The Pharma Innovation Journal 2023; 12(3): 5164-5173

www.ThePharmaJournal.com

The Pharma Innovation

ISSN (E): 2277-7695
ISSN (P): 2349-8242
NAAS Rating: 5.23
TPI 2023; 12(3): 5164-5173

www.thepharmajournal.com
Received: 05-12-2022
Accepted: 13-01-2023

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Incidence of virulence associated factors of *Staphylococcus aureus* isolates in subclinical bovine mastitis

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Abstract
Staphylococcus aureus is a common causative agent of bovine subclinical mastitis and it has worldwide public health significance. Here, we aimed to determine the incidence of virulence factors of *Staphylococcus aureus* isolated from milk of subclinical bovine mastitis. Milk samples were collected from 402 lactating animals (242 dairy cattle and 160 buffaloes) from different farms located in Rewa district of Madhya Pradesh (India). The collected samples were investigated for subclinical mastitis using indirect screening tests. A total of 96 milk sample from 76 cattle and 20 buffaloes were positive for subclinical mastitis. The total incidence of *Staphylococcus aureus* was 28.13% with 28.95% in cattle and 25.00% in buffaloes. A total of 27 *Staphylococcus aureus* isolates obtained from bovine subclinical mastitis and positive to catalase, were analyzed for production virulence factors. Out of 27 isolates, all the *Staphylococcus aureus* isolates (100%) were found positive for mannitol fermentation, coagulase production, clumping factor test, gram staining, novobiocin susceptibility and polymyxin-B resistance. Out of 27 isolates, the Voges-proskauer test, alpha, beta and alpha-beta haemolysin production were observed in 85.19%, 29.62%, 55.55% and 14.81% isolates respectively. Further the isolates were subjected to evaluation of virulence determinant *spa* genes. Virulence genes of *Staphylococcus aureus* strains isolated from bovine milk and *spa* genes were found in 100.0% isolates indicating the emergence, spread and pathogenicity of *Staphylococcus aureus*. Thus, the high incidence of *Staphylococcus aureus* mastitis is an important concern for diary industry of Madhya Pradesh (India), since the strains of this pathogen are with number of virulence factors and this is a concern for both animal and public health.

Keywords: *Staphylococcus aureus,* subclinical mastitis, milk, virulence, incidence

Introduction
Cattle and buffalo are backbone of rural economy and mainly reared for milk production. Bovine mastitis is a major disease that affects dairy industry and causes major economic loss worldwide. *Staphylococcus aureus* (*S. aureus*) is a common facultative pathogen that has long been conceded as a challenge in both human and veterinary medicine (Nemeghaire *et al.*, 2014) [1]. It is responsible for major sources of clinical and subclinical mastitis infection in dairy animals (Bradley *et al.*, 2007;Botrel *et al.*, 2010) [2, 3] leading to reduction in the milk production, alteration in the composition and quality of the produced milk, the need to discard the produced milk, early culling of infected animals, and high cost of treatment and control (Hussain *et al.*, 2012; Abdel-moein and Zaher, 2019; Martins *et al.*, 2019) [4, 5, 6].
S. aureus produces a variety of extracellular and cell wall associated virulence factors, which involved in survival of bacteria in the udder and pathogenesis of bovine mastitis, leading to chronic intra-mammary inflammation (Momtaz *et al.*, 2010; Bien *et al.*, 2011) [7, 8]. Virulence associated factors like, surface proteins that promote colonization of host tissues, invasions that promote bacterial spread in tissues (Hyaluronidase, Kinases, Leukocidin), surface factors that inhibit phagocytic engulfment (Protein A, Capsule), biochemical properties that enhance their survival in phagocytes (Catalase production), immunological disguises (Coagulase, Clotting factor, Protein A), inherent and acquired resistance to antimicrobial agents and membrane damaging toxins like haemolysins that lyse eukaryotic cell membranes (Panizzi *et al.*, 2004;Mubarack *et al.*, 2012) [9, 10].
S. aureus can be identified by traditional methods but it has been noted that this organism shows variations in phenotypic expressions (Ariyanti *et al.*, 2011) [11].

In such situations, molecular typing approaches have been reported to be of great advantageous in identifying and monitoring the spread of *S. aureus* strains (Diep *et al.*, 2003) [12]. Production of coagulase /coagulase /haemolysin are an important phenotypic feature used worldwide for the identification of *S. aureus* (Sanjiv *et al.*, 2008) [13]. Protein-A of *S. aureus* encoded by *spa* gene is considered as one of the important virulence factors in the severity and expansion of mastitis (Akinden *et al.*, 2001) [14]. The present study was carried out for the incidence of virulence associated factors of *S. aureus viz.* gram's staining, coagulase test, catalase test, voges-proskauer test, clumping factor test, mannitol fermentation, haemolysin properties, and further the detection of virulence associated *spa* genes in *S. aureus* isolates.

2. Materials and Methods

2.1 Detection of subclinical mastitis

Milk samples from suspected cases of subclinical mastitis in cattle and buffaloes from different places of Rewa district of Madhya Pradesh (India) were collected aseptically in sterilized vials. A total of 402 milk samples comprising of 242 cattle and 160 buffaloes were first processed for detection of subclinical mastitis by indirect screening tests *viz.* (Constable *et al.*, 2019) [15], Electrical Resistance (Siddique *et al.*, 2013; Galfi *et al.*, 2015) [16, 17], Somatic Cell Count (Schalm *et al.*, 1971; Harmon, 2001) [18, 19] and modified California Mastitis Test (David *et al.*, 2005) [20]. A total of 96 milk samples including 76 cattle and 20 buffaloes were positive for subclinical mastitis. Isolation and Identification of *S. aureus* was done as per the methods described by Buchanan and Gibson, 1974 [21], Cowan and Steel, 1974 [22] and Markey *et al.*, 2013 [23].

2.2 Virulence associated factors of *S. aureus*

2.2.1 Gram's staining

The isolates of *S. aureus*, were stained by gram stain to detect their response to stain, morphology and cellular arrangement. The staphylococci were seen as purple/blue coloured gram-positive cocci arranged singly, in pairs or in grape like bunches (Benson, 2002; Tille, 2014) [24, 25].

2.2.2 Coagulase Test

Citrated rabbit plasma diluted 1:5 was mixed with an equal volume of BHI broth culture then incubated at 37 °C. A tube of plasma mixed with sterile a broth is included as a control. For mating clots in 1-4 hr. indicates that the test is positive one (Forbes *et al.*, 2007) [26].

2.2.3 Catalase test

The isolates of *S. aureus* were transferred on the surface of a clean, dry glass slide. A drop of catalase reagent (3.00% H_2O_2) was added over the growth on the glass slide and positive results were indicated on copious amount of air bubbles formation (Harley and Prescott, 2007; Tille, 2014 [27, 25].

2.2.4 Haemolysin in Sheep blood agar

Haemolytic activity of the *S. aureus*, was detected on 5.00% sheep blood agar. The isolates were streaked on the medium and incubated aerobically overnight at 37 ^{0}C for 24 h. Results were interpreted after keeping all the plates at 4 ^{0}C for 1 to 2 h. Isolates showing haemolytic zones were taken as positive. Interpretation was made as per Markey *et al.* (2013) [23].

2.2.5 Mannitol fermentation test

The isolates of *S. aureus* were inoculated on the mannitol salt agar and incubated at 37 ºC for 48 h. The red to yellow coloration of colony along with media considered as mannitol fermenters (Markey *et al.*, 2013) [23].

2.2.6 Clumping factor test

A drop of physiological saline was placed on each end of a slide, or on two separate clean grease free slide. The small amount of isolates of *S. aureus* was picked and mixed with the saline in each half of the slide with the help of sterile loop to make two thick suspensions. A drop of rabbit plasma was added to one of the suspension and mixed gently. The sample showing clumping of the bacteria within 10 seconds was treated as positive (Tille, 2014) [25].

2.2.7 Voges-Proskauer test

The isolates of *S. aureus*, were inoculated into the voges-proskauer medium containing tube and incubated at 35 °C for 48 hours. The tube was mixed well after addition of 6 drops of 5.00% alpha -naphthol, and 2 drops of 40% potassium hydroxide. A pink-red color at the surface within 30 minutes was observed as positive reaction (Barritt *et al.*, 1936) [28].

2.2.8 Detection of virulence *spa* gene

Isolation of genomic DNA from the *S. aureus* isolated colony by Instagene Matrix (Bio-Rad) and Polymerase Chain Reaction (PCR) was carried out for the detection of Staphyloccoal Protein A (*spa)* gene (Stegger *et al.*, 2013) [29]. The genus level identification of *S. aureus* strains was performed by tracking the presence of the *spa* gene through PCR technique. The pair of primers *spa* F- TAA AGA CGA TCC TTC GGT GAG C (22-mer) and *spa* R- CAG CAG TAG TGC CGT TTG CTT (21-mer) were used and the PCR conditions was standardized to amplify the *spa* gene.

The PCR-amplified samples were analyzed by agarose gel electrophoresis by using a horizontal 2.00% (w/v) agarose gel in 1X TBE buffer (pH 8.3, 0.09 M Tris, 0.09 M boric acid, 2.0 mM EDTA) and with 0.003% (w/v) ethidium bromide incorporated for DNA staining. 8 µl of the PCR sample was loaded directly into the wells. Gels were run in 1 x TBE buffer at 80 V for 2 h. The PCR products were visualized and photographed on a Gel documentation unit (E-gel, Life Technologies). The 100 base pair ladder molecular weight marker was run in parallel with the samples. It is the method of identification of *S. aureus* and its important virulence determinant *spa* gene.

2.3 Statistical analysis

The data collected through questionnaire were entered into an excel sheet of Microsoft Office Excel and subjected to analysis by SPSS version 16.0. All the relevant data were labelled into sub heading and analyzed using descriptive statistics.

3. Results and Discussion

Mastitis is the most fearsome infectious disease affecting dairy animals and remains as a constant challenge in the dairy industry, which is often difficult to cure and is prone to resurgence. Successful management, prevention and treatment of bovine mastitis are great inevitable task for the dairy holders. *S. aureus* mastitis are highly variable and strain dependent features. The incidence of this pathogen is an

increasingly recognized and most frequently isolated etiology of bovine mastitis in most of the countries. Most of the dairy animal researchers consider this organism as the true mastitis pathogens with important virulence factors and the ability to cause chronic infections.

3.1 Milk profile of healthy and subclinical mastitis infected bovine

In the present study, bovine milk were screened for subclinical mastitis by milk pH, ER, SCC and mCMT. The mean value of milk pH, ER and SCC was 6.65±0.06, 370.00±18.26, $1.33\pm0.11\times10^5$ cells/ml, respectively in apparently healthy bovine, whereas the corresponding values significantly ($p<0.05$) changed to 7.57±0.04, 296.67±10.22 and $33.23\pm7.06\times10^5$ cells/ml in subclinical mastitis infected bovine (Table 1). These findings are close in accordance with the results of Ogola *et al.* (2007) [30], Elango *et al.* (2010) [31], Galfi *et al.* (2015) [17], Panchal *et al.* (2016) [32], Abdelazeem *et al.* (2020) [33] and Singh *et al.* (2021) [34].

3.2 Incidence of *S. aureus* in subclinical bovine mastitis

The present study was conducted on a total of 402 lactating animals (242 cattle and 160 buffaloes), out of these 96 lactating animals (76 cattle and 20 buffaloes) were positive for subclinical mastitis. A total of 96 subclinical mastitis positive bovine milk, 27 isolates were positive for *S. aureus.* Overall incidence of *S. aureus* in subclinical mastitis was found to be 28.13%. Considering species wise incidence, 25.00% buffaloes, whereas 28.95% cattle were detected positive for *S. aureus* (Table 2).

Purohit (1990) [35], Goswami (1998) [36], Hoque *et al.* (2018) [37], Maalik *et al.* (2019) [38] and Abdelazeem *et al.* (2020) [33] who found the incidence of *S. aureus* in subclinical mastitis to be 21.64, 51.29%, 37.5% 42.2% and 35.9%, respectively. It has also been reported that incidence of *S. aureus* in subclinical mastitis were higher in cattle in comparison to buffaloes (Kumar *et al.,* 2010;Sharma *et al.,* 2015;Maalik *et al.,* 2019) [39, 40, 38]. It clearly indicated the presence of *S. aureus* as most prevailing pathogen in cases of mastitis in dairy animals (Patel *et al.,* 2012;Awandkar *et al.,* 2013;Mohanty *et al.,* 2013;Charaya *et al.,* 2014;Patnaik *et al.,* 2014;Chandrasekaran *et al.,* 2015;Jena *et al.,* 2015) [41, 42, 43, 44, 45, 46, 47]. Being commensal to skin *S. aureus* are supposed to be first and foremost bacteria to enter in teat canal. However, the incidences can be reduced by maintaining proper hygienic conditions and pre and post milking sanitation of udder and its surroundings.

Similar to the present findings, Mengistie (2003) [48], Kivaria *et al.* (2005) [49], Ranjan *et al.* (2011) [50] and Jahan *et al.* (2015) [51] reported the incidence of staphylococcal mastitis to be 27.10%, 21.00%, 27.37% and 25.53%, respectively in cows. However, higher incidence of staphylococcal mastitis was reported by Wani and Bhatt (2003) [52], Thennarrasu *et al.* (2003) [53], Ghaleb *et al.* (2005) [54], Begum *et al.* (2007) [55], Patel (2007) [56], Tsegmed *et al,* (2007) [57], Morandi *et al.* (2009) [58], Gundogan and Avci (2014) [59], Abd El-Tawab *et al.* (2017) [60] and Wang *et al.* (2018) [61] who reported 45.00%, 47.06%, 68.30%, 75.00% 44.00%, 74.00%, 78.00% 56.00%, 80.00% and 46.2%, respectively in cows.

The higher incidence of *S. aureus* has also been reported by several workers in India (Tuteja, 1999;Kaya *et al.,* 2000;Sharma *et al.,* 2007;Yadav *et al.,* 2018) [62.63, 64, 65] and abroad (Hawari and Dabas, 2008;Tenhagen *et al.,* 2009; Nickerson, 2009; Zutic *et al.,* 2012;Cavicchioli *et al.,* 2015; Li *et al.,* 2015; Giacinti *et al.,* 2017) [66, 67, 68, 69, 70, 71, 72]. While lower incidence of *S. aureus* mastitis have also been reported by Donkor *et al.* (2007) [73] and Abd El-Tawab *et al.* (2017) [60] with 14.60% and 17.5%, respectively in raw milk. This difference in the incidence of pathogens is influenced by age, parity, lactation stage, milk yield, season, breed, species and place (Sharma *et al.,* 2007;Singh *et al.,* 2021) [64, 34]. Distribution of pathogens in mastitis changes over time, therefore, bacteriological examination at herd level must be taken regularly to monitor udder health.

3.3 Gram staining of the *S. aureus* isolates

The Gram stain is a very important preliminary step in the initial identification, characterization and classification of micro-organism. It is also a key procedure in the identification of micro-organism based on staining characteristics, enabling the micro-organism to be examined using a light microscope. Although the Gram staining is used for detection and differentiation of bacteria, other microorganisms, most frequently yeasts and fungi, can be seen on a Gram-stained smear (Rand and Tillan, 2006) [74]. Yeasts can appear Gram-positive or Gram-negative. Yeasts are generally at least 10-20 times the size of bacteria and may appear as single cells that may have buds, hence differentiation from bacteria is not a problem (Thairu *et al.,* 2014) [75].

In Gram staining, the organism revealed as gram positive cocci and arranged in grapes like cluster with violet colored, under light microscope. In the present study, 100% isolates were positive for gram staining (Table 3). This results are in consonance with Harmon *et al.* (1991) [76], Ashraf *et al.* (2015) [77], Jahan *et al.* (2015) [51], Abd El-Tawab *et al.* (2017) [60], Lucia *et al.* (2017) [78] and Shrivastava *et al.* (2017) [79] who reported that all *S. aureus* isolates were gram positive cocci arranged in cluster.

3.4 Coagulase test of the *S. aureus* isolates

Coagulase is a conventional marker for identifying *S. aureus* in the clinical microbiological laboratory. It is a protein produced by several micro-organisms that enables the conversion of fibrinogen to fibrin and they could protect themselves from host defenses by causing localized clotting. More importantly, *S. aureus* is normally coagulase-positive, meaning that a positive test would indicate the presence of *S. aureus.* In the present study, all isolates of *S. aureus* were subjected to tube coagulase test using rabbit plasma. The presence of 100% coagulase positive isolates in present study further suggests the increase in the number of pathogenic *S. aureus* in dairy animals (Table 3).

Present finding are in accordance with Pandya (1991) [80], Patel (2007) [56], Morandi *et al.* (2009) [58], Jahan *et al.* (2015) [51], Sharma *et al.* (2015) [40] and Shrivastava *et al.* (2017) [79] who reported all (100%) the *S. aureus* isolates to be positive for tube coagulase test. Our findings are also more closely related to Turutoglu *et al.* (2005) [81], Makwana *et al.* (2012) [82] and Parth *et al.* (2016) [83] who reported about 89.77%, 94.00% and 92.45%, respectively *S. aureus* isolates positive for tube coagulase test with subclinical mastitis.

The contrary findings by Kato and Kume (1980) [84], Wani and Bhatt (2003) [52], Boerlin *et al.* (2003) [85], Abd El-Tawab *et al.* (2017) [60], Pumipuntu *et al.* (2019) [86] and Abdeen *et al.* (2021) [87] who reported about 34.50%, 51.11%, 50.00%, 30.3%, 13.97% and 56.91%, respectively isolate of *S. aureus*

being coagulase-positive. Similarly, lower coagulase activity of *S. aureus* ranging from 31.30 to 53.33% from mastitis milk was also reported by Pankaj *et al.* (2013) [88], Ai-Jumaily *et al.* (2014) [89] and Patnaik *et al.* (2014) [45].

3.5 Catalase test of the *S. aureus* isolates

S. aureus is a gram positive, coagulase and catalase positive cocci and by far the most important pathogen among the staphylococci. It produces enzymes such as catalase which are considered to be virulence determinants and this enzyme allows bacteria to better resist intra and extra-cellular killing by hydrogen peroxide (Gruner *et al.*, 2007) [90]. In the present study, 100% *S. aureus* isolates were found positive for catalase test (Table 3). Our findings are in very close agreement with report of Jahan *et al.* (2015) [51] who found that 100% isolates were positive for catalase test. These findings are on par with earlier reports on isolation and characterization of *S. aureus* from clinical cases of mastitis (Carter *et al*, 1990;Kateete *et al*, 2010) [91, 92]. The results of present study is more or less similar to the findings of Ashraf *et al.* (2015)[77], Ankita (2015) [93], Abd El-Tawab *et al.* (2017) [60] and Yadav *et al.* (2018) [65], who reported about 80.00%, 90.66%, 80.00% and 91.12%, repectively *S. aureus* isolates positive for catalase test.

Although it is well known that nearly all strains of *S. aureus* have catalase activity. Catalase is a heme protein enzyme that degrades hydrogen peroxide produced by phagocytes. The catalase production does not appear to be essential for the growth of *S. aureus* in *vitro* and *in vivo* (Mandell *et al.*, 1975; Bertrand *et al.*, 2002) [94, 95] but it is a defense mechanism against destruction of the micro-organism in phagocytic cells (Kloos and Bannerman, 1999) [96]. On the other hand, there is good correlation between staphylococcal catalase activity and its lethality (Mandell *et al.*, 1975) [94].

3.6 Hemolysis patterns of the *S. aureus* isolates

Out of total 27 isolates, percentage of isolates showing alpha (α), beta (β) and alpha-beta haemolysin production were 29.62%, 55.55% and 14.81% respectively on sheep blood agar (Table 3). Our findings are very close in agreement with reports of Shrivastava *et al.* (2017) [79] with 21.42% isolates showed alpha, 57.14% beta and 24.56% both alpha and beta haemolysin, respectively in sheep blood agar. The present study is more or less similar to the findings of Pandya (1991) [80], Patel (2008) [97], Morandi *et al.* (2009) [58], and Dittmann *et al.* (2017) [98] who reported 11.71%, 27.50%, 6.00% and 19.70% alpha haemolysin, 54.68%, 48.75%, 54.00% and 43.94% beta haemolysin and 19.53%, 11.25%, 40.00% and 34.85% alpha-beta haemolysin, respectively.

In the present study, beta haemolysin (55.55%) producing *S. aureus* were found predominant in the subclinical mastitis. Which support the views of Aarestrup *et al.* (1999) [99], Larsen *et al.* (2002) [100] and Yadav *et al.* (2018) [65] opined that beta haemolysin production is a characteristic of animal strains of *S. aureus*. In contrast to these, Bhanderi (2007) [101] found that 62.79% alpha hemolytic *S. aureus* of animal origin, Stephan *et al.* (2001) [102] who found double (alpha-beta) hemolysin in 67.65% *S. aureus* isolated from cow milk samples and Morandi *et al.* (2009) [58], reported most of the *S. aureus* strains derived from goat dairy products (64.00%) showed double hemolysis.

3.7 Mannitol fermentation of the *S. aureus* isolates

Mannitol salt agar is frequently used as a selective and differential media for the isolation and identification of *S. aureus* from different clinic-pathological samples. It stimulate the growth of a group of certain micro-organism, while inhibiting the growth of others. Most pathogenic staphylococci, such as *S. aureus*, will ferment mannitol. While non-pathogenic staphylococci will not ferment mannitol. Thus, it is a species signature of *S. aureus*, and discriminates it from most other members of the genus (Kenny *et al.*, 2013) [103].

In the present study, 100% isolates of *S. aureus* were able to ferment mannitol (Table 3). Our findings rare very close in agreement with Makwana *et al.* (2012) [82], Jahan *et al.* (2015) [51] and Shrivastava *et al.* (2017) [79] who observed 100% mannitol fermentation by *S. aureus* and production of small yellow colonies. While Bhanderi (2007) [101], Abd El-Tawab *et al.* (2017) [60] and Yadav *et al.* (2018) [65] reported about 74.41% 67.80% and 94.12% *S. aureus* isolates produced mannitol fermentation, respectively. Quinn *et al.* (1994) [104] who reported that mannitol salt agar and Baired Parker medium were used and specifically in food microbiology.

3.8 Voges-proskauer test of the *S. aureus* isolates

In the present study, 85.19% isolates of *S. aureus* were found positive for voges-proskauer (Table 3). Our finding are very close in agreement with Abd El-Tawab *et al.* (2017) [60] and Yadav *et al.* (2018) [65] reported 73.8% and 85.30% *S. aureus* isolates positive for Voges-proskauer test. The test of acetoin production as a tool to differentiate the coagulase-positive *staphylococcu*s species. Voges-proskauer test (Acetoin test) be used as an additional means to differentiate *S. aureus* from coagulase-positive *S. hyicus* and S. *intennedius*. *S. intennedius* and *S. hyicus* do not produce acetoin (Schleifer, 1986) [105], while the majority of *S. aureus* strains do (Harmon *et al.*, 1991) [76].

3.9 Clumping factor test of the *S. aureus* isolates

S. aureus produces bound and free forms of coagulase, the bound forms coagulase, otherwise known as "clumping factor", can be detected by carrying out a slide coagulase test/ clumping factor test. It provides a simple and widely used test to identify *S. aureus* and distinguish it from the less virulent 'coagulase-negative' staphylococci (Ryan and Ray, 2004) [106]. In the present study, about 100% isolates of *S. aureus* were positive for clumping factor test (Table 3). Our findings are in close agreement with Yadav *et al.* (2018) [65] who reported 79.41% isolates positive in slide coagulase test. Coagulase is firmly bound to the surface of the micro-organism *S. aureus* and can cover its surface with fibrin upon contact with blood. The fibrin clot may protect the micro-organism from phagocytosis and isolate it from other protective defenses of the host. The fibrin coat can therefore make the bacteria more virulent (Tortora *et al.*, 2013) [107].

3.10 Novobiocin susceptibility and Polymyxin B resistant of the *S. aureus* isolates

The antibiotic-susceptibility profile of these antibiotics was prepared using the Kirby-Bauer disk diffusion method (Bauer *et al.*, 1966) [108]. In the present study, the interpretation to the susceptibility of organisms was observed by measuring zone

of inhibition. All (100%) the isolates (Table 3) were susceptible to Novobiocin (zone diameter ≥ 17 mm) and resistant to Polymyxin B (zone diameter ≤ 10 mm). Our finding are very close in agreement with Shrivastava *et al.* (2017) [79]. Novobiocin has higher efficacy against the gram-positive bacteria (most gram-negative bacteria are resistant), especially *S. aureus*. Novobiocin is used occasionally as an alternative to penicillins against penicillin-resistant *Staphylococcus* spp (Raad *et al.*, 1995; Walsh *et al.*, 1993 [109, 110], whereas Polymyxin B is considered a gram-negative antibiotic that does not diffuse well in medium, and resistance to this antibiotic is characteristic of *S. aureus* (Chuang *et al.*, 2012; Kowalski *et al.*, 2013) [111, 112].

3.11 Virulence determinant *spa* genes of the *S. aureus* isolates

The development and severity of mastitis depend on the production of virulent protein known as protein-A (Mitra *et al.*, 2013) [113]. The virulence-associated protein-A of *S. aureus*, encoded by *spa* gene, is one of the important virulence factors involved in the staphylococcal pathogenesis and severity of mastitis (Sharma *et al.*, 2000;Bhikane and Kawitkar, 2020) [114, 115]. Protein A is surface protein of *S. aureus* that binds to the IgG molecules by their Fc portion and interfere with opsonization and subsequent phagocytosis of bacteria and thus contributes to the development of the disease (Koreen, 2004) [116].

The present study was directed mainly to recognize virulence *spa* genes by molecular biological techniques (PCR) that may play a role in virulence of *S. aureus.* In this study, all the 27 isolates were positive (100%) for the *spa* gene. Our study are closely to the findings of Khan *et al.* (2013) [117], Marques *et al.* (2013) [118], Abdelazeem *et al.* (2020) [33] and Gomez *et al.* (2020) [119] who reported 98.50%, 100.00%, 86.6% and 80.00% incidence of *spa* gene in their study. Whereas, contrary result by Shakeri *et al.* (2010) [120], Suleiman *et al.* (2012) [121], Parth *et al.* (2016) [83] and Li *et al.* (2018) [122] have been reported for lesser incidence of *spa* types of gene in *S. aureus* isolates.

The results of present study indicate some virulent factors of *S. aureus* occur frequently in particular dairy herds. The presence of particular *S. aureus* genotypes in different herds could be due to increased resistance against the host's immune response (Moon *et al.*, 2007) [123]. The results of the current study indicated that some virulent factors of *S. aureus* genotypes were frequently observed in those herds that prevailed close to each other in particular area. The possible reason about the presence of these isolates could be linked to the poor management practices adopted by the farmers, existence of common pathogens in the same area, feeding and grazing of healthy and infected animals together and trading of animals among the herds.

Table 1: Milk profile of healthy and subclinical bovine mastitis

Testing of milk samples	Healthy bovine (n=10)	Subclinical bovine mastitis (n=96)
pH	6.65 ± 0.06^{a}	7.57 ± 0.04^{b}
ER	370.00 ± 18.26^{a}	296.67 ± 10.22^{b}
SCC (10^5cells/ml)	1.33 ± 0.11^{a}	33.23 ± 7.06^{b}
mCMT score	Negative	+/++/+++

[a, b]Values bearing different superscript differ significantly in row ($p<0.05$).

Table 2: Incidence of *S. aureus* in subclinical bovine mastitis

Species	Total no. of SCM positive bovine	No. of *S. aureus* positive isolate	Incidence *S. aureus* in SCM
Cattle	76	22	28.95%
Buffaloes	20	5	25.00%
Overall	96	27	28.13%

Table 3: Virulence factors of *S. aureus* isolates from subclinical bovine mastitis

Particular	Total no. of *S. aureus* positive isolate	No. of isolate positive for the test	Incidence
Gram staining	27	27	100%
Coagulase test	27	27	100%
Catalase test	27	27	100%
α Haemolysis	27	8	29.62%
β Haemolysis	27	15	55.55%
α and β haemolysis	27	4	14.81%
Mannitol fermentation	27	27	100%
Voges-proskauer test	27	23	85.19%
Clumping factor test	27	27	100%
Novobiocin susceptibility	27	27	100%
Polymyxin-B resistance	27	27	100%
spa gene	27	27	100%

4. Conclusion

It can be concluded from the results of the present study that *S. aureus* isolates obtained from bovine mastitic milk showed the high incidence of virulence factor and an important role of virulence factors in the pathogenesis of bovine mastitis. The presence of more virulence factors could increase the pathogenic ability of isolates. The findings of this present study warrants the need for strategic approach including dairy extension that focus on enhancing dairy farmers' awareness, practice of hygienic milking, regular screening for subclinical mastitis, dry cow therapy and culling of chronically infected milch animals.

5. Acknowledgement
The authors are thankful to the Dean, College of Veterinary & Animal Sciences, Rewa, Nanaji Deshmukh Veterinary Science University, Madhya Pradesh, India for rendering necessary facilities for successful completion of the research work.

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