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RESEARCH PAPER

Spatial distribution of coagulase-negative staphylococci (CNS) in parts of Bundelkhand: Relevance to the high incidence of subclinical mastitis

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Abstract: Coagulase-negative Staphylococci have emerged as a major group of pathogens in mastitis of dairy animals. The present work was performed to investigate the spatial distribution of milk-associated CNS pathogens in parts of Bundelkhand and to understand its relevance to the incidence of subclinical mastitis during the period 2016-2019. Etablished methods for culture and characterization of bacterial were used confirmation of HighStaph™ kit for identification of Staphylococci. Predominance of coagulase-negative Staphylococci among all bacterial pathogens in this region along with species distribution has been established. On considering CNS as a single group of pathogens, the CNS prevalence was found 39.20%, 38.40%, 22.40%, respectively for Jhansi, Jalaun, and Lalitpur. Since CNS distribution and subclinical mastitis incidences have high variability for different agro-climatic regions, this study will supplement the course of treatment and management of sub clinical mastitis (SCM) in the region through the development of understanding on causes and relationships of disease.

Keywords: Bundelkhand, Coagulase-negative, Mastitis, Staphylococci, Subclinical

Introduction

Mastitis is a complex and globally distributed disease, with significant economic losses (Ibrahim, 2017; Seifu and Tafesse, 2010). Mastitis is the udder inflammation in dairy animals and occurs either in the visible form called clinical mastitis or invincible form without any clinical signs termed as subclinical mastitis. Associated production losses and increasing expenditure on the treatments due to the delayed management of subclinical mastitis raising concerns on the profitability of farmers as well as degrading health of animals. Mastitis negatively impacts animal welfare as well as production losses due to associated deterioration of milk quality, increased mortality, reduced performance of production, and it may be caused by multiple microorganism groups (Khan *et al.,* 2021). This disease has been graded to have the potential of zoonosis due to emergence of the antimicrobial-resistant strains (Beyene *et al.,* 2017)

Many pathogens but CNS are very common to occur in mastitis infections & responsible for the preponderance of the disease. Among all bacterial pathogens associated with mastitis, three major pathogens are *Staphylococcus aureus, E.coli,* and *Streptococcus uberis* (Smulski *et al.* 2011). These bacteria are commonly found in most mastitic intramammary infections. But in recent times the importance of minor pathogens is increasing and has become a major challenge to the dairy sector, as reported in different studies (Leigh, 1999; Verbeke *et al.,* 2014). Coagulase-negative Staphylococcus sp. is the major group of minor pathogens responsible for the preponderance of the disease. However, the ubiquitous presence of these environmental pathogens like CNS is a major hurdle in dealing with this health issue (Hogan & Smith, 2012; Ruegg, 2012). Coagulase-negative Staphylococci had been reported as the most important emerging pathogen in subclinical bovine mastitis/Intra-mammary infections in various studies (Satu & Suvi, 2009). The distribution of CNS in India is very widespread and reported studies done by few authors for Dharwad region (Basappa *et al.,* 2011). More than 34 species of CNS had been reported by various authors in different parts of the world but few species are reported as endemic to some particular region. So data on the distribution of species may be effective in management and treatment concerning the occurrence of species-specific isolates in the regions as species-wise prevalence may specify the direction of treatment adopted by field veterinarians. The aspirational agrarian regions like Bundelkhand are still unexploited to have scientific data in this direction, and data-supported interventions can make a significant difference in improving clinical practices and the quality of milk production vis-à-vis animal health. Scientific data may also help in refining the course of treatment and avoid the long time consumption in the management of production diseases like mastitis. The objective of this study was to generate the primary data on spatial distribution of the coagulase-negative staphylococci in parts of Bundelkhand, and to understand its relevance in prevalence of subclinical mastitis.

Material and methods

Site selection for sampling: Nine representative villages, three from each of the districts of Jhansi, Jalaun, and Lalitpur were selected randomly during the period 2016-19. Karguan Ji, a suburban area around the Jhansi city was considered as representative of a mixed rural/urban economy. Two others were Shivaji Nagar and Merygaon was also included. Three villages from Jalaun were Ekon, Rampura, and Shahjadepur. Pawa, Piprai, and Bansi were villages from the Lalitpur district selected to collect samples. Consultation with farmers was done before selection and criterion of availability of samples with easiness was also adopted.

Animal selection: Smallholder dairy farms having at least one

Table 1. Spatial distribution of sites used to collect samples

Selected districts	Location/ Coordinates	Number of animals	Selected villages
Jhansi	25.4484^{0}N, 78.5685^0 E	1200/site	Karguan Ji Shivaji Nagar Merygaon
Jalaun	26.1271^0 N,79.4704^0 E	800/site	Ekon, Rampura, Shahjadepur
Lalitpur	24.6879^0 N,78.4120^0 E	600/site	Pawa, Piprai, Bansi

lactating animal either cow or buffalo were selected for the study in representative villages. Animal demographics, representation, and convenience in getting samples from farmers were considered as basic criteria in the selection of smallholder dairy farmers. Operational dairy farmers with average herd sizes from 10 to 18 with a median value of 14 were 154 in the selected villages during the study period. All the lactating animals including buffalo and cows were considered eligible for subclinical mastitis prevalence studies. Total number of 411 lactating animals (150 cows and 310 buffalo, however 49 samples get wasted) were used to collect milk samples.

Milk sampling: Preliminary survey for subclinical mastitis based on history and occurrence, was conducted in consultation with a local veterinary hospital assistant in Jhansi, Lalitpur, and Jalaun District. Villages were selected randomly. Contact was directly established with local farmers from villages and small dairy farms. The status of the cow for diseases was confirmed with the help of a veterinarian. The history and etiological factors were recorded by interviewing the concerned farmer.

The cleaning of the udder with water followed by 70% alcohol was done before collecting milk samples. After 5 minutes, 10 ml of milk sample was directly drawn into horizontally tilted falcon tube to avoid contamination due to skin shading. A thermal box filled with ice packs was used to transport the samples. Samples were processed immediately for bacteriology, somatic cell count (SCC) counting, Electrical conductivity. The remaining milk samples were stored at -80^{0}C.

Milk somatic cell count: Milk samples were processed for SCC by direct microscopy method. Direct screening of milk samples to determine mastitis status by counting somatic cells was done in lines with Newmann's staining with microscopy as per the procedure used by Schalm *et al.*(1971).

Bacteriological culture of milk: It was Conducted by culturing milk samples on different generalized and selective bacteriological media according to previous studies described by National Mastitis council, 1999, National Veterinary Institute Uppsala, Sweden, and other associated workers with local modifications wherever needed. In accordance to Ericsson *et al.,* 2009, samples positive for at least 3 cfu/ml were considered as bacteriologically positive for all bacterial genera. But the growth of a single colony was considered positive in the case of staphylococci.

Bacterial isolation: Samples positive for subclinical mastitis initially confirmed through California mastitis test (CMT) followed by somatic cell count (SCC), were subjected for bacteriological examination according to the procedure described by Sears *et al.,* 1993. Milk samples collected from each quarter were inoculated to the McConkey agar and Blood agar base enriched with 7% defibrinated sheep blood followed by aerobic incubation for 24 to 48 hours at 37^{0}C. Preliminary identification of bacteria was carried out based on bacterial growth characteristics and colony appearance in all cases. Identification of *Staphylococci* was done based on the outcome of the catalase test, coagulase test, morphological appearance/ staining, and sugar fermentation test. *Streptococci* were identified based on growth characteristic hemolytic pattern and esculin hydrolysis and Christie-Atkins-Munch-Peterson test. *E. coli* isolation was based on their growth and lactose fermentation characteristics and gas production.

Confirmation of coagulase-negative Staphylococci with KB1004, HiStaph™ identification kit: KB004 HiStaph™ identification kit supplied by Himedia Laboratories Pvt. Ltd., Mumbai was used for further characterization of presumptive coagulase-negative Staphylococci isolates. It has 12 different small-size wells to perform 12 tests i.e. VP Test, phosphate test, ONPG test, urease, and arginine utilization test simultaneously with sugar fermentation tests for lactose, sucrose, arabinose, raffinose, maltose, mannitol, and trehalose. As per instructions given by the manufacturer, 50 µL of 18hr old bacterial suspension culture equivalent with 0.5 on McFarland scale was laid down on the surface of each miniature well followed by 24 hr incubation at 37^{0}C. Reagents supplied with the kit for VP test and phosphatases were used in the procedure. Results were noted down following guidelines prescribed by the manufacturer. For speciation purposes, the results of biochemical tests were compared with the original index supplied by the manufacturer. Bacterial cultures used as reference cultures during the experimental part are given in Table 2.

Table 2. Reference culture species with catalog number

Species	Catalog no.
Staphylococcus aureus	96
Staphylococcus epidermidis	3382
S. saprophyticus	6155
S. haemolyticus	3383
S. chromogenes	3545

Results and discussion

All collected samples were initially screened for subclinical mastitis (SCM) by california mastitis test (CMT) and electrical conductivity (EC) Test followed by somatic cell count (SCC). Considering the established correlations, samples were subjected to bacterial isolation interpretation in line with SCC results.

Somatic cell count estimation: SCC includes neutrophils, lymphocytes, macrophages, and polymorpho-nuclear (PMN) cells, as well as secretory glandular desquamated epithelial cells, were also considered in SCC counting. The somatic cell count > 5 lakhs/ml, conventional criteria (Jashari *et al.,* 2016) were

considered to declare the milk samples as positive for subclinical mastitis. 172 (41.85%) out of 411 samples were found positive for SCM in the initial screening. The number of cases of subclinical mastitis was found slightly higer in the semi-urban area as compared to the rural area. Based on total counts of somatic cells, milk samples were divided into 4 different groups having several SCC in the particular range i.e. 0-1, 1-2, 2-5, and >5 lakhs cells/ml. 380 out of 415 milk samples were found to show the somatic cells count in the range of 0-5 lakhs. Individually, 89,68, 82 and 172 samples revealed SCC value in range of 0-1, 1-2, 2-5 and >5 lakhs/ml, respectively. Thus above showed the 41.85% prevalence of subclinical mastitis.

Bacterial isolation from milk samples: Although, 41.58% of samples were identified as positive for subclinical mastitis all the samples were subjected to bacterial isolation. Total 365 isolates belonging to different groups based on microscopic and cultural characteristics were identified (Table 3). Coagulase-negative Staphylococci and *Staphylococcus aureus* were found to correlate with the pattern of somatic cell count. The largest numbers of staphylococcal isolates were recovered from the samples showing SCC > 5 lakhs/ml. Three hundred and sixty-five (88.8%) out of 411, samples were found bacteriologically positive.

Characterization of isolates: Gram staining was performed on all the isolates by using 24-hour old pure culture in broth media. Isolates were found in cocci in chains, cocci in groups arranged as clusters and rods followed by biochemical characterization.

Catalase test: All staphylococcal isolates (167) and reference MTCC strains were found positive for catalase test by both methods i.e. Plate catalase test and slide catalase test.

Coagulase test: Based on the coagulase test with rabbit blood plasma isolates were further grouped into coagulase-negative and positive. In all, a total of 142 isolates were found negative for the coagulase test. Simultaneously, all the strains in reference cultures were found negative except Staphylococcus aureus. Further, these 142 isolates were processed for other biochemical characterization followed by molecular characterization. Moreover, only 12 isolates were found positive for the Thermonuclease test

Biochemical characterization with HiStaph™ Identification kit: After isolating the bacteria on culture media, all isolated presumptive pathogens were identified up to genus, based on the colony characteristics, the hemolytic pattern on blood agar, Coagulase test, Gram's staining, and catalase test. Further, to study speciation, all 132 isolates that were categorized as Coagulase-negative Staphylococci were further processed with HiStaph™ based identification system for biochemical test-based confirmation. Only 125 isolates were confirmed as coagulase-negative Staphylococci, belonging to 6 different species of the group on comparing the results with the interpretation chart supplied by the manufacturer.

Bacteriology of SCM positive milk samples: Only 41.8% of milk samples were found positive out of all samples collected and processed for bacterial isolation to confirm positivity to subclinical mastitis regarding common pathogens.

The bacterial pathogens identified in the collected milk samples based on biochemical characterization are shown in Table 3 Fig 1. On microbial and biochemical profiling of these milk samples, 365 different bacterial isolates were identified. These isolates were found to belonging to 6 different taxonomic classes of bacteria. Maximum numbers of isolates were found to belong to the group coagulase-negative Staphylococci (34.25%) followed by 13.42%, 11.51%, 11.51%, 8.77%, and 3.84% for *Bacillus sp., Staph. aureus*, *Escherichia coli, Strep. sp.* and *Corynebacterium bovis*, respectively. Sixty-one (16.71%) isolates were not identified with available biochemical tests and considered as others. Milk samples are collected from three districts of the Bundelkhand region *i.e.* Jhansi, Jalaun, and Lalitpur. The normal appearance of milk and absence of visible signs in the mammary gland tissue is the major barrier in the early detection of subclinical mastitis (Mishra *et al.,* 2018). SCM creates a reservoir of microorganisms that act as the source of infection to the other individuals and help in the preponderance of clinical mastitis (Thompson *et al.,* 2014). More than 137 organisms belonging to different classes and taxa have been identified as pathogens of bovine subclinical mastitis, including bacteria, viruses, fungi, algae, and mycoplasma (Watts, 1988). Staphylococcus aureus has been considered as the major causative agent (Verma *et al.,* 2017) but coagulase-negative Staphylococci has been reported by many authors in different countries as the most prevalent pathogen in bovine subclinical mastitis e.g. Poland, Iran, and India (Sztachanska *et al.,* 2016; Chavoshi & Hussaini, 2012; Hegde *et al.,* 2013). So the objectives

Table 3. Prevalence of different bacterial mastitis pathogens concerning respective SCC group

Bacterial groups identified	SCC per ml of the milk samples in lakhs/ml					Percentage
	0-1	1-2	2-5	≥5	Total	
	n=89	n=68	n=82	n=172	411	N=365
CNS	26	21	22	56	125	34.25
Staphylococcus aureus	04	06	11	21	42	11.51
Streptococcus sp.	0	0	05	27	32	8.77
Bacillus sp.	10	11	15	13	49	13.42
Corynebacteriumbovis	2	0	3	9	14	3.84
E. coli	5	13	7	17	42	11.51
Others	15	11	18	17	61	16.71
Total	62	62	81	160	365	

n= No. of samples; N= Total no. of isolates

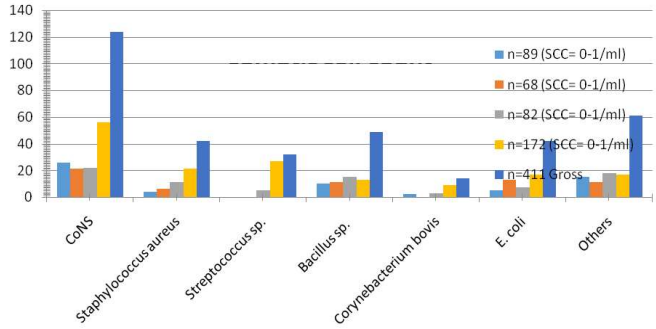

Fig.1. Relative numbers of isolates concerning different SCC values.

designed were in concurrence with the studies with a focus on the Bundelkhand region. A similar pattern of dominance of Coagulase-negative Staphylococci was also found in our study for the selected area. Further, genotypic diversity and speciation of coagulase-negative Staphylococci have emerged as a serious challenge in recent studies. As most of these organisms are commensals to human beings, so studies on the impact of single species on bovine udder health are possible only after the accurate identification of causatives agents (Zadocks & John, 2011). In our study, molecular identification and genotypic relationship studies reveal the speciation of coagulase-negative Staphylococci and the relationship of few randomly selected isolates to the existing databases of the group of Staphylococci. Our findings are similar to previous reports (Hegde, 2011), however, some of our results has minor difference with these studies. Possibly, these contradictions may be due to spatial and temporal separation from others. Data on bovine mastitis etiology is updated very fast in many developed countries like the USA, UK, and Newzealand (Harjanti *et al.,* 2018) but in India and especially in the study area it was not updated frequently, even no data is available to this region before the study was undertaken. Lack of updated data on the etiology of subclinical mastitis is a serious hurdle in the management of disease in our country. Quarter-wise milk sampling conducted for bacteriological isolation and prevalence studies is in agreement with previous studies (Dasohari *et al.,* 2017; Batavani *et al.,* 2007). Data presented in Table 3 reveals the number of bacterial isolates belonging to different classes and taxa. Total 365 bacterial isolates were identified by biochemical microbial culture followed by biochemical identification. Standard biochemical procedures were adopted as per Bergey's manual of determinative microbiology (Collee *et al.,* 1996). Mixed infections were found very common as 220 milk samples were found to infect with more than one bacterial group. The common occurrence of CNS, *Bacillus,* and Staphylococcus aureus in the collected samples is responsible for mixed infections. It is very important to screen the exact situation of subclinical mastitis pathogens in the dairy sector to control this disease but a very limited number of studies is carried out in India and no previous report was found in the literature for this particular region (Singh & Kumar, 2018).

Bacterial isolates in this study are very much similar to previous studies conducted in other parts of this country and other countries (Hegde, 2011; Saini *et al.,* 1994). Milk bacterial culture is the standard method to confirm mastitis infections for pathogens of common occurrence (Sudhan & Sharma, 2010). Quarter-wise 83.90% of milk samples were found positive for at least one mastic pathogen.. These results are in close agreement with Sharma, 1993 who have reported 85% of quarters as positive for subclinical mastitis by bacteriological examination and CMT. However, a higher rate of infection was observed in this study as compared to previous studies (Bulla *et al.,* 2006; Chavan *et al.,* 2007). This difference in the rate of infection may be due to unhygienic and bad management practices *viz.* unhygienic water, feed, lake of regular cleaning, infrequent change of animal bedding, adopted in the area as evidenced from previous studies that the higher occurrence may also be due to changed environmental conditions (Schultze, 1985). Comparatively, bad management practices might be the major cause behind the higher prevalence and bacterial infections in this particular region.

Different authors from different parts of the world and in India have conducted similar kinds of studies and reported the occurrence of CNS, *S. aureus*, *Bacillus sp.,* Corynebacterium sp., *Streptococcus sp., E. coli, Proteus,* and *Micrococcus sp.*

Table 4. Sector/site wise CNS isolates in different SCC groups

Source		Relationship between CNS isolates and SCC value (S=Number of samples, N=Number of isolates)							
		SCC:- 0-1 lakh / ml		SCC: 1-2 lakh / ml		SCC: 2-5 lakh / ml		SCC e"5 lakh / ml	
		S	N	S	N	S	N	S	N
Jhansi	Site1	10	02	07	01	10	03	22	09
	Site2	12	03	06	02	12	05	24	08
	Site3	12	01	04	02	07	03	28	10
	Total	34	06	17	05	29	11	74	27
Jalaun	Site 4	11	02	06	03	08	02	25	11
	Site 5	09	01	07	00	09	05	15	09
	Site 6	08	01	17	01	11	02	17	11
	Total	28	04	30	04	28	09	57	31
Lalitpur	Site 7	05	00	07	01	05	01	13	09
	Site 8	15	00	06	02	07	01	12	06
	Site 9	07	01	08	00	13	00	16	07
	Total	27	01	21	03	25	02	41	22
Grand total (n)	89	11	68	12	82	22	172	80	
%	—	12.35	—	17.65	—	26.83	—	46.51	

Table 5. Species distribution of coagulasenegative *staphylococci* isolates with respect to sites and districts

Area Species		Jhansi				Jalaun				Lalitpur				Gross
		1	2	3	n=49	4	5	6	n=48	7	8	9	n=28	N=125
S. epidermidis	No.	05	03	09	17	04	03	04	11	04	01	01	06	34
	%	14.70	8.83	26.47	34.69	11.76	8.82	11.76	22.92	11.76	2.94	2.94	21.42	27.20
S. chromogenes	No.	04	06	03	13	03	04	03	10	01	02	01	04	27
	%	14.81	22.22	11.11	26.53	11.11	14.81	11.11	20.83	3.70	7.41	3.70	14.28	21.60
S. saprophyticus	No.	02	04	02	08	02	03	01	06	02	01	02	05	19
	%	10.53	21.05	10.53	16.33	10.53	15.79	5.26	12.50	10.53	5.26	10.53	17.86	15.20
S. simulans	No.	02	03	01	06	03	02	01	06	02	00	03	05	17
	%	11.76	17.65	5.88	12.24	17.65	11.76	5.88	12.50	11.76	0.00	17.65	17.86	13.60
S. haemolyticus	No.	00	01	01	02	02	00	01	03	00	02	00	02	07
	%	0.00	14.28	14.28	4.08	28.57	0.00	14.28	6.25	0.00	28.57	0.00	7.14	5.60
S. xylosus	No.	02	01	00	03	04	03	05	12	02	03	01	06	21
	%	9.52	4.76	0.00	6.12	19.05	14.29	23.81	25.00	9.52	14.29	4.76	21.43	16.80
Total CoNSN=125	No.	15	18	16	49	18	15	15	48	11	09	08	28	
	%	12.00	14.44	12.80	39.20	14.40	12.00	12.00	38.40	8.80	7.20	6.40	22.40	

Note: data given is on the basis of biochemical profiling followed by molecular confirmation with PCR, 1-9; Sites of sample collection

as predominant sp. in milk samples from bovine subclinical mastitis (Pal *et al.,* 2017; Abdalhamed *et al.,* 2018; Biruke & Shimeles, 2015). Some of the important studies were also conducted in different parts of India and reported almost similar results (in Rewa, M.P. by Singh *et al.,* 2016, in Chhatisgarh by Ottalwar *et al.,* 2018, in Maharashtra by Joshi & Gokhle, 2006). In partial agreement with these studies, the present investigation isolated and identified the CNS, as predominant mastitis pathogens with a gross prevalence of 34.55% followed by *Bacillus sp., Staphylococcus aureus, E. coli, Streptococcus sp*. and *Corynebacterium bovis*. Further, 16.71% of isolates were unidentified and categorized as others. Findings for CNS were found in full agreement with 37% prevalence reported in Hyderabad (Anusha *et al.,* 2017). One hundred sixty isolates out of a total of 365 were reported from milk with SCCe"5 lakhs/ml and emerged dominant group supported by a previous study (Hegde *et al.,* 2013). The highest prevalence of CNS isolates in SCM cases agreed with old reports (Belachew, 2016). Percentage of isolation for CNS was found much higher in the selected region than the previous reports of 19.5%, 11.9%, and 22.2%, respectively in the studies conducted by Abdalhamed *et al.,* 2018, Pal *et al.,* 2017, Ngu *et al.,* 2020 and Belachew, 2016 at different locations. Higher prevalence of CNS maybe because of the higher occurrence of acute mastitis infections in the region. Following Pyorolla, about 30% of cases of acute subclinical mastitis maybe because of CNS infection which is in full agreement with our results. Factually, no consideration of clinical mastitis may be attributed to low prevalence with 11.51% for Staphylococcus aureus found higher than 4.90% (Sahoo *et al.,* 2009) but in agreement with 16% (Ahire *et al.,* 2008). The similar prevalence reported for E. coli is in agreement with 13% reported in Jammu (Bhat *et al.,* 2017). They also reported a very high prevalence of S. aureus with 60% more than our findings. Prevalence was less than 21% as reported in Ludhiana (Karanvir *et al.,* 2018) and 28% reported in Bikaner, Rajasthan (Gangwal & Kashayap, 2017). Minor occurrence of *E.coli* in this region may be associated with low-producing animals As higher occurrence of this pathogen in animals was found to be associated with high production (Hogan & Larry, 2003). 3.84% prevalence of *Corynebacterium bovis* as reported in the study may be associated with latent infections in few animals. Findings are in agreement with the 3.3% prevalence reported for this pathogen in similar studies conducted in Ethiopia (Tesfaheywet & Gerema, 2017).

Coagulase-negative *Staphylococcus* sp. Isolates: Coagulase-negative isolates were further divided into four different groups based on respective SCC counts of milk samples from which isolate was originated. Data in correlation with SCC 0-1, 1-2, 2-5, and more than 5 lakhs/ml of milk. Study results showed the 11 (12.35%), 12 (17.65%), 22 (26.83%) and 80 (46.51%) isolate belonging to groups I, II, III, and IV, respectively. All isolates were further confirmed by tuf gene-based PCR up to genus level followed by groEL gene amplification and RFLP of the amplicon with restriction enzymes Alu I and Hind III. Total 132 CNS isolates were identified by biochemical characterization but only 125 were confirmed with PCR-RFLP studies. Final data on CNS was analyzed by considering the isolates that were confirmed by the PCR-RFLP of the *gro* EL gene. The remaining seven CNS isolates were categorized as bacteria belonging to other groups.

Distribution of coagulase-negative staphylococcal isolates: The distribution and percent prevalence of confirmed staphylococci isolates found as negative with the coagulase test is presented in Table 4. One hundred twenty-five isolates confirmed as CNS with coagulase test were further identified by PCR of tuf gene amplification up to genus level followed by PCR-RFLP of *gro*EL gene for species determination and are tabulated concerning sites and districts. In speciation studies, as presented in Table 5, isolates were found to belong to 6 different species of coagulase-negative staphylococci. Identified species along with percent distribution were *Staphylococcus epidermidis* (27.20%), *S. chromogenes* (21.60%), *S. saprophyticus* (15.20%), *S.simulans* (13.60%), *S.haemolyticus* (5.60%), and *S.xylosus* (16.80%), throughout the study area. Further, *S. epidermidis* isolates were highest in the Jhansidistrict followed by Jalaun and Lalitpur with percentages of 34.69%, 22.92%, and 21.42%, respectively.

Similarly, most of the isolates of *Staphylococcus chromogenes* were also reported from the Jhansi district followed by Jalaun and Lalitpur with the respective percentage of 26.53%, 20.83%, and 14.28%. The pattern of distribution for *Staphylococcus saprophyticus* differed, as the highest number of isolates (17.86%) was found in Lalitpur followed by Jhansi and Jalaun with percentages of 16.33% and 12.50%, respectively. The highest number of isolates of Staphylococcus simulans (17.86%) and *Staphylococcus haemolyticus* (7.14%) were found in Lalitpur followed by Jalaun and Jhansi with the respective percentage of 12.50%, 12.24% and 6.25%, 4.08%, respectively. Differed with all, highest prevalence of *Staphylococcus xylosus* (25%) was found in Jalaun district followed by Lalitpur and Jhansi with 25% and 6.12%, respectively. If CNS is considered as a single group, the highest number of isolates, 49 (39.20%) was found to be distributed in Jhansi district followed by Jalaun and Lalitpur with 48 (38.40%) and 28 (22.40%), respectively.

Given the rapidity, specificity, and reliability, this method based on the amplification of chaperonin *gro*EL was used in this study to investigate and confirm the presumptive CNS isolates found on culturing the milk samples. All 125 isolates were yielded the 550 bp DNA fragment on PCR amplification of the *gro*EL gene. Further restriction digestion also yielded the desired fragments of the *gro*EL gene. On comparing the results with previous studies in this field, isolates were found to belong to 6 different species of CNS *viz., Staphylococcus epidermidis, S. chromogenes, S. xylosus, S. haemolyticus, S. simulans & S. saprophyticus*. These results were fully supported by the findings of Santos *et al.,* 2008 and Korcan*et al.,* 2015, Al-Haddadi *et al.,* 2020.

The present study revealed the predominance of coagulase-negative staphylococci among all bacterial pathogens in this region which is in agreement with studies conducted in different parts of the country (Thakur *et al.,* 2018; Krishnamoorthy *et al.,* 2021). High occurrence of CNS can be correlated with the bad management practices and lack of routine mastitis diagnosis in the selected region, as *staphylococci* mastitis has been reported as the most common and contagious mastitis where the pathogen can transmit from one cow to another cow through hand and equipment or environment (Bagley, 1997).

Overall results were in agreement with the findings of authors based on the partial study, a very high prevalence of 51.22% was reported (Singh & Kumar, 2018). The difference was found, maybe because of the low sample size in the previous study, and only biochemical characterization of isolates was performed. Some of the isolates were not confirmed as CNS. Molecular studies show that changing dynamics in associated bacterial populations may also be an important factor as; study includes the temporal and spatial separation of the samples collected.

Conclusion

Study indicated the predominance of subclinical mastitis caused by coagulase-negative staphylococci with a low occurrence of common mastitis pathogens belonging to the groups *S. aureus*, *Streptococci, E coli, Bacillus*, and *Corynebacterium*. A very high incidence of CNS in the milk samples in the area of the study indicates its probable role as an emerging pathogen in subclinical mastitis. The occurrence of different mastitis pathogens in the study area indicated the unhygienic and bad management practices at the farm level. Hence, recommended the adoption of good management practices in the study area to avoid the occurrence of disease. However, a systematic study for the complete region of Bundelkhand at the village level to make a complete database on the prevalence of pathogens of both clinical and subclinical needs to be undertaken.

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