *et al*. (2012) [2], Ranjan *et al.* (2011) [26], Kurjogi and Kaliwal (2011) [17], Unnerstad *et al.* (2009) [35], Dutta *et al*. (2007) [11], Ambore *et al.* (2005) [5], Balakrishnan *et al*. (2004) [6].

In the present investigation mono infection was present in 18 quarters (78.26%) and mixed infections were in 5 quarters (21.73%), (Table-7 and figure-9) which indicated that the mono infection was more noticed or responsible for mastitis than mixed infections. Similar findings were reported by Siddiki *et al*. (2019) [33], Mandial *et al.* (1999) [18], Bhattacharya (2002) [8] and Patel (2008) [8]. These workers reported 80%, 75.43%, 88.88% and 70.83% mono infections, respectively. The mono and mixed infections in quarters have been depicted in Table-7 and Figure-9.

Monomicrobial infection with *Staphylococcus aureus* was noticed in a total of 8/23 (34.78%) quarters, *E. coli* in 3/23 (13.04%), *Streptococcusagalactiae* 4/23 (17.39%), *Streptococcus dysgalactiae* 1/23 (4.34%) and *Klebsiella pneumoniae* in 2/23 (8.69%) quarters.

Table 2: Relative frequency of bacterial isolates from clinical mastitis infected quarters by culture isolation

Sl. No.	Bacterial isolate	No. of quarter	Percentage (%)
1.	*Staphylococcus aureus*	8	34.78
2.	*Escherichia coli*	3	13.04
3.	*Streptococcus agalactiae*	4	17.39
4.	*Streptococcus dysgalactiae*	1	4.34
5.	*Klebsiella pneumonia*	2	8.69
6.	*Staphylococcus aureus + Streptococcus agalactiae*	1	4.34
7.	*Staphylococcus aureus + Streptococcus dysgalactiae*	1	4.34
8.	*Staphylococcus aureus + E. coli*	2	8.69
9.	*Klebsiella pneumoniae + E. coli*	1	4.34
	Total	23	100

Mixed infection was recorded in 5 quarters. In mixed infection combination of two genera were found. The combination of organisms isolated in one quarter (4.34%) *Staphylococcus aureus* and *Streptococcus agalactiae*, *Staphylococcus aureus* and *Streptococcus dysgalactiae* was in 1(4.34%) quarter. In another 2 (8.69%) quarters *Staphylococcus aureus* and *E. coli* were recorded. One (4.34%) quarter showed the presence of *E. coli* and *Klebsiella pneumoniae.*

In the present study 16 clinical mastitic animals having 23 affected quarters were divided into two groups containing 8 animals each. In group I, total of 12 quarters were infected and in group II, total of 11 quarters were infected.

Group I

In group-I, mono microbial infection was noticed in 10/12 (83.33%) quarters and mixed infection was seen in 2/12 (16.66%) quarters when milk samples were collected on 0th day of presentation and subjected to cultural examination.

Mono microbial infection with *Staphylococcus aureus* was observed in 5/12 (41.66%) quarters and that of *E. coli*, *Streptococcus agalactiae*, *Streptococcus dysgalactiae* and, *Klebsiella pneumoniae* were seen in 1/12 (8.33%), 2/12 (16.66%), 1/12 (8.33%) and 1/12 (8.33%) quarters respectively.

Similarly, mixed infection with *Staphylococcus aureus* and *streptococcus agalactiae* was observed in 1/12 (8.33%) quarters while a mixed infection of *Staphylococcus aureus* and *E. coli* was seen in 1/12 (8.33%) quarter (Table-8 and Annexure-II).

Table 3: Quarter wise presence of organisms causing mastitis in group I (n=12)

Sl. No.	Bacteria isolated	No. of infected quarters	Percentage (%)
1	*Staphylococcus aureus*	5	41.66
2	*Escherichia coli*	1	8.33
3	*Streptococcus agalactiae*	2	16.66
4	*Streptococcus dysgalactiae*	1	8.33
5	*Klebsiella pneumoniae*	1	8.33
7	*Staphylococcus aureus+ Streptococcus agalactiae*	1	8.33
8	*Staphylococcus aureus + E. coli*	1	8.33

Group II

In the group-II, mono microbial infection was noticed in 8/11 (72.72%) quarters and mixed infection was seen in 3/11 (27.27%) quarters when milk samples were collected on 0th day of presentation and subjected to cultural examination.

The mono microbial infection with *Staphylococcus aureus*, *E. coli*, *Streptococcus agalactiae* and *Klebsiella pneumoniae* was seen in 3/11 (27.27%), 2/11 (18.18%) 2/11 (18.18%) and 1/11 (9.09%) quarters respectively. It was also revealed that mixed infection with *Staphylococcus aureus* and *Streptococcus dysgalactiae*, *Staphylococcus aureus* and *E. coli*, *E. coli* and *Klebsiella pneumoniae* were observed in 1/11 (9.09%) quarters each (Table-9 and Annexure-III).

Table 4: Quarter wise presence of organisms causing mastitis in group II (n=11)

Sl. No.	Bacteria isolated	No. of infected quarters	Percentage (%)
1	*Staphylococcus auus*	3	27.27
2	*Escherichia coli*	2	18.18
3	*Streptococcus agalactiae*	2	18.18
4	*Klebsiella pneumoniae*	1	9.09
5	*Staphylococcus aureus+ Streptococcus dysgalactiae*	1	9.09
6	*Staphylococcus aureus + E. coli*	1	9.09
7	*E. coli + Klebsiella pneumoniae*	1	9.09

5. Conclusion

Animals were selected based on abnormalities found in milk and udder on physical examination.On cultural examination of mastitic milk of 23 quarters of 16 cattle, 18 quarters (78.26%) were having single bacterial infection, whereas 5 quarters (21.73%) were having mixed infection. The bacteria isolated in this study were *Staphylococcus aureus*, accounting for 12 of the isolates (42.85%), followed by 6 (21.42%) *E. coli*, 5 (17.85%) *Streptococcus agalactiae*, 3 (10.71%) *Klebsiella pneumoniae* and 2 (7.14%) *Streptococcus dysaglactiae*, respectively. In mixed infection combination of four genera were found. Organisms isolated in mixed infection were *Staphylococcus spp., Streptococcus spp., Klebsiella pneumoniae* and *E. coli.* It is concluded that Clinical mastitis was observed high in 4th parity, early lactation stage and in hind quarters. Major pathogens found were *Staphylococcus spp., Streptococcus* spp, *Klebsiella pneumonia* and *E. coli* on bacteriological culture.

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