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Standardization of multiplex PCR for identification of ten *staphylococci* species and their prevalence in bovine mastitis

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ABSTRACT

To assess the clinical impact, epidemiology of intra-mammary infections, accurate diagnosis, and choosing appropriate antimicrobial therapy, the staphylococci group should be studied correctly up to the species level. In this study, a multiplex PCR (mPCR) was developed to identify 10 important *Staphylococcus* species that was applied to study the prevalence of *staphylococci* species in bovine mastitis from five agro-climatic regions of Punjab, India. A two tube mPCR assay consisted of 5 species each, with one reaction including primer pairs of *S. epidermidis*, *S. hemolyticus, S. hominis, S. warneri* and *S. saprophyticus,* and another reaction consisted primer pairs of *S. aureus*, *S. intermedius, S. chromogenes, S. sciuri* and *S. capitis* was standardized. Prevalence study revealed *S. aureus* (35.76%) as most prevalent species among all *staphylococci* followed by *S. chromogenes* (16.56%), *S. haemolyticus* (15.23%), *S. epidermidis* (5.30%), *S. sciuri* (1.99%), *S. capitis* (0.66%), *S. hominis* (0.66%), *S. saprophyticus* (0.66%) and rest were other staphylococci (23.18%). The mPCR developed in this study could be used for identification of staphylococci of various origins including those from human specimens. The high prevalence of *S. chromogenes* and *S. haemolyticus* in the present study suggest emergence of these non-*aureus* staphylococci in bovine mastitis in Punjab (India).

Keywords: Bovine mastitis, Multiplex PCR, Non-*aureus staphylococci*

The species within genus *Staphylococcus* were classified into coagulase-negative staphylococci (CoNS) and coagulase-positive staphylococci (CoPS) based on their ability to produce coagulase enzyme. Condas *et al.* (2017) proposed new classification of *Staphylococcus* genus into *S. aureus* and non-*aureus* staphylococci species (NAS) on the basis of various limitations in the classification into CoPS and CoNS. The genus *Staphylococcus* consists of 47 validated species and 23 subspecies, majority of which are coagulase-negative (38 species) (Becker *et al.* 2014). Although, *Staphylococcus aureus* is one of the most common and important CoPS isolated from bovine mastitis, more than 15 CoNS species have also been reported to cause IMI in dairy cows; *S. chromogenes, S. epidermidis, S. haemolyticus, S. hyicus, S. simulans,* and *S. xylosus* being the most prevalent ones (Veerle *et al.* 2011, Srednik *et al.* 2015). Identification of individual *Staphylococcus* species is laborious and elaborative, and time-consuming microbiological tests including commercial biochemical identification kits have been devised for correct identification of staphylococci species (Cunha *et al.* 2004).

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However, limitations in conventional phenotypic approaches have shifted the focus towards more accurate and superior DNA based genotypic identification methods. DNA sequence based identification is regarded as gold standard but is costly than PCR based methods, that are simple, reliable and economical. In the last few years, several genotypic methods have been developed for accurate identification of *Staphylococcus* species (Bes *et al.* 2000, Braem *et al.* 2011). Also several multiplex PCR (mPCR) protocols have been developed identifying varying number of *Staphylococcus* spp., such as 4 spp. (Morot-Bizot *et al.* 2004), 7 spp. of CoPS (Sasaki *et al.* 2010), 6 spp. (Shome *et al.* 2011), 9 spp. (Hirotaki *et al.* 2011).

Therefore, present study was devised to develop a mPCR protocol that could identify 10 important *Staphylococcus* species in a shortest possible time so as to minimize the delay in identification of correct species for proper diagnosis. In India, limited research has been carried out to understand the epidemiology of staphylococci associated with bovine mastitis. Furthermore, the mPCR was used to study the prevalence of *Staphylococcus* spp. in different agro-climatic regions of Punjab, India.

MATERIALS AND METHODS

Standard bacterial strains used: The mPCR assay was developed using 12 Microbial Type Culture Collection

(MTCC) bacterial strains (IMTECH, Chandigarh, India), i.e. *S. epidermidis* (MTCC 3382), *S. hemolyticus* (MTCC 3383), *S. chromogenes* (MTCC 3545), *S. simulans* (MTCC 3610), *S. hominis* (MTCC 4435), *S. warneri* (MTCC 4436), *S. capitis* (MTCC 6151), *S. intermedius* (MTCC 6152), *S. sciuri* (MTCC 6154), *S. saprophyticus* (MTCC 6155), *S. xylosus* (MTCC 8309), *S. urealyticus* (MTCC 9794) and 1 *S. aureus* (ATCC 33591; Manassas, VA, USA) reference strains.

Collection of milk samples and microbial analysis: Lactating cows (250) from five different agro-climatic regions (50 from each region) of Punjab, India, were screened for subclinical mastitis (SCM) by California Mastitis test. Out of 250 cows screened, 108 were found positive for CMT and were included in the study for bacterial isolation. Quarter fore milk (10 mL) from SCM cows was collected aseptically in sterile 15 ml glass test tubes and carried to laboratory in icebox for bacterial analysis. The staphylococci were presumptively identified using standard microbial procedures (Hogan *et al.* 2009). Staphylococci isolates were stored at –20°C in trypticase soy broth (30% glycerol) for future use.

DNA extraction: Individual bacterial colony was inoculated in 1 ml brain heart infusion broth (BHI, HiMedia), and incubated overnight. The broth was pelleted at 7,500 rpm for 5 min in refrigerated centrifuge (Heraeus Biofuge Primo R, Thermo Scientific), followed by DNA extraction as per guidelines of QIAamp DNA mini kit (Qiagen). The DNA eluted was stored at –20°C until further use.

PCR detection of Staphylococcus genus (*16S rDNA*, i.e. *ribosomal DNA*): *Staphylococcus* genus (16S rDNA, i.e. ribosomal DNA) PCR was carried out using the protocol and primers given in Table 1 (Strommenger *et al.* 2003).

Standardization of mPCR for identification of 10 different staphylococcal species: The primers for 10 different staphylococcal species were selected from the published literature (Table 1) (Sasaki *et al.* 2010, Hirotaki *et al.* 2011, Brakstad *et al.* 1992). A two tube mPCR assay consisted of 5 species each, with one reaction including primer sets of *S. epidermidis*, *S. hemolyticus, S. hominis, S. warneri* and *S. saprophyticus,* while another reaction comprised primer pairs of *S. aureus*, *S. intermedius, S. chromogenes, S. sciuri* and *S. capitis* was developed successfully. The standardized mPCR assay comprised of 1.5 U of Taq polymerase, 0.2 mmol/L dNTPs, 1 X reaction buffer, 25 mmol MgCl and 10 pmol of each of the primers in a total of 25 µl reaction. Amplification was carried, once at 94°C for 5 min; followed by 35 cycles at 94°C for 30 sec; 58°C for 30 sec; 72°C for 45 sec; and final extension at 72°C for 7 min. Electrophoresis was carried out in 1.5% agarose gel containing ethidium bromide (10 µg/ml) and gel visualized

Table 1. List of primers used in the study

Organisms/ Target	Primer	Oligonucleotide sequence (5′–3′)	Amplicon size (bp)	Reference
Staphylococcus genus	*16S r DNA-F* *16S rDNA-R*	CAG CTC GTG TCG TGA GAT GT AAT CAT TTG TCC CAC CTT CG	420	Strommenger *et al.* (2003)
S. aureus	*Nuc-F* *Nuc-R*	GCGATTGATGGTGATACGGTT AGCCAAGCCTTGACGAACTAAAGC	280	Brakstad *et al.* (1992)
S. intermedius	in-F in-R	CATGTCATATTATTGCGAATGA AGGACCATCACCATTGACATATTGAAACC	430	Sasaki *et al*. (2010)
S. epidermidis	epid-F epid-R	AAGAGCGTGGAGAAAAGTATCAAG TCGATACCATCAAAAAGTTGG	130	Shome *et al.* (2011)
S. hemolyticus	hem-F hem-R	CAAATTAAATTCTGCAGTTGAGG AGAGCCCCATTGTTCTTTGA	214	
S. chromogenes	chrom-F chrom-R	GCGTACCAGAAGATAAACAAACTC CATTATTTACAACGAGCCATGC	222	
S. sciuri	ssci-F ssci-R	GATTCCGCGTAAACGGTAGAG CATCATTTAATACTTTAGCCATTG	306	
S. hominis	hom-F hom-R	TACAGGGCCATTTAAAGACG GTTTCTGGTGTATCAACACC	177	Hirotaki *et al.* (2011)
S. warneri	war-F war-R	CGTTTGTAGCAAAACAGGGC GCAACGAGTAACCTTGCCAC	999	
S. saprophyticus	sap-F sap-R	TTTTGGATGCGATAGATTGG TCTTCAGACTTTTCAAAGGC	843	
S. capitis	cap-F cap-R	ACTACGCCTATGATTATTGC GAYGCTTCTTTACCATAGGG	525	
PCR-RFLP	Dnaj-F Dnaj-R	GCCAAAAGAGACTATTATGA ATTGYTTACCYGTTTGTGTACC	Variable	Hauschild and Stepanoviæ (2008)

using the Molecular Imager® ChemiDoc™ XRS + imaging system (Bio-Rad). The *Staphylococci* species isolated from quarter milk sample were used for validation of mPCR protocol and their prevalence was recorded.

Confirmation by restriction fragment length polymorphism-PCR (*PCR-RFLP*): For confirmation of *staphylococci* spp., PCR-RFLP was carried out on the amplified products of *dnaj* gene (primers given in Table 1) as per protocol of Hauschild and Stepanoviæ (2008).

Statistical analysis: Prevalence of staphylococci was shown as frequency distribution and region wise prevalence of staphylococcal quarter infections in SCM cows was checked for statistical significance using chi square test.

RESULTS AND DISCUSSION

Molecular confirmation at genus level was done using *Staphylococcus* genus (16S rDNA, i.e. ribosomal DNA) PCR (Fig. 1). The mPCR for 10 different staphylococcal species was standardised successfully amplifying exact DNA amplicons (Fig. 2), and was used to identify *Staphylococcus* spp. in mastitic milk samples resulting in identification of only 8 species, which were further confirmed by PCR-RFLP of *dnaj* gene amplicons using *ApoI* restriction enzyme (New England BioLabs Inc.) (Fig. 3). All the species identified by mPCR gave consistent band pattern upon digestion by *ApoI* restriction enzyme, confirming amplification by species specific primers in the mPCR. Several mPCR protocols have been developed by various researchers, identifying different number of *Staphylococcus* spp. (Morot-Bizot *et al.* 2004, Hirotaki *et al.* 2011), however, mPCR developed in the present study detects 10 important *Staphylococcus* spp. associated with bovine mastitis. The mPCR developed in this study proved easy, cost effective, and time saving approach. Due to increased role of *Staphylococcus* spp. in bovine mastitis, it is important to identify individual species for correct diagnosis, and choosing antibiotic of choice. This mPCR assay could be used for staphylococci species identification

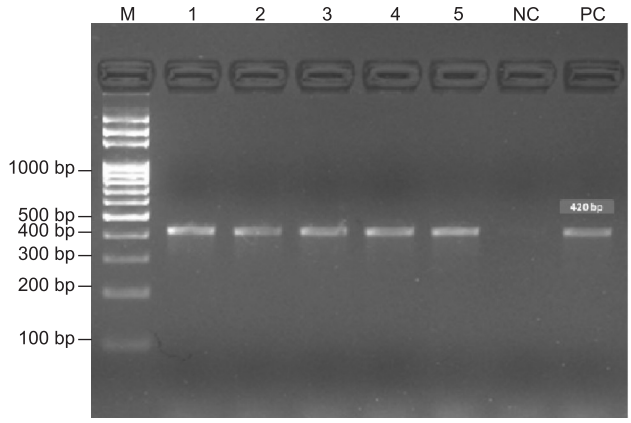

Fig. 1. Electrophoresis image of *Staphylococcus* genus targeting 16s rDNA (420 bp) gene. Lanes 1–5 indicate *Staphylococci* spp. isolated from bovine mastitis milk samples. NC, negative control; PC, positive control *S. aureus* (ATCC 33591). M indicate SMOBio ExcelBand 100 bp DNA ladder (DM2100).

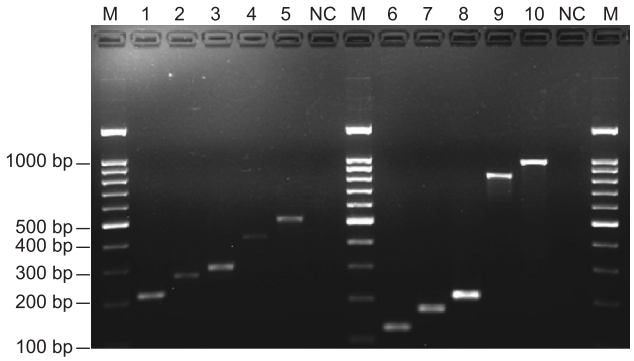

Fig. 2. Electrophoresis image of mPCR for identification of 10 *Staphylococcus* spp. Two tube multiplex PCR showing simultaneous amplification of 1: *S. chromogenes* (220 bp), 2: *S. aureus* (280 bp), 3: *S. sciuri* (306 bp), 4: *S. intermedius* (430 bp), 5: *S. capitis* (525 bp), 6: *S. epidermidis* (130 bp), 7: *S. hominis* (177 bp), 8: *S. hemolyticus* (214 bp), 9: *S. saprophyticus* (843 bp) 10: *S. warneri* (999 bp). M indicate Excel Band 100 bp DNA ladder (DM2300, SMOBio) and NC is negative control.

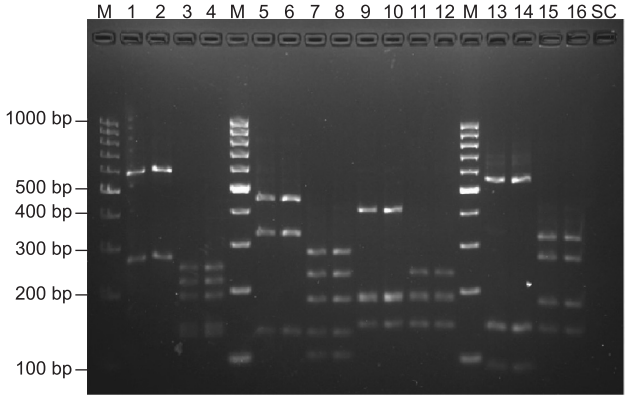

Fig. 3. Electrophoresis image of PCR-RFLP using *Apo*I restriction digestion of *DNAj* gene of staphylococci. Lanes 1, 2: *S. chromogenes*; 3, 4: *S. hemolyticus*; 5, 6: *S. epidermidis*; 7, 8: *S. capitis*; 9, 10: *S. saprophyticus*; 11, 12: *S. hominis*; 13, 14: *S. sciuri* and 15, 16: *S. aureus.* M, GeneRuler 100 bp DNA ladder (SM0243, Thermo Scientific); NC, negative control.

of various origins, including staphylococci from human specimens, and therefore, will help in accurate treatment and prevention of *Staphylococcus* infections, epidemiological investigations, and continuous monitoring.

Total 284 bacterial isolates were isolated, out of which 169 were presumptively identified as *Staphylococcus* spp. and on applying *Staphylococcus* genus specific PCR, 151 were confirmed. Out of these 151 isolates, 116 were identified up to species level (8 species) using mPCR protocol and 35 *Staphylococcus* species were unidentified staphylococci (Table 2). *S. aureus* was found most prevalent (35.76%) species followed by *S. chromogenes* (16.56%), *S. haemolyticus* (15.23%) and *S. epidermidis* (5.30%). Region wise no statistical significant difference was observed in the prevalence of staphylococci isolated from cows ($p<0.05$, Table 3). High prevalence of staphylococci spp. in bovine mastitis has been reported by several workers over the years (Gillespie *et al.* 2009, Cheng *et al.* 2019). Our findings fall in line with previous studies that reported

Table 2. mPCR based prevalence of staphylococci isolated from SCM in cows

Staphylococcus species	Cow	
	Number	Per cent
S. aureus	54	35.76
S. chromogenes	25	16.56
S. epidermidis	8	5.30
S. hemolyticus	23	15.23
S. sciuri	3	1.99
S. capitis	1	0.66
S. saprophyticus	1	0.66
S. hominis	1	0.66
S. warneri	0	0.00
S. intermedius	0	0.00
Unidentified	35	23.18
Total	151	100.00

Table 3. Region wise prevalence of staphylococcal quarter infections in SCM cows

Region**	Cow		
	Quarters	Isolates	Per cent
UPZ	94	28[a*]	29.79
CPZ	86	37[a]	43.02
SMUZ	90	28[a]	31.11
WZ	77	26[a]	33.77
WPZ	75	32[a]	42.67
Total	422	151	35.78

*The number of isolates with same superscripts did not differ significantly between the regions (P< 0.05). **UPZ, Upper plain zone; CPZ, Central plain zone; SMUZ, Sub-Mountainous undulating zone; WZ, Western zone; WPZ, Western plain zone.

S. aureus and *S. chromogenes* as common mastitis pathogens along with other species such as *S. epidermidis, S. hyicus, S. simulans* and *S. haemolyticus* (Srednik *et al.* 2015, Persson *et al.* 2011, Thorberg *et al.* 2009, Qu *et al*. 2019). *S. aureus* has been reported predominant contagious pathogens from both clinical and subclinical bovine mastitis (Mir *et al.* 2014, Luini *et al.* 2015), contributed mainly to its frequent colonization of teats, ability to exist intracellularly, localization within micro abscesses in the udder parenchyma, and recurrence under certain occasions (MacDonald 1997). Its ability to establish chronic or subclinical infections acts as source of infection to other healthy cows. *S. chromogenes* has been reported as a predominant NAS in recent studies using molecular identification methods (Condas *et al.* 2017, Qu *et al.* 2019, Traversari *et al.* 2019), however, earlier studies which were mainly based on phenotypic methods have reported *S. hyicus* as more predominant NAS. The higher prevalence in earlier studies could be due to its phenotypic similarity with *S. chromogenes* and low accuracy of phenotypic methods (Sampimon *et al.* 2009). High prevalence of *S. choromogenes* has been attributed to its potential to cause persistent infections, adaptation to bovine skin microbiota, and emergence of resistance to antibiotics and teat disinfectants (Fry *et al.* 2014). Our results were contrary to few studies reporting NAS such as, *S. sciuri, S. capitis, S. intermedius*, and *S. hominis* as frequent isolates of intramammary infections (Sampimon *et al.* 2009). The variation in the prevalence of NAS species has been attributed to variation in identification methods, age or parity, regional differences, and different management practices or housing conditions, or other cow factors (Thorberg *et al.* 2009, Persson *et al.* 2011, Piepers *et al.* 2013, Condas *et al.* 2017). Usually NAS infections remain subclinical but sometimes may flare up to (mild) clinical mastitis (Piepers *et al.* 2013). The importance of NAS in mastitis has tremendously increased in recent years owing to adoption of various preventive measures to control major contagious mastitis pathogens such as *S. aureus*. This trend is also evident from the results of the present study reporting higher prevalence of NAS, and therefore, the study highlights the need for accurate species level identification of staphylococci associated with bovine mastitis.

The mPCR developed in present study leads to correct identification of 10 important staphylococci species and therefore, this assay could be used for identification of staphylococci of various origins including those from human specimens. The high prevalence of *S. chromogenes* and *S. haemolyticus* in the present study suggest emergence of these NAS in bovine mastitis in Punjab (India). The study highlights the need for species level identification and requirement of thorough investigations to understand the epidemiology of staphylococcal mastitis, and also continuous monitoring of dairy herds for emergence of newer pathogens.

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REFERENCES

Becker K, Heilmann C and Peters G. 2014. Coagulase-negative staphylococci. *Clinical Microbiology Reviews* **27**(4): 870–926.

Bes M, Guerin-Faublee V, Meugnier H, Etienne J and Freney J. 2000. Improvement of the identification of staphylococci isolated from bovine mammary infections using molecular methods. *Veterinary Microbiology***71**: 287–94.

Braem G, De Vliegher S, Supré K, Haesebrouck F, Leroy F and De Vuyst L. 2011. (GTG)5-PCR fingerprinting for the classification and identification of coagulase negative *Staphylococcus* species from bovine milk and teat apices: A comparison of type strains and field isolates. *Veterinary Microbiology* **147**: 67–74.

Brakstad O D D G, Aasbakk K and Maeland J A. 1992. Detection of *Staphylococcus aureus*_nuc pcr und primer. *Journal of Clinical Microbiology* **30**(7): 1654–60.

Cheng J, Qu W, Barkema H W, Nobrega D B, Gao J, Liu G, De Buck J, Kastelic J P, Sun H and Han B. 2019. Antimicrobial

resistance profiles of 5 common bovine mastitis pathogens in large Chinese dairy herds. *Journal of Dairy Science* **102**(3): 2416–26.

Condas L A Z, De Buck J, Nobrega D B, Carson D A, Roy J P, Keefe G P, DeVries T J, Middleton J R, Dufour S and Barkema H W. 2017. Distribution of non-aureus staphylococci species in udder quarters with low and high somatic cell count, and clinical mastitis. *Journal of Dairy Science* **100**: 5613–27.

Cunha M L, Sinzato Y K and Silveira L V A. 2004. Comparison of methods for the identification of coagulase-negative staphylococci. *Memorias do Institutes Oswaldo Cruz* **99**: 855–860.

Fry P R, Middleton J R, Dufour S, Perry J, Scholl D and Dohoo I. 2014. Association of coagulase-negative staphylococcal species, mammary quarter milk somatic cell count, and persistence of intramammary infection in dairy cattle. *Journal of Dairy Science* **97**(8): 4876–85.

Gillespie B E, Headrick S I, Boonyayatra S and Oliver S P. Prevalence and persistence of coagulase-negative *Staphylococcus* species in three dairy research herds. *Veterinary Microbiology* **134**(1–2): 65–72.

Hauschild T and Stepanovic S. 2008. Identification of *Staphylococcus* spp. by PCR-restriction fragment length polymorphism analysis of dnaJ gene. *Journal of Clinical Microbiology* **46**(12): 3875–79.

Hirotaki S, Sasaki T, Kuwahara-Arai K and Hiramatsu K. 2011. Rapid and accurate identification of human-associated staphylococci by use of multiplex PCR. *Journal of Clinical Microbiology* **49**(10): 3627–31.

Hogan J S, Gonzales R N, Harmon R J, Nickerson S P, Oliver J W, Pankey J W and Smith K L. 2009. *Laboratory Handbook on Bovine Mastitis*. National Mastitis Council, Madison, WI.

Luini M, Cremonesi P, Magro G, Bianchini V, Minozzi G, Castiglioni B and Piccinini R. 2015. Methicillin-resistant *Staphylococcus aureus* (MRSA) is associated with low within-herd prevalence of intra-mammary infections in dairy cows: Genotyping of isolates. *Veterinary Microbiology* **178**(3–4): 270–74.

MacDonald J S. 1997. Streptococcal and staphylococcal mastitis. *Journal of American Veterinary Medical Association* **170**: 1157.

Mir A Q, Bansal B K and Gupta D K. 2014. Subclinical mastitis in machine milked dairy farms in Punjab: Prevalence, distribution of bacteria and current antibiogram. *Veterinary World* **7**(5): 291–94.

Morot-Bizot S C, Talon R and Leroy S. 2004. Development of a multiplex PCR for the identification of *Staphylococcus* genus and four staphylococcal species isolated from food. *Journal of Applied Microbiology* **97**(5): 1087–94.

Persson Waller K, Aspán A, Nyman A, Persson Y and Grönlund Andersson U. 2011. CNS species and antimicrobial resistance in clinical and subclinical bovine mastitis. *Veterinary Microbiology* **152**(1–2): 112–16.

Piepers S, Schukken Y H, Passchyn P and De Vliegher S. 2013. The effect of intramammary infection with coagulase-negative staphylococci in early lactating heifers on milk yield throughout first lactation revisited. *Journal of Dairy Science* **96**(8): 5095–5105.

Qu Y, Zhao H, Nobrega D B, Cobo E R, Han B, Zhao Z, Li S, Li M, Barkema H W and Gao J. 2019. Molecular epidemiology and distribution of antimicrobial resistance genes of *Staphylococcus* species isolated from Chinese dairy cows with clinical mastitis. *Journal of Dairy Science* **102**(2): 1571–83.

Sampimon O C, Barkema H W, Berends I M G A, Sol J and Lam T J G M. 2009. Prevalence and herd-level risk factors for intramammary infection with coagulase-negative staphylococci in Dutch dairy herds. *Veterinary Microbiology* **134**(1–2): 37–44.

Sasaki T, Tsubakishita S, Tanaka Y, Sakusabe A, Ohtsuka M, Hirotaki S, Kawakami T, Fukata T and Hiramatsu K. 2010. Multiplex-PCR method for species identification of coagulase-positive staphylococci. *Journal of Clinical Microbiology* **48**(3): 765–69.

Shome B R, Mitra S D, Bhuvana M, Krithiga N, Velu D, Shome R, Isloor S, Barbuddhe SB and Rahman H. 2011. Multiplex PCR assay for species identification of bovine mastitis pathogens. *Journal of Applied Microbiology* **111**(6): 1349–56.

Srednik M E, Grieben M A, Bentancor A and Gentilini E R. 2015. Molecular identification of coagulase-negative staphylococci isolated from bovine mastitis and detection of ß-lactam resistance. *Journal of Infection in Developing Countries.* **9**(9): 1022–27.

Strommenger B, Kettlitz C, Werner G and Witte W. 2003. Multiplex PCR assay for simultaneous detection of nine clinically relevant antibiotic resistance genes in Staphylococcus aureus. *Journal of Clinical Microbiology* **41**(9): 4089–94.

Thorberg B M, Danielsson-Tham M L, Emanuelson U and Persson Waller K. 2009. Bovine subclinical mastitis caused by different types of coagulase-negative staphylococci. *Journal of Dairy Science* **92**(10): 4962–70.

Traversari J, Van Den Borne B H P, Dolder C, Thomann A, Perreten V and Bodmer M. 2019. Non-aureus staphylococci species in the teat canal and milk in four commercial swiss dairy herds. *Frontiers in Veterinary Science* **6**(Jun): 11–14.

Veerle P, Van Coillie E, Verbist B, Supré K, Braem G, Van Nuffel A, De Vuyst L, Heyndrickx M and De Vliegher S. 2011. Distribution of coagulase-negative *Staphylococcus* species from milk and environment of dairy cows differs between herds. *Journal of Dairy Science* **94**: 2933–44.