The Pharma Innovation Journal 2022; 11(8): 46-53

www.ThePharmaJournal.com

The Pharma Innovation

ISSN (E): 2277-7695
ISSN (P): 2349-8242
NAAS Rating: 5.23
TPI 2022; 11(8): 46-53

www.thepharmajournal.com
Received: 13-05-2022
Accepted: 29-07-2022

M Latha Mala Priyadharshini
Vaccine Research Centre-Bacterial Vaccine, Centre for Animal Health Studies (CAHS), Tamil Nadu Veterinary and Animal Sciences University, TANUVAS, Chennai, Tamil Nadu, India

S Manoharan
Vaccine Research Centre-Bacterial Vaccine, Centre for Animal Health Studies (CAHS), Tamil Nadu Veterinary and Animal Sciences University, TANUVAS, Chennai, Tamil Nadu, India

K Viswanathan
Translational Research Platform for Veterinary Biologicals, Centre for Animal Health Studies (CAHS), Tamil Nadu Veterinary and Animal Sciences University, TANUVAS, Chennai, Tamil Nadu, India

K Porteen
Department of Veterinary Public Health and Epidemiology, Madras Veterinary College, Chennai, Tamil Nadu, India

B Samuel Masilamoni Ronald
Department of Veterinary Microbiology, Madras Veterinary College, Chennai, Tamil Nadu, India

G Dhinakar Raj
Department of Animal Biotechnology, Madras Veterinary College, Chennai, Tamil Nadu, India

Corresponding Author:
S Manoharan
Vaccine Research Centre-Bacterial Vaccine, Centre for Animal Health Studies (CAHS), Tamil Nadu Veterinary and Animal Sciences University, TANUVAS, Chennai, Tamil Nadu, India

Molecular detection of bio-film formation property of *Staphylococcus* spp. of bovine mastitis origin

M Latha Mala Priyadharshini, S Manoharan, K Viswanathan, K Porteen, B Samuel Masilamoni Ronald and G Dhinakar Raj

Abstract
In this report, the biofilm forming character of the *Staphylococcus* spp isolates were characterized based on bacteriological and molecular techniques. For this study, initially milk samples were collected from lactating cows, both apparently normal and clinically affected by mastitis. Totally 110 mastitis milk samples was collected and cultured in Tryptic Soy Broth. Among this 93 samples (84.5%) showed turbidity and we found only 76 (81.7%) samples produced viable pathogens by isolation. Based on cultural morphology many types of bacterial isolates were obtained like *Micrococcus* spp (12); *Escherichia coli* (3), Klebsiella spp (2), Pseudomonas spp (5) and Bacillus spp. (7). So other viable bacterial species accounted for was 29 (26.4%) and along with *Staphylococcus* spp viable bacterial isolates was 110. The *Staphylococcus* spp. isolates obtained were 81 (87.1%) from 93 samples. Out of this 81 isolates 69 (85.2%) were from sub-clinical milk and remaining (14.8%) from clinical mastitis milk. The Antibiotic susceptibility test (AST) was done using 12 antibiotics for all isolates along with PCR typing of the bio-film forming genes in iCAD *operon*. The result showed that specific bio-film forming genes was present in *Staphylococcus* spp. and if they got amplified for at least 2 out of the 4 genes screened for the iCAD operon it had a positive correlation by exhibiting drug resistance to more than two antibiotics. Based on the amplifications of biofilm genes in *Staphylococcus* spp. 10 were positive for *ica-A* gene, 25 for *ICA-D* gene, 30 for *ica-D* gene and only 2 for *ICA-A* gene. The study results concluded that, the presence of biofilm gene indicates the tendency for increased resistance to antibiotics that are used in regular mastitis treatment and thereby cause recurrence.

Keywords: Bovine mastitis, AST, PCR, Bio-film, iCAD ADBC operon, *Staphylococcus* spp.

Introduction
India is one of the important dairying countries of the world. Livestock sector provides employment to many accounting for the major source of income for an estimated 27.6 million people and nearly 70 per cent of them are women [1]. The productivity of Indian milch animals is only about 5 to 8 liters of milk per day/animal which is relatively less [2]. Over the past 125 years, mastitis has been one of the most studied devastating disease conditions in terms of economic losses, occurring throughout the world [3]. Calculations of economic losses resulting from mastitis vary among countries. Therefore mastitis is a scourge to the dairy industry and a nation's economy. Mastitis is a multi-factorial multi-etiological inflammatory condition of the udder, characterized by physical, chemical and microbiological changes in the milk and pathological changes in the glandular tissues of the udder [4]. Besides health disorders of the mammary gland, significant losses in milk yield, alterations in its quality both nutritive and organoleptic properties, mastitis causes fertility disorders and even systemic diseases [5]. Moreover, causative agents of mastitis with zoonotic potential may represent a health risk for human populations via the food chain [6].
Mastitis in milch animals is initially triggered as a sporadic or opportunistic infection, but depending on the causative agent, its persistence and prevalence exists in a herd. Many pathogens are well equipped with an arsenal of virulence factors and strategies that aid them in establishing an ascending infection in the udder. The two most important forms are contagious mastitis caused by *Staphylococcus* spp and Streptococcus spp as a sub-clinical infection gradually progressing to a chronic form and *Escherichia coli*, Klebsiella spp. Pseudomonas spp. etc., which are opportunistic pathogens giving rise to clinical condition.
S. aureus has been designated as a causative agent of both clinical [7] and subclinical [5] mastitis [8] initiated research on biofilms in1978, since it is related to pathogenicity and it has been proposed that *Staphylococcus* bio-films are major causes of recurrent and chronic mastitis in dairy cattle [9].

Staphylococcus spp. bio-film formation mechanisms are complex and include the participation of many kinds of proteins expressed by many genes. One among them is the *icaADBC* operon which encodes enzymes involved in the synthesis of PIA [10]. Research is focusing on the mechanisms of *Staphylococcus* biofilm formation and most have used standard strains instead of clinical isolates.

In India, research on bio-films is limited and is focused on *Staphylococcus* spp. from clinical samples of humans. The aim of the present study was to test the bio-film-forming character and analyze the distribution of bio-film-associated genes in the *Staphylococcus* spp. isolates causing bovine mastitis.

Materials and Methods

Ethical Statement / Ethical approval

Ethical approval is not required for this type of work, wherein no invasive procedure is applied. But the milk samples were collected as per standard procedures without harming the animals.

All the 110 isolates used in this study were recovered from milk samples taken from cows presenting sub-clinical and clinical mastitis between October 2019 and March 2020 from different farms and private backyard holdings in and around Chennai and Tiruvallur districts of Tamil Nadu.

Sample Collection

The samples were taken shortly before to milking. Both apparently normal and cows exhibiting clinical signs of mastitis were sampled. Prior to taking the samples, the udder was washed and the teats were disinfected with cotton soaked in 70% ethanol. Three mid-stream milk jets from each teat were collected and stored in sterile vials. The samples were properly marked and stored in a thermos with ice packs (4 °C to 8 °C) during transport to the microbiology laboratory for further processing. The bacteria were identified and preserved in our laboratory as glycerol stocks at -70 °C till further use.

Isolation and Bacterial Identification

The cultivation of the samples for isolation was performed by inoculation in Nutrient broth or Tryptic Soy Broth and incubating at 37 °C @ 170 rpm for 16-18 hours. All the turbid broth samples were streaked onto Nutrient agar plates. Gram's staining was done for colonies showing different morphological features in the nutrient agar. Based on the microscopic picture showing a mixed staining, it was sub-cultured onto Mannitol Salt agar, China Blue Lactose agar Baird Parker Agar, MacConkey agar, Eosin Methylene Blue agar, and Sabouraud agar. Direct streaking of milk was done on Blood agar supplemented with 5% sheep blood. All the plates were incubated at 37 °C for 24 hours, except for Sabouraud agar (incubated at both 25 °C and 37 °C) which was cultivated for one week with the first evaluation after 48 h of growth. Prevalence of putative *S. aureus* in individual cow milk samples with distinct morphological colonies suggestive of the species were further examined and distinguished as coagulase positive (CoPS) and negative Staphylococci (CoNS) by the coagulase test. The microorganisms were identified according to the morphological and staining characteristics described by Markey *et al*. 2013 [15].

Biochemical Tests for Identification of *Staphylococcus* spp isolates

After incubation, the colonies were evaluated according to the morphological characteristics and hemolysis patterns. Gram-staining and Catalase tests were performed, the latter separates the likely *Staphylococcus* and *Micrococcus* strains (catalase-positive) from *Streptococcus* and *Enterococcus* strains (catalase-negative). The isolates that were Gram positive and Catalase test positive was grown on Mannitol salt agar. The Catalase negative isolates underwent the Bile esculin test, Urease utilization and tolerance to NaCl 6.5% to characterize the species. [19]. For isolates which were Gram-negative, the tests used were Catalase and Oxidase test, Motility, Indole production, Methyl Red, Voges Proskauer test (IMViC test panel), Citrate utilization test, urea utilization test, Triple sugar Iron slants and Eijkman's tests

Antimicrobial Susceptibility Testing (AST)

The AST was carried out using the Kirby-Bauer disc diffusion method [11]. For each isolate, one colony from the selective plate was streaked onto a non-selective Tyrptic Soy (TS) agar plate. One colony from the TS agar plate was used to inoculate 10 ml of nutrient broth and statically incubated for 18–24 hours (overnight). About 600 µL–1000 µL from each suspension of overnight nutrient broth culture was adjusted with 5 ml of sterile saline to match the visual opacity with that of 0.5 - McFarland standard, containing approximately 1–2 × 10^6 colony forming units (CFU)/mL of the *Staphylococcus* spp. The saline suspension (50 µL) and 100 µL of the culture was spread onto the surface of a Mueller-Hinton Agar plate with a sterile swab (Himedia). The antimicrobial discs (Himedia) containing antibiotics of veterinary importance were dispensed onto the surface of the Mueller Hinton Agar plates at least 24 mm apart from the centre and to each other. The plates were incubated at 37 °C for 16-18 hours. The diameters of the inhibited zones were measured, including the diameter of the discs to the nearest whole millimetre using sliding callipers and interpreted using standard break points [21].

The minimal inhibitory concentration (MIC) [24] for the resistant antibiotics to the resistant *Staphylococcus* spp isolates was assessed using a Hi-comb strip method for confirmation of resistance.(CLSI, 2008) [22]. The inoculum was spread onto Mueller Hinton Agar plates (Himedia) as above. The specific Hi-comb strips containing the resistant antibiotic of interest in gradually decreasing gradients of the drug concentration in Strip A and Strip B was placed on the plate and incubated for 16-18 hours before visually taking the readings.

Genotyping of isolates for Bio-film producing strains of *Staphylococcus* spp.

The *S. aureus* possesses the capacity to forms bio-film (Dhanawade *et al*., 2010) [14] of which the iCAD operon is common and is directly related to the formation of bacterial biofilms as an essential factor. It consists of four genes (*icaA*, iCAD, *icaB* and *icaC*) and their coding product co-synthesizes the key material, PIA, for *Staphylococcus* adhesion in the process of bio-film formation. In this study, all the Staphylococcal isolates from mastitis milk samples were subjected to molecular analysis by genotyping for their

23S rRNA for genus specificity, *coa* gene for differentiating the (CoPS) and (CoNS), iCAD *ADBC* genes for biofilm formation and also for *Staphylococcus* spp. speciation. Furthermore, a few isolates that carried *ica* could not form a biofilm, likely because *ica* expression is regulated by multiple accessory regulators, or it may be due to the existence of other *ica*-independent genes.

DNA Extraction

Genomic DNA extraction by Boiling Method**:** In this method, 5 µl of bacteria were inoculated in 5 ml of TSB culture medium, which was then cultured at 37 °C overnight for 16 h with shaking at 120 rpm/sec in a shaker incubator. About 1.5 ml overnight culture was transferred to a microfuge and centrifuged at12000 r/min for 3 min. The supernatant was discarded and the bacterial cell pellet was washed once with isotonic 1 X PBS again by centrifugation. The washed pellet was dissolved in 100 µl NFW and subjected to boiling at 100 °C in a water bath for 10min. It was then snap chilled on crushed ice and centrifuged @ 10,000 rpm for 10 min. The supernatant containing the liberated genomic DNA was collected and stored at -80 °C in suitably labeled microfuges and the cell debris was discarded. The purity of the DNA was estimated at 260/280 nm in the TECAN nano-quant plate.

Genotyping for Staphylococcal 23S rRNA, *coa* virulence gene and speciation

Using the bacterial genomic DNA as templates, thermal cycling was carried to confirm for the 23S rRNA at the genus level for all the 81 suspected Stpahylococcus spp isolates. Also, the *coa gene* for virulence was genotyped to differentiate between coagulase positive and negative isolates. Each PCR was performed in a total volume of 15 µl containing 12.5x Red Dye Master Mix (Genei) PCR buffer and one unit of Taq DNA polymerase, 94 µM of each dNTP and 0.5 µM of each primer. *Staphylococcus* speciation was also done to identify 4 different species by a Multiplex PCR with a total volume of 25 µl containing 12.5x Red Dye Master Mix (Genei) PCR buffer and one unit of Taq DNA polymerase, 94 µM of each dNTP and 0.5 µM of each primer per reaction. The Primers and cycling conditions for the PCR programme are given in Table No 3. And Table No 4.

Molecular typing of iCAD *operon*- ADBC genes involved in bio-film formation by PCR

Molecular characterization to amplify a specific sequence of each of the 4 gene of the iCAD *operon* was carried out. Totally 81 *Staphylococcus* spp. isolates were screened for the PIA genes encoded by *ica-A/ICA-A* and *ica D/ICA-D* genes of the iCAD *operon* were tested using published primers and the list and PCR cycling conditions are given in Table No.5

Each PCR was performed in a total volume of 25 µL containing 12.5X Red Dye Master Mix (Genei) PCR buffer and one unit of Taq DNA polymerase, 94 µM of each dNTP and 0.5 µM of each primer. The PCR amplification was performed in a Thermal Cycler (Bio- Rad) under the following conditions. Amplified products were separated by electrophoresis using 1.5% agarose containing 0.5 mg Ethidium bromide in 0.5X Tris- acetate EDTA electrophoresis buffer at 100 V, documented and photographed under UV illumination in a Bio-Rad Gel documentation system.

Results

A total of 110 mastitis milk of bovine origin was collected, out of which 79 samples (71.8%) showed turbidity upon inoculation in Tryptic Soy broth. Out of these only 101 samples (84.2%) produced viable bacteria by cultural isolation. Out of these 79 viable samples, totally 69 *Staphylococcus* spp. (80.2%), 03 *Escherichia coli* (9.9%) and 03 Klebsiella spp (100%) was isolated. The details of isolates obtained from sub-clinical and clinical mastitis milk samples are tabulated in Table No 1. All the isolates were subjected to Antibiotic Sensitivity Test (AST). The Staphylococcal isolates were resistant to Penicillin by (93.3%) to Methicillin by (6.8%) and to Streptomycin by (10.5%). They were sensitive to Enrofloxacin by (93.3%), to Ceftrioxne by (86.7%) and to Gentamicin by (73.3%). The results of AST are tabulated in Table No. 2 and the images of sensitive and resistant isolates were shown in Figure 1and 2.

A total of 81 *Staphylococcus* spp isolates were obtained from the mastitis milk. Out of this 69 isolates were got from sub-clinical mastitis and 12 from clinical mastitis. Totally 69 isolates were positive by 23S rRNA and it was shown in Figure 3. The remaining 12 which did not amplify for 23S rRNA was *Micrococcus* spp. The coagulase positive (*coa*) *Staphylococcus* spp were 26, which exhibited different banding pattern ranging from 180bp, 390bp, 510bp, 600bp, 750bp, 800bp and 1200bp for different isolates, each exhibiting a minimum of two bands and it was shown in Figure 4 and 5. The reason for multiple banding is the polymorphic nature of the *coa* gene which exists as different alleles. The frequently encountered coa banding pattern was 510bp, 570bp, 595bp, 600bp which was often found in mastitis milk samples, as in another study by (Faizun *et al*., 2018) [7]. The *Staphylococcus* spp. isolates were characterized for their speciation by a Multiplex PCR. Of this, CoNS totaled to 55 isolates (79.7%) from which 18 were *S.chromogenes* (222bp) (32.7%); 3 were *S. haemolytica* (531bp) (5.45%); 15 were *S. epidermidis* (130bp) (27.3%) and 11 were *S. scuirii* (306bp) (20%). Rest of the 08 isolates (14.5%) were non-type able with the limited 4 primers used in speciation, Details of Molecular characterization of the *Staphylococcus* spp isolates for speciation and the iCAD operon for its bio-film forming capacity is given in Table No.6 and the gel image was shown in Figure 6.

The molecular typing of *Staphylococcus* spp for the bio-film forming capacity was carried by amplifying the 4 intracellular adhesion genes (*ica*) by PCR. Each of the iCAD *operon* genes that got singly amplified were as *ica-A* gene (1315bp) in 10 isolates; *ICA-D* (198bp) gene in 25 isolates; *ica –D* gene (381bp) in 30 isolates and *ICA-A* gene (188bp) in 2 *Staphylococcus* spp. isolates respectively. Combination of each of these bio-film forming genes that got amplified in same isolates is tabulated in Table No. 7

There was a definite link between the presence of a bio-film forming gene and its correlation to antibiotic resistance in the isolates. The *Staphylococcus* spp isolates carrying the iCAD operon genes and exhibiting phenotypic resistance to antibiotics is in Table No.8

Table 1: Details of the Bacterial isolates obtained from Bovine mastitis milk

	Details of cultural isolation	Total	%	Mastitis milk	
1	Bovine mastitis milk collected	110		SCM	CM
2	Growth on culture medium	93	84.5		
3	No growth on culture media	17	15.5		
4	Cultures that produced viable growth	76	81.7		
5	No. of different bacterial isolates got from turbid culture	110	-	-	-
a	Isolates from sub-clinical mastitis milk	155	76.4	155	-
b	Isolates from clinical mastitis milk	48	23.6	-	48
5	Total suspected Gram + ve coccid isolates	93	-	74	19
6	Total Micrococcus spp.	12	12.9	05	07
7	Total *Staphylococcus* spp.	81	87.1	69	12
8	Total suspected Gram –ve rods	10		04	06
9	Total *Escherichia coli* isolates	03	30	01	02
10	Total Klebsiella spp. isolates from clinical mastitis milk	02	20	-	02
11	Total *Pseudomonas* spp. isolates	05	50	03	02
12	Total G +ve rods – i.e., *Bacillus* spp. isolates	07	0.07	03	04
		-	-	155	48

Key: SCM- Sub-clinical mastitis; CM- Clinical mastitis

Table 2: Sensitivity and Resistant patterns of bacterial isolates to antibiotics by AST

Drug class	Drug sub-class	ABST pattern in %		
		Sensitive	Inter mediate	Resistant
		Staphylococcus spp. Isolates Staph		
Beta-lactams	Penicillin-G (10-IU)	4.6	-	93.3
	Methicillin (5 µg)	93.2	-	6.8
Aminoglycosides	Streptomycin (5 µg)	10.5	82.2	60.5
	Gentamicin (10 µg)	73.3	68.4	-
Fluoroquinolones	Enrofloxacin (10 µg)	93.3	7.9	-
	Ofloxacin-(10 µg)	71.1	-	-
Cephalosporins	Ceftrioxne (30 µg)	86.7	15.8	-
	Cefixime -(30 µg)	46.67	52.63	47.36
	Amoxyclav (30 µg)	-	7.89	92.10
Macrolide	Erythromycin (15 µg)	-	5.5	87.9
Tetracyclines	Oxytetra-cycline (30 µg)	28.94	-	71.11
	Doxycycline Hcl (30 µg)	66.67	31.57	-

Table 3: Genes and primers used for identification of Staphylococcal spp. 23S rRNA and *coa* virulence gene by PCR and its operational conditions

No	Gene	Primer Sequence (5'-3')				Amplicons (bp)
1	23S rRNA (Stuar-4) -F	ACG-GAG-TTA-CAA-AGG-ACG-AC				1250
	23S rRNA (Stuar-6) - R	ACG-TCA-GCC-TTA-ACG-AGT-AC				
2	*coa*-F	ATA-GAG-ATG-CTG-GTA-CAG-G				Polymorphic 600-950
	coa-R	GCT-TCC-GAT-TGT-TCG-ATG-C				
	PCR Cycling Programme					
Genes	Initial denaturation	Cycles	Denaturation	Annealing	Extension	Final Extension
23S rRNA Stuar	94 ºC-10 sec	37	94 ºC-40 sec	64 ºC-1 min	72 ºC-75 sec	72 ºC-10 min
Coa	94 ºC-1-3 min	30	94 ºC-1 min	58 ºC-1 min	72 ºC-1 min	72 ºC-5 min

Table 4: Genes and primers used in Speciation of *Staphylococcus* spp isolates by m-PCR and the Cycling conditions with their respective product size

No	Genes	Region	Primer Sequence(5'-3')				Product size (bp)
1	*Sod-a*	SCHS-1F	GCG-TAC-CAG-AAG-ATA-AAC-AAA-CTC				222
		SCHS-1R	CAT-TAT-TTA-CAA-CGA-GCC-ATG-C				
2	*sod A*	SHS2-F	CAA-ATT-AAA-TTC-TGC-AGT-TGA-GG				531
		SHS-2-R	GGC-CTC-TTA-TAG-AGA-CCA-CAT-GTT-A				
3	*rdr*	SER-F	AAG-AGC-GTG-GAG-AAA-AGT-ATC-AAG				130
		SER-R	TCG-ATA-CCA-TCA-AAA-AGT-TGG				
4	*gap*		GAT-TCC-GCG-TAA-ACG-GTA-GAG				306
			CAT-CAT-TTA-ATA-CTT-TAG-CCA-TTG				
		Multiplex PCR Programme					
Genes as above		Initial denaturation	Cycles	Denaturation	Annealing	Extension	Final Extension
		94 ºC-5 min	30	94 ºC-30 sec	58 ºC-30 sec	72 ºC-45 sec	72 ºC-5 min

Table 5: Genes and primers for ICAD *operon* typing of *Staphylococcal spp* by PCR and the Cycling conditions with their respective product size (Vasudevan. P *et al*., 2003) [20].

No	Gene	Primer Sequence(5'-3')			Product size
1	*ica-A* –F	CCT-AAC-TAA-CGA-AAG-GT-G			1315bp
	ica-A-R	AAG-ATA-TAG-CGA-TAA-GTG-C			
2	*ICA-D*-F	ATG-GTC-AAG-CCC-AGA-CAG-AG			198bp
	ICA-D-R	CGT-GTT-TTC-AAC-ATT-TAA-TGC-AA			
3	*ica-D*-F	AAA-CGT-AAG-AGA-GGT-GG			381bp
	ica-D-R	GGC-AAT-ATG-ATC-AAG-ATA-C			
4	*ICA-A*-F	TCT-CTT-GCA-GGA-GCA-ATC-AA			188bp
	ICA-A-R	TCA- GGC-ACT-AAC-ATC-CAG-CA			
Gene	**Initial denaturation**	**Cycles**	**Denaturation**	**Annealing**	**Extension**
ica-A	94 ºC-5 min	40	94 ºC 60 sec	49 ºC- 60 sec	72 ºC-60sec
ICA-D					
ica-D	92 ºC-5 min	40	92 ºC -60 sec	49 ºC -60 sec	72 ºC -60 sec
ICA-A	94 ºC-5 min	50	94 ºC -30 sec	55.5 ºC-30sec	72 ºC-30 sec

Table 6: Molecular characterization of the *Staphylococcus* spp isolates for speciation and the iCAD operon for its bio-film forming capacity

Sample Source	23S rRNA	*coa*	C	H	E	G	*ica A*	*ICA-D*	*ica D*	*ICA-A*
			CoNS (*coa*–ve) =55 (67.9%), Untypeable CoNS=08 (14.5%)							
Product size (bp)	1250bp		222	531	130	306	1315bp	198bp	381bp	188bp
Thapalpetti- 07	05	1	2	-	1	1	-	1	2	-
Aavin sheds-16	13	2	4	-	3	2	1	4	2	-
VUPH- 09	08	3	2	1	-	2	2	-	6	-
Pattalam-12	12	3	2	-	2	2	3	4	8	
LAC/OP/M-23	21	3	6	1	7	2	4	12	9	1
Private Farms-14	12	2	2	1	2	2	-	4	3	1
Total= 81, Micrococcus -12	69	14	18	03	15	11	10	25	30	2
% amplified	85.1	20.3	32.7	5.5	27.3	20	14.5	36.2	43.5	2.9

(i) *coa*- Coagulase gene- polymorphic multiple bands- (390bp, 600bp, 750bp, 800bp 1200bp)
(ii). C- *S.chromogenes*-222bp (iii). H- *S.haemolytica*-531bp (iv). E- *S.epidermidis*-130bp
(v). G- *S. scurii*-306bp (vi).*ica A* and *D* & *ICA-D* and *A*- Intracellular adhesion genes

Table 7: The different combination of bio-film genes amplified in *Staphylococcus* spp isolates

+ve for 1 gene and size	In **iCAD** operon	+ve for 2 and more genes	Genes and Amplicon size (bp)	+ve Isolates %
ica-A (1315bp)	10	+ve for 2 genes	*ica-A + ica D=(1315+381)bp*	08 ()
ICA-D (198bp)	25	+ve for 3 genes	*ica-A + ICA-D+ ica D=(1315+198+ 381)bp*	22 ()
ica D (381bp)	30	+ve for 3 genes	*ICA-D+ ica D+ICA-A(198+381+188)bp*	08 ()
ICAA (188bp)	02	+ve for 4 genes	*ica-A + ICA-D+ ica D+ICA-A (1315+198+381+188)bp*	15 ()
+ve isolates	67			
Other iCAD genes combinations & Amplicon Size		*ica-A* & *ICA-D*	1315bp & 198bp	30 - ()
		ica-A & *ica -D*	1315bp & 381bp	35 - ()
		ICA-D & *ica-D*	198bp & 381bp	49 - ()
		ICA-D & *ICA-A*	198bp &188bp	18 - ()
		ica-D & *ICA-A*	381bp & 188bp	14 - ()

Table 8: *Staphylococcus* spp (CoNS and CoPS) isolates carrying the iCAD operon genes and exhibiting phenotypic resistance to antibiotics

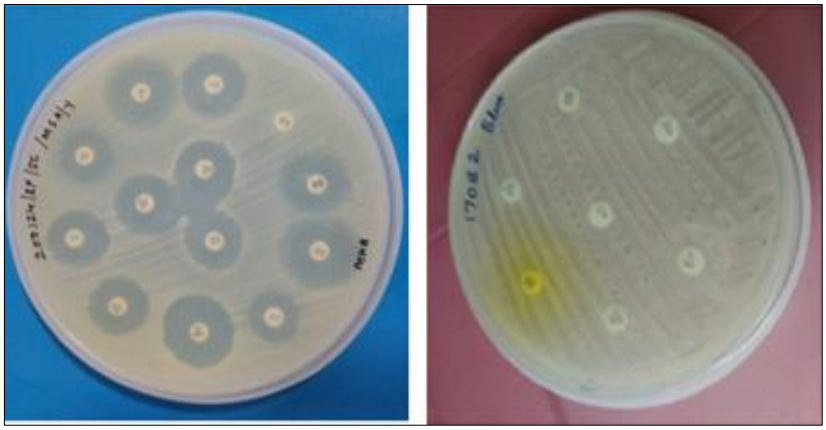
No	Details of the iCAD genes that got amplified in CoNS and CoPS isolates						
I	**CoNS (*coa* -ve) (55+ve)**	***ica- A***	***ICA-D***	***ica-d***	***ICA-A***	**iCAD +ve in CoNS**	**Untypeable CoNS**
1	*S. chromogenes* - (18+ve)	4	5	6	-	15	03
2	*S. haemolytica* - (03+ve)	-	-	2	-	02	01
3	*S. epidermidis*- (15+ve)	2	4	5	2	13	02
4	*S. scurii* - (11+ve)	2	4	4	-	10	02
	iCAD total+ves in CoNS	07	13	17	02	40	08
II	CoPS (*coa*+ve) (14+ve)	3	5	3	-	-	02 *coa*+ve did not carry any ICAD genes
	iCAD total +ves in CoPS	3	5	3	-	11	

Table 9: Total Resistant Antibiotics in CoNS and CoPS isolates

Antibiotics & Dose (µgm)	P (10 - IU)	A/clv (30)	MET (5)	CFM (30)	CTR (30)	CTX (30)	DoHcl (30)	E (15)	EX (10)	GEN (10)	OF (10)	OTC (30)	S (10)	Total MDR
S. chromogenes	7	-	-	4	2	-	3	7	-	1	2	3	3	32
S. epidermidis	4	3	3	2	1	2	3	6	1	2	1	2	6	36
S. scurii	3	2	-	2	-	1	-	3	1	2	-	2	3	19
Total CoNS resistance	14	5	3	8	3	3	6	16	2	5	3	7	12	87
CoPS (*coa* + ve) resistance	09	06	-	03	-	-	-	07	-	-	02	03	06	36
Total CoNS and CoPS resistance	23	11	03	11	03	03	06	23	02	05	05	10	18	123

4 isolates of *S. chromogenes*, had *icaA* gene and it carried antibiotic resistance to one each of Penicillin, Erythromycin Cefixime and Ceftrioxne. Also, 5 isolates crried ICA-D gene with antibiotic resistance to one each of Streptomycin, Gentamicin and Ceftrioxne and two each for resistance against Penicillin, Ofloxacin, and 3 each of Erythromycin, Oxytetracycline and Cefixime.

6 isolates of *S. chromogenes* carried the ica-d gene with resistance to Penicillin for 4, Erythromycin for 3 and Streptomycin for 2 and Docycline Hydrochloride for 3 of the isolates



a b

Fig 1: Images of *Staphylococcus epidermidis* Sensitive (a) and Resistant isolate (b)

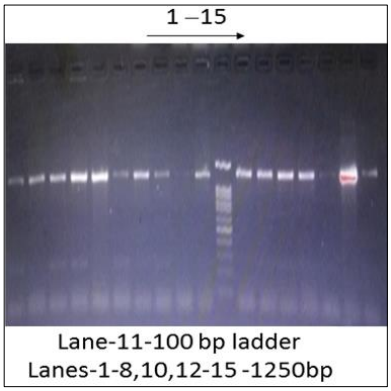


Fig 2: Images of 23 SrRNA typing of the *Staphylococcus* spp. isolates (1250bp)

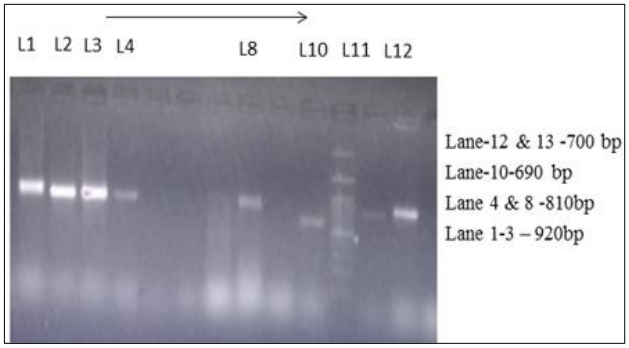


Fig 3: Images of *Staphylococcus* spp. typing of *coa* gene products of 690 bp, 700 bp, 810 bp and 920 bp in different lanes

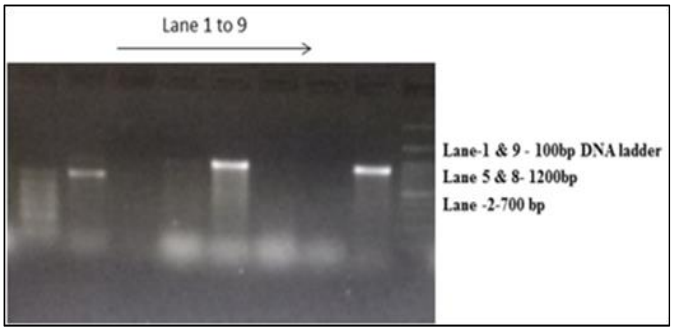


Fig 4: Images *Staphylococcus* spp. typing of *coa* gene products of 690 bp, 700 bp, 810 bp and 920 bp in different lanes

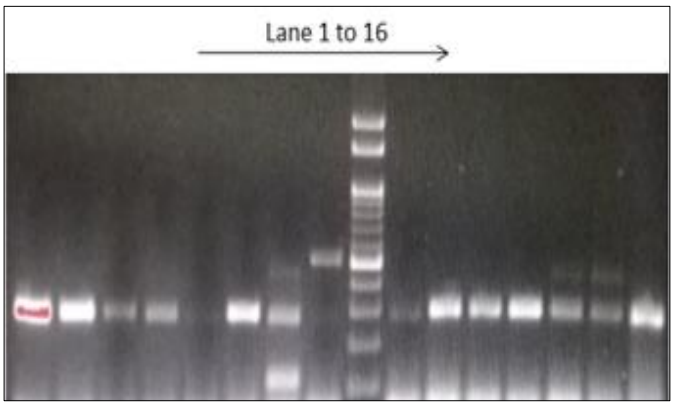


Fig 5: Images of Speciation m-PCR of *Staphylococcus* spp showing S. *epidermidis* (130bp), *S. haemolytica* (531bp) and *S. scurii* (306bp)

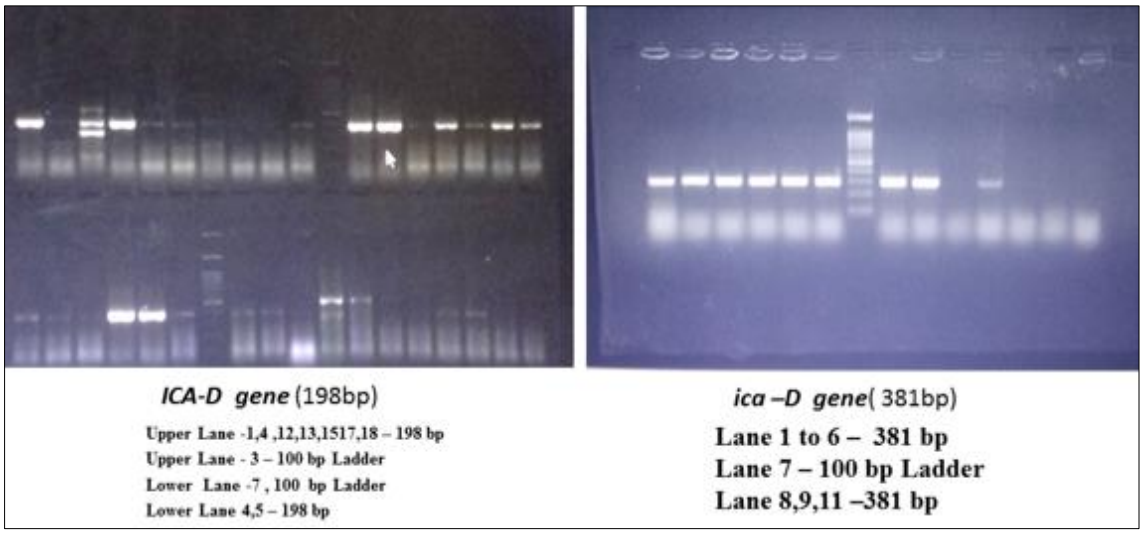


Fig 6: Images of biofilm formation genes in ICAD operon

Discussion

The mammary gland epithelium lines the milk compartment of the udder and is widely exposed to invading pathogens. Mastitis can appear in clinical and subclinical forms, the latter being commonly found in most herds [5, 7]. An increased milk somatic cell count (SCC) is the only sign of subclinical IMI. There is a known relationship between particular pathogens and the form of the disease. For example, *S. uberis*, *Escherichia coli*, *Klebsiella* spp., *Pseudomonas aeruginosa* and pyogenic bacteria are mainly considered as causative agents of clinical mastitis. On the other hand, *S. agalactiae*, Coagulase-negative staphylococci (CoNS) and *Enterococcus* spp. are associated with sub-clinical mastitis. However, *S. aureus* has been designated as a causative agent of both clinical and subclinical mastitis. CoNS are a minor group of udder pathogens of increasing importance. Mastitis caused by CoNS usually displays relatively mild clinical signs and these bacteria can therefore affect milk quality for a long period before being noticed.

Clinical mastitis may cause serious damage to the udder and even systemic disorders leading to the culling of affected animals. In the present study, a high frequency of isolation of *Staphylococcus spp* was observed, generally in subclinical for which unlike clinical mastitis, is milder, more insidious, difficult to detect and is widely spread among dairy herds wherein the cows appear sound and the udder inflammation and milk appear normal despite an elevated somatic cell count and detection of microorganisms. CoNS are the most prevalent cause of subclinical mastitis, and in this study also total of 4 different CoNS species were identified among which 11 isolates (20%) were *S. scuirii*; 15 were (27.3%) *S. epidermidis*; 18 were (32.7%) *S. chromgenes* and 3 were (5.5%) *S. haemolytica totaling* to 55 CoNS (79.7%). A total of 08 isolate (14.5%) were non-typeable using only the 4 primers for speciation in this study. Table No.1 gives the details of the bacterial isolates obtained from bovine mastitis milk

In recent times, an increasing antimicrobial resistance rate has been recognized in *Staphylococcus spp* isolated from bovine mastitis milk [16] and the results documented in this study are in compliance as well. Table No. 2 gives the Sensitivity and Resistant patterns of an Antibiotic Sensitivity Test done for *Staphylococcus* spp. isolates with a dozen antibiotics categorized under 6 major drug groups.

The *Staphylococcus* spp was 93.3% resistance beta-lactam drugs like Penicillin-G and 6.8% to Methicillin/Oxacillin which is an indicator antibiotic that expresses the presence of an altered or inefficient Penicillin binding protein (PBP2a) which is encoded by the *mec-A* gene in coagulase positive *S. aureus* isolates. Also, the *Staphylococcus* spp isolates were highly susceptible to the Fluoroquinolones, followed by Cephalosporins, Aminoglycosides, Tetracyclines and Macrolide in the decreasing order of susceptibility. The isolates were considered to be resistant if they exhibited resistance to two or more antibiotics of different drug groups. The expression of phenotypic resistance in MHA plate indicates the growing resistance of bacterial strains towards commonly used antibiotics in mastitis treatment. The isolates that showed phenotypic resistance also carried bio-film forming genes of the *icaADBC* operon in this study. Table No.6 shows the different combination of bio-film genes that were amplified in the *Staphylococcus* spp. isolates.

Biofilm production enables adhesion of bacteria to the epithelium of mammary glands and facilitates their persistence in the host tissue by protecting the bacterial cells against the mechanisms of the host defense [18]. Production of biofilm requires the presence of the intracellular adhesion locus gene cluster *icaADBC* [17] and strains harbouring the *icaADBC* cluster are potential biofilm producers. The *icaADBC* cluster, is an operon present in *Staphylococcus epidermidis* etc. and participates in bio-film formation by encoding proteins involved in the synthesis of a biofilm matrix polysaccharide (named PIA/PNAG) composed of linear -1-6-linked *N*-acetyl glucosamine residues[11]. Involved in intercellular adhesion [11]. The existence of bacteria in a biofilm matrix is a survival strategy and a virulence feature s well. The presence and amplification of a considerate number of genes under the iCAD operon in the *Staphylococcus* spp isolates is an indication of a persistent infection making them arbitrary to commonly used antibiotics under regular treatment regime.

In this study 26 *Staphylococcus* spp isolates were coagulase positive (CoPS), and 55 were coagulase negative (CoNS).The biofilm forming genes in the *icaADBC* operon were distributed in different numbers among the CoPS and CoNS isolates, but their presence was more in CoNS isolates, which

was similar to the study done by Faizan. J. *et al*., 2018 [26]. Consequently, in the current milk production chain, it is important to educate producers about good hygiene practices, as well as the harmful effects of low quality milk, such as the transmission of diseases, the associated economic and human costs, as well as decreased quality of dairy products, among others.

Acknowledgments
The authors wish to acknowledge the support provided by Tamil Nadu Veterinary and Animal Sciences University, for carrying out the research work.

Competing Interests
The authors declare that they have no competing interests.

References
1. Patel SJ, Patel MD, Patel JH, Patel AS, Gelani RN. Role of women gender in livestock sector: A review. J Livestock Sci. 2016;7:92-96.
2. Debabrata Paula, Chandel BS. Improving Milk Yield Performance of Crossbred Cattle in North-Eastern States of India. Agricultural Economics Research Review. 2010;23(1):69-75.
3. Bangar YC, Singh B, Dohare AK, Verma MR. A systematic review and meta-analysis of prevalence of subclinical mastitis in dairy cows in India. Trop Anim Health Prod. 2015;47(2):291-7.
4. Le Marechal C, Thiery R, Vautor E, *et al*. Mastitis impact on technological properties of milk and quality of milk products a review. Dairy Science & Technol. 2011;91(3):247-282.
5. Awale MM, Dudhatra GB, Avinash Kumar, Chauhan BN, Kamani DR. Bovine Mastitis: A Threat to Economy. Scientific reports. 2012;1:295.
6. Bradley AJ. Bovine mastitis: An evolving disease. Veterinary Journal. 2002;164(2):116-128.
7. Gruet P, Maincent P, Berthelot X, Kaltsatos V. Bovine mastitis and intramammary drug delivery: review and perspectives. Advanced Drug Delivery Reviews. 2001;50(3):245-259.
8. Costerton JW, Stewart PS, Greenberg EP. Bacterial biofilms: a common cause of persistent Infect. Science. 1999;284(5418):1318-1322.
9. Melchior MB, Fink-Gremmels J, Gaastra W. Extended antimicrobial susceptibility assay for *Staphylococcus* aureus isolates from bovine mastitis growing in biofilms. Vet Microbiol. 2007;125(1-2);141-149.
10. Frank KL, Patel R. Poly-N-acetylglucosamine is not a major component of the extracellular matrix in biofilms formed by *icaADBC* positive *Staphylococcus lugdunensis* isolates. Infect. Immunity. 2007;75(10):4728-4742.
11. Bauer AW, Kirby WM, Sherris JC, Turck M. Antibiotic susceptibility testing by a standardized single disk method. Am J Clin Pathol. 1966;45(4):493-496.
12. Freeman DJ, Falkiner FR, Keane CT. New method for detecting slime production by coagulase negative staphylococci. J Clin. Pathol. 1989;42(8):872-874.
13. Mariana NS, Salman SA, Neela V, Zamberi S. Evaluation of modified Congo red agar for detection of biofilm produced by clinical isolates of methicillin resistance *Staphylococcus* aureus. African Journal of Microbiology Research. 2009;3(6):330-338.
14. Dhanawade NB, Kalorey DR, Srinivasan R, *et al.* Detection of intercellular adhesion genes and biofilm production in *Staphylococcus aureus* isolated from bovine subclinical mastitis. Vet Res Commun. 2010;34(1):81-89.
15. Markey B, Leonard F, Archambault M, Cullinane A, Maguire D. Clinical veterinary microbiology e-book. Elsevier Health Sciences; c2013.
16. Wang SC, Wu CM, Shen JZ, Wu YN, Wang Y. Hypermutable *Staphylococcus* aureus strains present at high frequency in subclinical bovine mastitis isolates are associated with development of antibiotic resistance. Veterinary Microbiology. 2013;165(3-4):410-15.
17. Cramton SE, Gerke C, Schnell NF, Nichols WW, Gotz F. The intercellular adhesion (*ica*) locus is present in *Staphylococcus aureus* and is required for biofilm formation. Infect. Immun. 1999;67(10):5427-5433.
18. Melchior MB, Vaarkamp H, Fink-Gremmels J. Biofilms: a role in recurrent mastitis infections? Vet J. 2006;171(3):398-407.
19. Mac Faddin JF. Biochemical Tests for Identification of Medical Bacteria. The Williams & Wilkins, Baltimore; c1976. p. 312.
20. Vasudevan P, Nair MKM, Annamalai T, Venkitanarayanan KS. Phenotypic and Genotypic characterization of bovine mastitis isolates of *Staphylococcus aureus* for biofilm formation. Vet Microbiol. 2003;92(1-2):179-185.
21. Andrews JM. BSAC standardized disc susceptibility testing method (version 7). J Antimicrob Chemother. 2008;62(2):256-78.
22. Clinical and Laboratory Standards Institute (CLSI): Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals; approved standard-third edition. CLSI document M31-A3; c2008.
23. Kumar Shukla S, Rao TS. Dispersal of *bap*-mediated *Staphylococcus* aureus biofilm by proteinase K. J of Antibiotic. 2013;66(2):55-60.
24. Oliveira L, Langoni H, Hulland C, Ruegg PL. Minimum inhibitory concentrations of *Staphylococcus* aureus recovered from clinical and subclinical cases of bovine mastitis. Journal of Dairy Science. 2012;95(4):1913-1920.
25. Faizan Javid, Anil Taku, Mohd Altaf Bhat, Gulzar Ahmad Badroo, Mir Mudasir, Tanveer Ahmad Sofi. Molecular typing of *Staphylococcus aureus* based on coagulase gene. Veterinary World. 2018;11(4):423-430.