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Identification of bacterial pathogens in dry bovine udder and their antimicrobial susceptibility#

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Abstract

Mastitis is a multi-etiological production disease of dairy cows worldwide. The likelihood of developing clinical mastitis in subsequent calving is enhanced by intramammary infections with pathogens during the late dry and post-calving period. There is an increase in the occurrence of intramammary infections during early and late dry period. Thirty apparently healthy, pregnant cross-bred dairy cows were screened for the presence of contagious mastitis pathogens in the secretions taken after 48 h of cessation of milking. A total of 118 bacterial isolates were obtained from the secretions collected from 108 quarters, and identified using morphological and cultural characteristics. Among them, 50.8 per cent were coagulase negative Staphylococci (CNS), 16 per cent were coagulase positive Staphylococci (CPS) and 27.1 per cent were Streptococci isolates. Micrococci and E. coli constituted 2.5 per cent each and Klebsiella spp. formed 0.8 per cent of the total isolates obtained. The CNS isolates identified included 22 S. chromogens, 14 S. xylosus, 12 S. saprophyticus, six S. hominis and S. epidermidis each. Among the CPS isolated 11.8 per cent were S. aureus and 4.2 per cent were non-aureus coagulase positive Staphylococcus which included three S. hyicus and two S. pseudointermedius. Molecular confirmation of S. aureus, CNS isolates, St. agalactiae were done by targeting nuc, cns and 16SrRNA gene respectively. Identification of bacterial pathogens that are present during dry period can help in formulating an effective dry cow therapy. Antibiogram studies of the isolates obtained were done using in vitro antibiotic sensitivity testing method.

Keywords: *Mastitis, dry period, bacterial pathogens, molecular confirmation*

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Mastitis is an economically devastating disease of dairy herds throughout the world. Dry period is important in dairy cows because the intramammary infections (IMI) acquired during the previous lactation persists during this period and can result in occurrence of mastitis in subsequent lactation. The gland is especially vulnerable to new infections during the early and late dry periods, which correspond to involution and colostrogenesis, respectively (Smith *et al.,* 1985, Green *et al.,* 2002). A major factor that results in the increased occurrence of intramammary infections during dry period is the delay in formation of teat plug in the streak canal (Dingwell *et al*., 2002). Even though dry period is an optimum time to cure an existing IMI, it is also a period of greatest susceptibility to new infection. Various studies done on dry period secretions of bovines showed that the most common bacteria present in dry period secretions were *Staphylococcus* spp. (Kulangara *et al*., 2017; Freu *et al*., 2020; Nickerson *et al.,* 2020). Streptococci and Staphylococci invaded 24 per cent of uninfected quarters during the initial few weeks of dry period and half of these infection persists and transferred to next lactation and among those, half led to clinical mastitis (Neave *et al*., 1950). The infections that are present during the lactating period would be transferred to dry period resulting in increased occurrence of *Staphylococci* in dry period secretions (Radostits *et al*., 2007). Hence, the present study was carried out to isolate and identify the contagious mastitis pathogens present during the dry period in cows in Wayanad district.

Materials and methods

The present study was conducted in 30 pregnant cross bred dairy cows which were selected from organized dairy farms and rural households in Wayanad district during the period from December 2021 to September 2022. Dry period secretions were collected separately from all the four quarters of the pregnant cows after 48 hrs of cessation of milking. Epidemiological data regarding age, breed, parity, stage of pregnancy, herd and managemental practices and history of occurrence of mastitis in the farms were collected. *In vitro* antibiotic sensitivity of the clinical isolates was studied using Kirby Bauer disc diffusion method, as per the Clinical and Laboratory Standards Institute guidelines.

Isolation and identification of bacterial isolates

Dry period secretions collected were streaked on to brain heart infusion (BHI) agar for primary culturing. From the total of 120 quarters selected for the study 108 quarters yielded bacterial growth, which yielded a total of 118 bacterial isolates. These isolates were identified using colony morphology, Gram staining and biochemical tests. Various conventional technique like catalase, oxidase, coagulase, indole, MR, VP, citrate utilisation test, urease test, nitrate reduction test and sugar fermentation test were used to characterise Gram positive bacterial isolates.

Based on the preliminary examination using Gram's stain, the isolates were further sub cultured on to the selective media and a presumptive identification was done according to their colony characteristics. All Gram positive cocci were streaked on to mannitol salt agar. The colony characteristics of the culture were observed after incubation at 37°C for 24-48 hrs. Biochemical test including Catalase, Oxidase, Coagulase, Indole test, Methyl red, Voges Proskauer test, Citrate utilization test, urease, nitrate reduction test and sugar fermentation tests were done. The presumptively identified *Staphylococcus* isolates were further confirmed as *Staphylococcus aureus* (*S. aureus*) by direct streaking and subsequent incubation at 37°C for 24hr on Baird Parker agar and DNase agar. Gram negative colonies were identified using selective medias like MaConkey agar, EMB agar and IMViC test.

Molecular confirmation of the isolates

Molecular confirmation of the staphylococcus and streptococcus isolates were done using polymerase chain reaction (PCR). The details of the primers used are given (Table 1). The PCR conditions for the amplification of the *Staphylococcus* spp., cns, *S. aureus*, *St. agalactiae* were as per the reference quoted in the table.

Table 1. Details of primers used for molecular confirmation of organisms

Organism	Genes	Primer sequence	Amplicon size (bp)	References
Staphylococcus spp.	*16S rRNA*	AACTCTGTTATTAGGGAAGAACA CCACCTTCCTCCGGTTTGTCACC	756	McClure *et al*. (2006)
S. aureus	*nuc*	AACTCTGTTATTAGGGAAGAACA CCACCTTCCTCCGGTTTGTCACC	359	Sasaki *et al*. (2010)
Coagulase negative *Staphylococcus*	*cns*	TATCCACGAAACTTCTAAAACAACTTTACT TCTTTAGATAATACGTATACTTCAGCTTTGAATTT	204	Okolie *et al*. (2015)
St. agalactiae	*16s rRNA*	GCTAATACCGCATAAGAGTTAACA GGTAGATTTTCCACTCCTACCAA	317	Shome *et al*. (2011)

Antibiogram studies

In vitro antibiotic sensitivity of the clinical isolates was studied using Kirby Bauer disc diffusion method. Mueller Hinton agar was used to study the antibiotic sensitivity pattern of the isolates. Antibiotic discs with known concentration in microgram (µg) or international unit (IU) per disc were used in the study. Antibiotics discs used include ceftriaxone tazobactam, amoxicillin clavulanate, ceftiofur, gentamicin, cefoperazone, tetracycline and enrofloxacin. The antibiotic discs were applied under aseptic conditions. The discs were deposited with a distance of at least 24 mm apart. The plates were incubated immediately at 37□ and examined after 14-18 hrs or later if necessary. The clear zones were measured and the diameter of the zones to the nearest mm was recorded. The zones were compared with the standard inhibition zone chart provided (CLSI, 2020).

Results and discussion

Occurrence of bacterial pathogens during dry period

Occurrence of intramammary infection was mostly identified in the age group between two to four years (Table 2). Compton *et al*. (2014) observed a high incidence of intramammary pathogens in dry period between three to four years of age.

Occurrence of intramammary pathogens was highest in the animals in first parity (Table 3). This result is in accordance with Dingwell *et al.* (2002), who also reported highest (43.7 per cent) occurrence of intramammary pathogens in dry period cows in first parity. Similar findings were also reported by Elbably *et al*. (2013) in mastitis cases, with a high incidence of mastitis in parity ranging from two to four years, followed by cows with parity up to two. Generally, increase in incidence of mastitis pathogen increased with number of parities which may be associated with factors like milk yield, age related changes in udder and milking practices (Bhat *et al*., 2017).

Isolation and identification of bacteria

Among 118 bacterial isolates, 79 were Gram positive cocci which were arranged in bunches of grapes. Three were Gram positive cocci arranged in tetrads and 32 were cocci arranged in chains. Four isolates obtained were Gram negative bacilli. All the 79 cocci were catalase positive and oxidase negative, which shows that these are Staphylococci. Among 79 Staphylococcal isolates, 19 isolates (16.1 per cent) were mannitol fermenters and 60 isolates (75.9 per cent) were non-fermenters. *S. aureus* has the ability to ferment mannitol, which causes the media to become acidic, resulting in yellow colonies with yellow zones, whereas CNS and *Micrococc*us spp. which are not mannitol fermenters, produced pink or red colonies with no change in the colour of the media (Davies *et al.,* 2008). Based on positive coagulase reaction on tube coagulase test, and growth in BP agar with black colonies the 19 isolates were identified as coagulase positive *Staphylococcus* (CPS) and 60 isolates were negative for coagulase test and were identified

Table 2. Occurrence of mastitis pathogens in the dry period cows based on age

Sl.No.	Age (yr)	Number of affected animals	Per cent (%)
1	2-4	14	46.6
2	5-7	10	33.3
3	Greater than 7	6	20
	Total	30	100

Table 3. Occurrence of mastitis pathogens in dry period cows based on parity

Sl. No.	Parity	Number of affected animals	Per cent (%)
1	1st	12	40
2	2nd	9	30
3	3rd	7	23.3
4	4th	1	3.3
5	5th	1	3.3
Total		30	100

as coagulase negative Staphylococcus (CNS). Among the 19 isolates,14 showed growth in DNase agar with zone of clearance around the growth and was identified as *S. aureus*.

Among five CPS isolates three were *S. hyicus* and two were *S. pseudointermedius*. From the 60 isolates of CNS, 22 were *S. chromogens*, 14 were *S. xylosus* and 12 were *S. saprophyticus*. Six isolates each were *S. hominis* and *S. epidermidis*. This observation is in accordance with Nickerson *et al.* (2020) where different species of CNS was identified using API Staph test. From the remaining Gram positive cocci 32 isolates were identified as *Streptococcus* spp. and three isolates were identified as *Micrococcus* spp. From the Gram negative bacilli isolates three were *E. coli* and one was *Klebsiella* spp.

Out of the 114 total Gram positive cocci isolated, 60 were CNS (50.8 per cent), 19 were CPS (16.1 per cent), 32 were *Streptococcus* spp. (27.1 per cent), and three were *Micrococcus* spp. (2.5 per cent). Out of the four Gram negative bacilli isolates obtained three were *E. coli* (2.5 per cent) and one was *Klebsiella* spp. (0.8 per cent) (Fig. 1). The result obtained is in agreement with Nickerson *et al.* (2020) who reported that more than 70 per

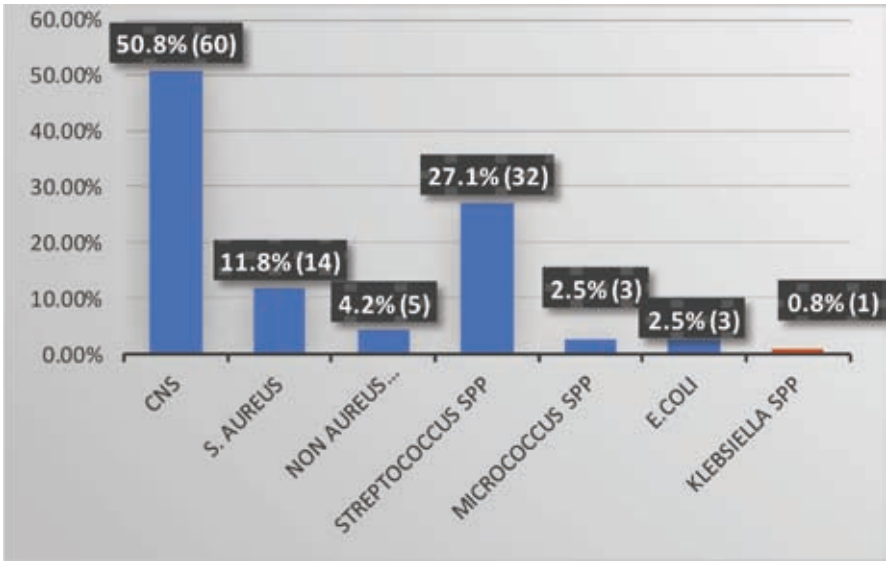

Fig. 1. Occurrence of bacterial pathogens in dry period cows

cent of the isolates obtained from dry period secretion were *Staphylococcus* spp. which mainly included *S. hyicus*, *S. chromogenes*, *S. aureus* and *S*. *xylosu*s. The low prevalence of coliforms in dry period secretions in the present study is in accordance with Gundelach *et al.* (2011) who isolated only 0.5 per cent of coliforms from dry period secretions. Low incidence of enterobacterial organisms was associated with increased presence of lactoferrin in the dry period secretions which inhibit the growth of enterobacterial organisms by reversibly binding to iron making it unavailable to the bacteria (Todhunter *et al.,* 1985). The observation made from this study was not in agreement with Green *et al*. (2002) who reported infection with *E*. *coli* being common during dry period.

` Primer targeting *16SrRNA* was employed for *Staphylococcus* spp. in the present study and all the 79 isolates were positive for this gene and showed an amplicon size of 759 bp (Fig. 2). These findings are in agreement with Amrithapriya (2019) who used the *16SrRNA* to identify *Staphylococcus* spp. in bovine mastitis milk. In the present study PCR targeting *nuc* gene was used for the identification of *S. aureus* and 14 phenotypically identified *S. aureus* isolates showed an amplicon size of 359 bp (Fig. 3). Multiplex PCR method used for the identification of CPS by targeting thermonuclease (*nuc*) gene was 99.8 per cent sensitive and 100 per cent specific*. S. aureus* produced an amplicon size of 359 bp and other CPS produced different amplicon sizes with primer pair which are specific for each species (Sasaki *et al.,* 2010). Sixty isolates which were identified as CNS showed the presence of *cns* gene with an amplicon size of 204 bp (Fig. 4). In the present study, phenotypically identified 32 isolates of *Streptococcus* spp. were confirmed as *St. agalactiae* by primer targeting the gene *16SrRNA* (Fig. 5).

Antibiogram of coagulase positive Staphylococcus isolates

Among the 19 CPS isolates, 12 isolates (63.2 per cent) were resistant to ceftriaxone tazobactam, nine isolates (47.3 per cent) were resistant to gentamicin, eight isolates (42.1 per cent) were resistant to enrofloxacin and seven isolates (36.8 per cent) were resistant to tetracycline. Resistance towards cefoperazone and ceftiofur was shown by five isolates each (26.3 per cent). Amoxicillin clavulanate was found to be the least resistant antibiotic with only two isolates (10.5 per cent) being resistant. Fourteen isolates among the 19 CPS were *S. aureus*, and these isolates showed highest resistance towards Ceftriaxone tazobactam with nine isolates (64.2 per cent) showing resistance and least resistance was shown towards amoxicillin clavulanate with two isolates (14.28 per cent) showing resistance. Similar observation was made by

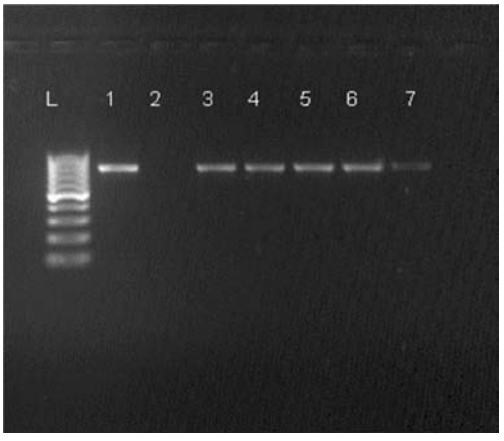

Fig. 2. Agarose gel electrophoresis showing *Staphylococcus* spp. with amplicon size 759 bp
Lane L- ladder
Lane 2- negative control
Lane 1, 3-7- positive control

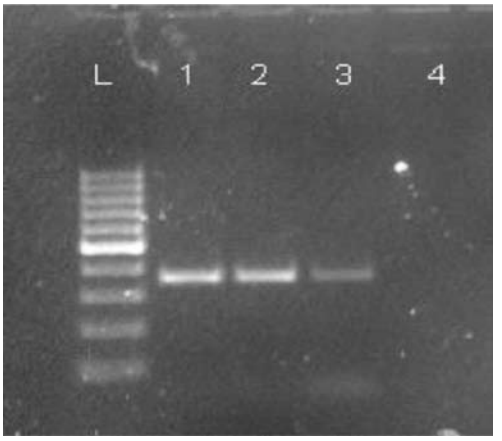

Fig. 3. Agarose gel electrophoresis showing *S. aureus* with amplicon size of 359 bp
Lane L- ladder
Lane 4- negative control
Lane 1-3- positive control

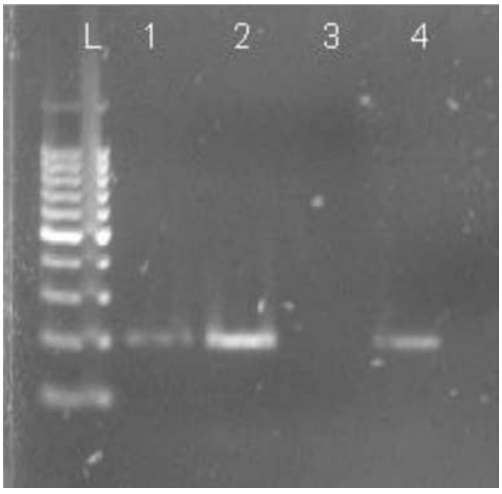

Fig. 4. Agarose gel electrophoresis showing CNS with amplicon size of 204 bp
Lane 3- negative control
Lane1,2 & 4- positive control

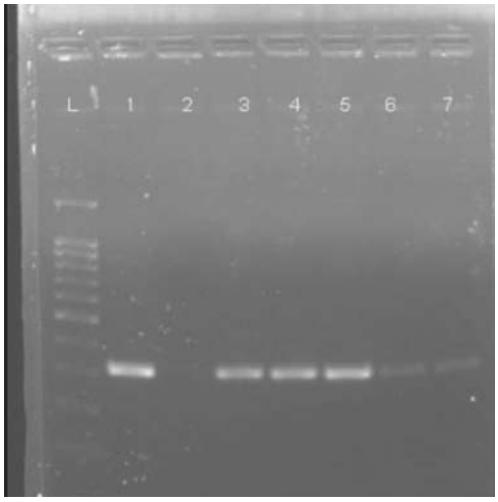

Fig. 5. Agarose gel electrophoresis showing *St. agalactiae* with amplicon size o 317 bp
Lane 2- negative control
Lane1,3-7 – positive control

Hamid *et al.* (2017) who reported that 50 per cent of *S. aureus* isolates showed resistance towards ceftriaxone. Study done by Jose *et al*. (2021) reported a higher sensitivity of *S. aureus* isolates from mastitis cases towards ceftriaxone which is not in accordance with the result obtained in the present study. Study done by Molineri *et al.* (2021) studied antimicrobial resistance of *S. aureus* isolates obtained from bovine mastitis and reported low incidence of antimicrobial resistance of the isolates towards ceftiofur. Study done by Beyene (2016) reported 92 per cent sensitivity of *S. aureus* isolates towards amoxicillin. The result obtained in the present study was not in agreement with Elias *et al*. (2020) who reported 47.06 per cent of the *S. aureus* isolates as resistant to ceftiofur and a higher sensitivity of *S. aureus* isolates was shown towards gentamicin. Antibiotic resistance in *S. aureus* strains has emerged as a result of the improper usage of antibiotics (Bhagya *et al*., 2021).

Antibiogram of coagulase negative Staphylococcus isolates

Among 60 CNS isolates obtained, highest number of isolates showed resistance towards tetracycline (45 per cent) followed by ceftriaxone tazobactam (43.3 per cent), gentamicin (38.3 per cent) and cefoperazone (31.6 per cent). Least resistance was shown towards amoxicillin clavulanate with only 11.6 per cent of isolates being resistant, 36.6 per cent isolates showed resistance towards ceftiofur. This result is in agreement with Kulangara *et al*. (2017) who reported higher rate of antimicrobial resistance (76.3 per cent) towards tetracycline and a high rate of sensitivity towards cefoperazone. Kaliwal *et al*. (2011) noticed a high susceptibility of CNS isolates towards ceftriaxone and least susceptibility towards gentamicin and amoxicillin clavulanate.

Antibiogram of Streptococcus agalactiae

Streptococcus agalactiae isolates in the present study showed least resistance to amoxicillin clavulanate (15.6 per cent) and highest resistance to enrofloxacin (53.1 per cent), followed by gentamicin (50 per cent), tetracycline (40.6 per cent), ceftriaxone and cefoperazone (37.5 per cent each) and ceftiofur (28.1 per cent). This result showed a similarity with work done by Kaczorek *et al.* (2017) were kanamycin, tetracycline and gentamicin were the least effective antibiotics. Study done by Krishnaveni *et al.* (2014) on antibiogram profile of *St. agalactiae* isolated from bovine mastitis cases reported a high rate of occurrence of resistance among isolates with 81 per cent resistance towards enrofloxacin and 88 per cent of isolates resistant towards ceftriaxone. This result obtained was not in accordance with Elias *et al.* (2020), who reported that only 13.5

per cent of the isolates showed susceptibility to amoxicillin. However, the proportion of the isolates showing resistance to ceftiofur is in agreement with the current study.

Antibiogram of Micrococci and other Gram negative isolates

Among the three *Micrococci* spp. isolates one isolate (33.3 per cent) showed resistance towards gentamicin, ceftriaxone tazobactam, ceftiofur and cefoperazone. Among the three *E. coli* isolates one (33.3 per cent) showed resistance towards tetracycline, ceftriaxone tazobactam and ceftiofur and two isolates (66.6 per cent) showed resistance towards gentamicin. *Klebsiella* spp. isolate showed resistance against gentamicin only.

Conclusion

The present study isolated and identified bacterial pathogens in the dry bovine udder. *St. agalactiae* (27.1 per cent) and *S. aureus* (11.8 per cent) were identified as the major contagious bacterial pathogens present during this period. A higher per cent of CNS (50.8 per cent) were also identified. The increased occurrence of these bacterial pathogens indicates the persistence of these bacteria in the bovine udder which could be transferred from the intramammary infections present during the earlier lactation. This may also result in increased occurrence of clinical mastitis in the subsequent lactation. The occurrence of Gram negative bacteria in the dry period secretion was extremely low. Coagulase negative Staphylococcus which reside on the surface of the teat get easy access to mammary glands during the dry period if teats are not closed. Phenotypic antibiogram studies revealed that antibiotic resistance among bacterial isolates are increasing and least antibiotic resistance of isolates was seen towards amoxicillin clavulanate. Our findings recommend proper hygienic and management practices in dairy farms along with dry cow therapy.

Conflict of interest

The authors declare that they have no conflict of interest

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