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QUANTIFICATION OF *Staphylococcus aureus* AND *Escherichia coli* FROM BOVINE SUBCLINICAL MILK SAMPLES BY CONVENTIONAL PCR

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Quantification

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ABSTRACT

The present study explores the application of conventional PCR and Mole v/s Avogadro's number using single copy genes of *Staphylococcus aureus* and *Escherichia coli* for assessing bacterial load in milk samples from mastitis cases. The *nuc* and *uid*A genes produced the lowest detection limit for *Staph. aureus* and *E. coli* respectively. The present findings estimated that, as few as 60 and 89 genome copies or organisms of *Staph. aureus* and *E. coli* were detected, respectively. Based on the LOD of molecules, standard graphs for these genes were generated and this knowledge was applied to milk samples from the field. Spiking known amount of genomic DNA (which in turn indicates organisms based on Avogadro's number) in LB broth and pasteurized milk was carried out to compare the input with output and simulating field conditions. A total of 90 samples from subclinical cases of mastitis were collected from four organized farms located at various villages. Out of 90 samples, 29 (32.22 %) were showed culture and duplex PCR positive. Of these, 12 (13.33 %) were found to be positive for *E. coli* alone, nine (10 %) were found to be positive for *Staph. aureus* alone, and eight (8.88 %) carried a mixed infection for both. In conclusion, this study suggests that quantification of bacteria directly from milk by the conventional PCR can be an alternative to time consuming conventional culture method and expensive real-time PCR, but requires extensive standardization.

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1 Introduction

Mastitis continues to be the most important economical in modern dairy industry affecting the quality of milk, milk production, farm economics, and animal welfare. Subclinical mastitis (SCM) is considered as the main form of mastitis in modern dairy herds, affecting 20 per cent to 50 per cent of cows in given herds (Pitkala et al., 2004; Birhanu et al., 2017). In bovines, disease is most frequently caused by microbial infection, commonly associated with *Staphylococcus aureus* (*Staph. aureus*) and *Escherichia coli* (*E. coli*) which are causative agents of contagious and environmental mastitis, respectively. Detection of SCM is based on conventional tests like Somatic cell count (SCC), California mastitis test (CMT), Electrical conductivity (EC) and Bromothymol blue test (BTB) Whereas, these tests are qualitative, indirectly reflect the infection without exact etiological agent and complicated to interpret, and do not truly indicate the severity of infection (Preethirani et al., 2015). Estimation of the bacterial load could enable us to understand the severity of infection.

Currently employed techniques for quantification of bacteria are plate count method and most-probable-number (MPN) method (Pascual & Calderon 2000). The disadvantage of plate count technique is negative culture (Phuektes et al., 2001) and time-consuming (Hogan et al., 1999) whereas MPN is also laborious and time-consuming (Pascual & Calderon 2000).

Considering the limitations with special reference to longer time, manpower, and cost ineffectiveness associated with the above mentioned techniques, the use of DNA-based assays has become popular recently. The Polymerase Chain Reaction (PCR), which is highly sensitive, specific and so far have been reported only for the qualitative detection or identification of bacteria at earlier stages of infection, but application to gauge the bacterial load which inturn indicates the severity of the infection are lacking. However, real-time PCR can be used to quantify bacterial DNA and thus has potential for accurate enumeration of microorganisms, but high cost is a limiting factor for its wide applicability.

In the present study, the concept of Mole v/s Avogadro's number is explored for quantitative estimation of *Staph. aureus* and *E. coli* in bovine subclinical milk samples by conventional PCR assay. Avogadro's number is the number of elementary units such as atoms, molecules or ions in one mole of a substance. Here single copy genes, *nuc* for *Staph. aureus* and *uid*A for *E. coli* were selected based on its minimum detection limit as mentioned by Chandrashekar et al. (2015).

2 Materials and Methods

2.1 Standardization of duplex PCR

2.1.1 Strains and genomic DNA

Reference cultures of *Staph. aureus* (Accession No. JN247783) (MTCC No. 96) and *E. coli* (Accession No. JF926686), were obtained from Microbial Type Culture Collection, Chandigarh, India. The genomic DNA from reference strains were extracted from *Staph. aureus* and *E. coli* bacteria using the AMpurE Bacterial gDNA Mini Spin kit (Amnion Biosciences Pvt. Ltd., Bangalore), as per the manufacturer's recommendations. Whereas, genomic DNA from the neat and diluted field milk samples were extracted by using Cremonesi method (Cremonesi et al., 2006). The purity and concentration of the genomic DNA was estimated by using nanodrop spectrophotometer (Thermo Scientific, U.S.A.)

2.1.2 Duplex PCR

Five µl of the extracted genomic DNA from the standard reference strains were subjected to PCR containing 2.5 µl of 10X Taq Pol Assay Buffer, 0.5 µl (3 U / µl) of Taq Polymerase, 1.0 µl (10 mM / µl) of dNTP mix (i.e., 2.5 mM of each of the four dNTPs), 0.5 µl (10 picomoles / µl) of each of the forward and reverse primers, in a final volume of 25 µl (Chern et al., 2011; Hegde et al., 2013). The primers and cycling conditions are detailed in Table 1. Amplification was performed for 30 cycles in all cases. The amplified products (15 µl) were subjected to electrophoresis in 2% agarose gel stained with ethidium bromide, and visualized and documented by using automatic gel doc system (Gel Doc XR; Bio-Rad., U.S.A). The above procedure was applied for the genomic DNA extracted from field milk samples.

Table 1 Primers and cycling conditions used to amplify the single copy genes of *Staph. aureus* and *E. coli* for duplex PCR

Gene	Primer sequence 5` - 3` (Forward, Reverse)	Cycling conditions	Amplicon (bp)	Reference
nuc	GTGCTGGCATATGTATGGCAATTGT TACGCCGTTATCTGTTTGTGATGC	94^0C for 10 min 94^0C / 30 sec 57^0C / 30 sec 72^0C / 30 sec 72^0C for 10min	181	Hegde *et al*., 2013
*uid*A	ATCACCGTGGTGACGCATGTCGC CACCACGATGCCATGTTCATCTGCC		486	Chern *et al*., 2011

2.2 Sample collection

A total of ninety milk samples were collected from four organized farms (Farm A, B, C and D) and various villages (Doddamandiganahalli, Bittagowdanahalli, Kudrugundi, Aladahalli, Rachenahalli, Doddapura, Cheeranahalli, Guddenahalli) in and around Hassan based on CMT. Milk samples were collected in 10 mL sterile tubes following strict aseptic measures and immediately transported to the laboratory in refrigerated condition and processed for SCC.

Forty samples which having high SCC (> 5,00,000 cells / mL of milk) (Table 2) were log diluted in LB broth, 100 µl from each dilution was plated on MSA (*Staph. aureus*) and MCA (*E. coli*) and genomic DNA was extracted from the rest of the remaining dilutions by Cremonesi method and quantified as above (Cremonesi et al., 2006). Duplex PCR for single copy genes of *Staph. aureus* (*nuc*) and *E. coli* (*uid*A) was carried out. Quantification of bacterial load in the milk samples were estimated based on the concentration, amplicon size and size of the genomic DNA (assuming *Staph. aureus* and *E. coli* to contain 2.81 Mbp and 4.68 Mbp long genomes) by using the Avogadro`s number, formula as follows (Hein et al., 2001; Taponen et al., 2009).

Weight in Dalton (gram / mol) = (base pair size of DNA) X (330 Dalton / nucleotide X 2 nucleotides / base pair)

$$\text{Copy number} = \text{grams / molecule} = \frac{\text{gram / mole}}{\text{Avogadro's number (i.e. } 6.02214199 \times 10^{23})}$$

$$\text{No. of molecules in the reaction} = \frac{\text{Concentration of DNA (gram / µl)}}{\text{Copy number}}$$

3 Results

Out of 90, forty (44.44 per cent) and fifty (55.55 per cent) samples were found to be positive and negative respectively for subclinical mastitis based on SCC. Among these 40 positive samples, 29 (72.5 per cent) samples were found to be positive for *Staph. aureus* and *E. coli* by duplex PCR. Of these, 12 (41.38 per cent) were found to be positive for *E. coli* alone, nine (31.03 per cent) were found positive for *Staph. aureus* alone, and eight (27.59 per cent) carried a mixed infection of *E. coli* and *Staph. aureus*. Expected amplicon sizes of 181 bp and 468 bp were observed for *nuc* and *uid*A genes of *Staph. aureus* and *E. coli*, respectively in reference strains (Figure 1) and in all 40 field samples which were positive for SCM by SCC, here we have shown only three samples representing infection of *Staph. aureus* (Figure 2), *E. coli* (Figure 3) and mixed infection of both organisms (Figure 4). Bacterial load of *Staph. aureus* and *E. coli* in subclinical positive field milk samples were estimated based on the concentration of extracted genomic DNA from milk samples (Table 2).

4 Discussion and Conclusion

Subclinical mastitis, which is defined as mastitis that cannot be diagnosed by clinical examination, instead relying on the analysis of inflammatory products in the milk (Ceron-Munoz et al., 2002; Schukken et al., 2003). In most cases, direct or indirect measurement of the milk SCC is used to diagnose SCM. This is often combined with bacteriological examination of the milk to arrive at an etiological diagnosis.

The detection of bacterial etiology is primarily based on conventional isolation and characterization. Routine quantification of organisms according to International Standardization Organization and International Dairy Federation methods (Anonymous, 1995a; Anonymous, 1995b; Anonymous, 1997) apply conventional microbiological technique such as the plate count method based on the use of selective media for direct enumeration or the recovery of isolates after enrichment in selective broth for 24–48 h at 37˚C. This technique requires up to 6 days for quantification and detection, thus being time-consuming. In addition, culture-based assays can only enumerate viable organisms in a sample that can be cultured under laboratory conditions. Other methods for quantification include most-probable-number (MPN), a rather laborious and time-consuming procedure (Pascual & Calderon, 2000), and the quantification of the enzymatic activity of the bacterial phosphatase (Fang, 1996), which is not specific and can therefore be applied only to pure cultures.

Considering that the above approaches are time-consuming and can produce ambiguous results for species-level identification, PCR has been developed recently for rapid and precise identification of predominant mastitis causing bacteria, especially for *Staph. aureus*, *E. coli* and *Streptococcus* species (Jayarao et al., 1996; Forsman et al., 1997; Kim et al., 2001; Riffon et al., 2001; Daly et al., 2002; Meiri-Bendek et al., 2002; Phuektes et al., 2001; Phuektes et al., 2003; Shome et al., 2011; Sundareshan et al., 2012; Nithinprabhu et al., 2013; Hegde et al., 2013; Preethirani et al., 2015). Whereas these PCR-based approaches have so far been reported for the qualitative detection of bacteria, additional estimation of bacterial load could be of a more practical significance. Currently, enumerating bacteria in mastitic milk is being accomplished by culture-based methods to determine the colony forming units (Hogan et al., 1999). Recently, real-time PCR was developed for rapid enumeration of bacterial load (Koskinen et al., 2010; Gaibani et al., 2013). Other PCR-based methods such as hybridization can also improve the sensitivity of

Table 2 Details of the samples Viz SCC, Positive by culture and duplex PCR, Quantification based on plating and concentration of genomic DNA

Sl. No.	Sample No.	SCC / ml	Results based on culture and duplex PCR	Quantification based on Culturing (CFUs/ ml)	Quantification based on concentration of genomic DNA Nanogram per µl (Organisms per ml)
1	2	29,00,000	*Staph. aureus*	2.7×10^6	19.6 (3.9×10^8)
2	4	35,50,000	*Staph. aureus*	3×10^6	21.7(4.4×10^8)
3	5	5,50,000	*E. coli*	3×10^5	7.8 (0.8×10^8)
4	7	9,50,000	Negative	Negative	Negative
5	10	39,50,000	Mixed	E - 5×10^5 S - 2×10^6	26.2 (2.9×10^8) 26.2 (5×10^8)
6	12	5,50,000	Negative	Negative	Negative
7	14	19,50,000	*E. coli*	8×10^6	14.1 (1.6×10^8)
8	16	23,00,000	*Staph. aureus*	3.7×10^5	17.9 (3.6×10^8)
9	18	53,50,000	Mixed	E - 4×10^5 S -2.3×10^6	27.3 (3.1×10^8) 27.3 (5.5×10^8)
10	20	6,00,000	Negative	Negative	Negative
11	22	7,00,000	Negative	Negative	Negative
12	23	6,50,000	*Staph. aureus*	15×10^4	11.7 (2.37×10^8)
13	25	41,00,000	Mixed	E - 7×10^5 S - 2.1×10^5	25.9 (2.9×10^8) 25.9 (5×10^8)
14	27	50,00,000	*E. coli*	1.3×10^6	27.5 (3.1×10^8)
15	28	9,00,000	Negative	Negative	Negative
16	30	48,00,000	Mixed	E - 6×10^5 S - 2.3×10^6	21.6 (2.4×10^8) 21.6 (4.3×10^8)
17	31	39,50,000	*E. coli*	5×10^6	20.7(2.3×10^8)
18	33	5,00,000	*E. coli*	6×10^5	11.8(1.3×10^8)
19	36	22,50,000	*Staph. aureus*	2.7×10^5	16.4(3.3×10^8)
20	38	5,50,000	Negative	Negative	Negative
21	39	34,50,000	Mixed	E - 7×10^6 S - 1.3×10^6	22.9(2.6×10^8) 22.9(4.6×10^8)
22	41	16,50,000	*E. coli*	8×10^5	16.2(1.8×10^8)
23	44	5,00,000	Negative	Negative	Negative
24	46	29,00,000	*Staph. aureus*	S - 6×10^5	20.3(4.1×10^8)
25	48	16,00,000	*Staph. aureus*	S - 2×10^5	13.8 (2.8×10^8)
26	50	5,50,000	Negative	Negative	Negative
27	53	36,00,000	Mixed	E - 7×10^5 S - 2.7×10^5	20.5(2.3×10^8) 20.5(4.1×10^8)
28	54	4,50,000	*Staph. aureus*	8×10^4	7.2(1.4×10^8)
29	58	16,50,000	*E. coli*	5×10^6	16.7(1.9×10^8)
30	61	6,00,000	Negative	Negative	Negative
31	63	23,00,000	*Staph. aureus*	1.4×10^5	18.9 (3.8×10^8)
32	67	40,00,000	*E. coli*	1×10^6	14.8(1.6×10^8)
33	69	50,00,000	Mixed	E - 9×10^6 S - 2×10^6	27.1(3×10^8) 27.1(5×10^8)
34	71	11,50,000	Negative	Negative	Negative
35	73	7,50,000	*E. coli*	6×10^5	12.3(1.4×10^8)
36	76	40,00,000	Mixed	E - 8×10^6 S -1.1×10^6	23.4 (2.6×10^8) 23.4 (4.7×10^8)
37	79	24,00,000	*E. coli*	7×10^5	19.3(2.2×10^8)
38	83	7,50,000	Negative	Negative	Negative
39	87	30,00,000	*E. coli*	8×10^6	21.7(2.4×10^8)
40	90	23,00,000	*E. coli*	1×10^6	19.8(2.2×10^8)

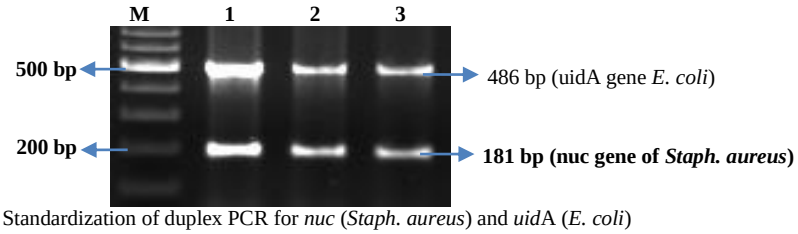

Figure 1 Standardization of duplex PCR for *nuc* (*Staph. aureus*) and *uid*A (*E. coli*) Genes (Lane M: 100 bp ladder; Lane 1 to 3: Genomic DNA of reference strains)

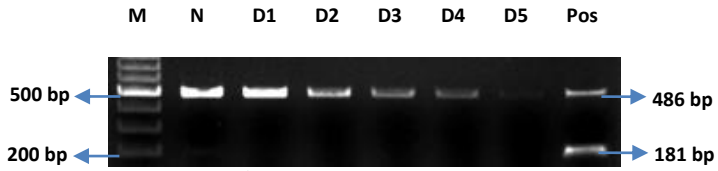

Figure 2: Duplex PCR applied for *E. coli* (Sample no. 31) (*Here - Lane M: 100 bp ladder; Lane N: 18.92 × 10^6 CFUs / 5 µl; Lane D1: 2.28 × 10^6 CFUs / 5 µl; Lane D2: 0.45 × 10^6 CFUs / 5 µl; Lane Pos: Positive control)*

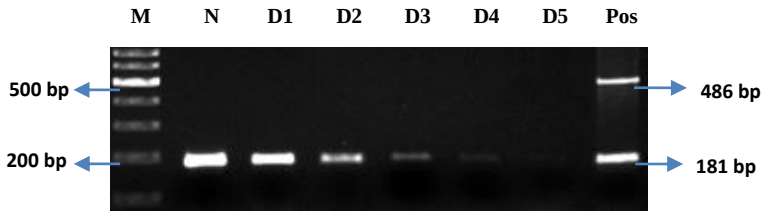

Figure 3: Duplex PCR applied for *Staph. aureus* (Sample no. 48) (*Here - Lane M: 100 bp ladder; Lane N: 22.4 × 106 CFUs / 5 µl; Lane D1: 1.46 × 106 CFUs / 5 µl; Lane D2: 0.81 × 106 CFUs / 5 µl; Lane Pos: Positive control*)

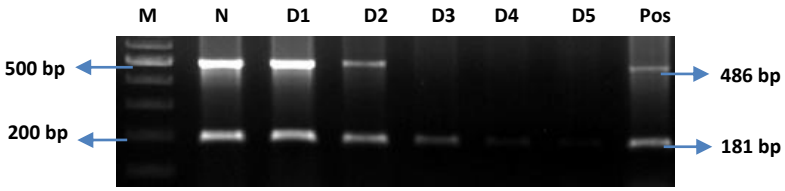

Figure 4: Duplex PCR for *uid*A of *E. coli* and *nuc* gene of *Staph. aureus* in mixed infection (Sample no. 10) (*Here - Lane M: 100 bp ladder; Lane N: E= 23.9 × 10^6 CFUs / 5 µl; S= 42.5 × 10^6 CFUs / 5 µl; Lane D1: E= 1.91 × 10^6 CFUs / 5 µl; S= 3.4 × 10^6 CFUs / 5 µl; Lane D2: E= 0.63 × 10^6 CFUs / 5 µl; S= 1.13 × 10^6 CFUs / 5 µl; Lane Pos: Positive control*)

LOD manifold and could greatly enhance analytical sensitivity. However, tedious standardization procedures, and high cost of equipment and reagents are deterrents for its routine application in the laboratory.

Polymerase chain reaction (PCR)-based methods provide a promising option for the rapid identification of bacteria. In addition, PCR can also provide information about the load of bacteria in clinical samples. Importantly, sensitivity, specificity and the LOD can be improved by adapting PCR-based technologies. This is especially important while studying the etiology and microbial load in bacterial SCM. In this context, it is important to note that identification of bacterial species as well as the load have typically depended on culturing of the organisms involving a prior enrichment step. This overnight enrichment using non-selective media is not only likely to lead to selective growth of particular organisms due to stoichiometric competition but is also expected to lead to erroneous extrapolations of the original bacterial load due to differential growth of the various species. It is therefore important to identify and enumerate the number of bacteria directly in suspected milk sample. One of the best ways to do so is to employ PCR-based techniques.

In order to translate data from studies based on LOD of PCR for determination of bacterial load, it is important to know the number of genomic copies of the genes being targeted for amplification. While multiple copy genes are useful in increasing the analytical sensitivity and hence may be useful for qualitative assays, reducing the number of genomes would be challenging with end-point titration assays targeting genes with multiple copies, especially those with unknown or variable copy numbers among different strains. Hence, targeting a gene which is carried in known number of copies by all the members of a population is critical. We chose three genes for each of these organisms as it was possible that the LOD could be better with one than with the others (Chandrashekar et al., 2015).

In this study, we used conventional PCR assay for the quantification of *Staph. aureus* and *E. coli* in bovine subclinical masitic milk samples. For this, DNA was extracted from bacteria suspension, and the concentration in ng / µl was measured, in order to calculate genome-copies / mL based on molarity (using Avogadro's number). The application of this method assumes that (a) a single viable bacterial cell produces a single CFU; (b) there is absolutely no loss of genomic DNA during DNA extraction procedure, (c) each cell contains only a single complement of the genome (Huggett et al., 2013). Assuming these to be true, and applying the method to a gene which exists only as a single copy per genome, the ratio of the concentration in CFU / mL to the concentration in genome-copies / mL would be one.

Avogadro`s number is used to generate a standard curve by calculating the number of copy for absolute quantification of bacteria by real-time PCR. In all previous studies, absolute quantification was done by using plasmid (with the molecular weight of the plasmid and insert gene was known) (Vondrakova et al., 2014) or by using single copy gene (Karpowicz et al., 2010).

In conclusion, results with conventional PCR lay the foundation for further work on the standardization of assay with *nuc* and *uid*A genes to indirectly enumerate *Staph. aureus* and *E. coli*, respectively, directly in milk, and to correlate bacterial load with clinical or subclinical mastitis.

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Conflicts of interest

No conflicts of interests are declared by authors for the contents in this manuscript.

References

Anonymous (1995a) Microbiology of food and animal feeding stuffs-horizontal method for the enumeration of coagulase positive staphylococci (*Staphylococcus aureus* and other species) by colony-count technique at 35°C/37°C. Part 1. Technique with confirmation of colonies. International Standardization Organization document 6888–1. International Standardization Organization, Geneva, Switzerland.

Anonymous (1995b) Microbiology of food and animal feeding stuffs—horizontal method for the enumeration of coagulase positive staphylococci (*Staphylococcus aureus* and other species) by colony-count technique at 35°C/37°C. Part 2. Technique without confirmation of colonies. International Standardization Organization document 6888–2. International Standardization Organization, Geneva, Switzerland.

Anonymous (1997) Milk and milk-based products: enumeration of coagulase positive staphylococci: colony count technique. International Dairy Federation standard 154A:1997. International Dairy Federation, Brussels, Belgium.

Ceron-Munoz M, Tonhati H, Duarte J, Oliveira J, Munoz-Berrocal M, Jurado-Gamez H (2002) Factors affecting somatic cell counts and their relations with milk and milk constituent yield in buffaloes. Journal of Dairy Science 85: 2885–2889.

Chandrashekar KM, Isloor S, Veeresh BH, Hegde R, Rathnamma D, Murag S, Veeregowda BM, Upendra HA, Hegde NR (2015)

Limit of detection of genomic DNA by conventional PCR for estimating the load of Staphylococcus aureus and Escherichia coli associated with bovine mastitis. Folia Microbiologica 60: 465-72.

Chern EC, Siefring S, Paar J, Doolittle M, Haugland RA (2011) Comparison of quantitative PCR assays for Escherichia coli targeting ribosomal RNA and single copy genes. Journal of Applied Microbiology 52: 298–306.

Cremonesi P, Castiglioni B, Malferrari G, Biunno I, Vimercati C, Moroni P, Morondi S, Luzzana M (2006) Technical note: improved method for rapid DNA extraction of mastitis pathogens directly from milk. Journal of Dairy Science 89: 163-169.

Daly P, Collier T, Doyle S (2002) PCR-ELISA detection of Escherichia coli in milk. Journal of Applied Microbiology 34: 222–226.

Fang W (1996) Quantification of *Staphylococcus aureus* and *Escherichia coli* in the liquid medium by fluorimetry and its use in phagocytosis assay. Journal of Applied Bacteriology 80: 577–582.

Forsman A, Tilsal-Timisjarvi PA, Alatossava T (1997) Identification of Staphylococcal and Streptococcal causes of bovine mastitis using 16S–23S rRNA spacer regions. Journal of Microbiology 143: 3491–3500.

Gaibani P, Mariconti M, Bua G, Bonora S, Sassera D, Landini MP, Mulatto P, Novati S, Bandi C, Sambri V (2013) Development of a broad-range 23S rDNA real-time PCR assay for the detection and quantification of pathogenic bacteria in human whole blood and plasma specimens. International Journal of Biological Medical Research 2013: 264651.

Hein I, Lehner A, Rieck P, Klein K, Brandl E, Wagner M (2001) Comparison of different approaches to quantify *Staphylococcus aureus* cells by real-time quantitative PCR and application of this technique for examination of cheese. Applied Environmental Microbiology 67: 3122–3126

Hegde R, Isloor S, Nithin Prabhu K, Shome BR, Rathnamma D, Suryanarayana VVS, Yatiraj S, Renuka Prasad C, Krishnaveni N, Sundareshan S, Akhila DS, Gomes AR, Hegde NR (2013) Incidence of subclinical mastitis and prevalence of major mastitis pathogens in organized farms and unorganized sectors. Indian Journal of Microbiology 53: 315–320.

Hogan JS, Gonzalez RN, Harmon RJ, Nickerson SC, Oliver SP, Pankey JW, Smith KL (1999) Laboratory handbook on bovine mastitis. National Mastitis Council, Madison, Pp.222.

Huggett J, Laver T, Tamisak S, Nixon G, O'sullivan D, Elaswarapu R (2013) Considerations for the development and application of control materials to improve metagenomic microbial community profiling. Accreditation and Quality Assurance 18: 77–83.

Jayarao BM, Gillespie BE, Oliver SP (1996) Application of randomly amplified polymorphic DNA finger printing for species identification of bacteria isolated from bovine milk. Journal of Food Protection 59: 615–620.

Karpowicz E, Novinscak A, Ba¨Rlocher F, Filion M (2010) qPCR quantification and genetic characterization of Clostridium perfringens populations in biosolids composted for 2 years. Journal of Applied Microbiology 108: 571– 581.

Kim CH, Khan M, Morin DE, Hurley WL, Tripathy DN, Kehrli M, Oluoch AO, Kakoma I (2001) Optimization of the PCR for detection of *Staphylococcus aureus* nuc gene in bovine milk. Journal of Dairy Science 84: 74-83.

Koskinen MT, Wellenberg GJ, Sampimon OC, Holopainen J, Rothkamp A, Salmikivi L, Van Haeringen WA, Lam TJGM, Pyorala S (2010) Field comparison of real-time Polymerase Chain Reaction and bacterial culture for identification of bovine mastitis bacteria. Journal of Dairy Science 93: 5707–5715.

Meiri-Bendek, Lipkin E, Friedman A, Leitner G, Saran A, Friedman S, Kashi Y (2002) A PCR based method for the detection of *Streptococcus agalactiae* in milk. Journal of Dairy Science 85: 1717-172.

Birhanu M, Leta S, Mamo G, Tesfaye S (2017) Prevalence of bovine subclinical mastitis and isolation of its major causes in Bishoftu Town, Ethiopia. BMC Research Notes10:767. doi: 10.1186/s13104-017-3100-0.

Nithinprabhu K, Isloor S, Hegde R, Rathnamma D, Veeregowda BM, Murthy HNN, Shome R, Suryanarayana VVS (2013) Development of polymerase chain reaction for detection of predominant streptococcal isolates causing subclinical mastitis. Indian Journal of Biotechnology 12: 208 – 212.

Pascual MR, Calderon V (2000) Investigation y recuento de *Staphylococcus aureus*. In: Microbiologia Alimentaria (2nd Edn) ed. Dıaz de Santos, Madrid, Spain, Pp. 77–87.

Pheuktes P, Mansell PD, Browning GF (2001) Multiplex Polymerase Chain Reaction assay for simultaneous detection of *Staphylococcus aureus* and Streptococcal causes of bovine mastitis. Journal of Dairy Science 84: 1140 – 1148.

Phuektes P, Browning GF, Anderson G, Mansell PD (2003) Multiplex polymerase chain reaction as a mastitis screening test for *Staphylococcus aureus*, *Streptococcus agalactiae*, *Streptococcus dysgalactiae* and *Streptococcus uberis* in bulk milk samples. Journal of Dairy Research 70: 149–155.

Pitkala A, Haveri M, Pyorola S, Myllys V, Buzalski HT (2004) Bovine mastitis in Finland 2001-Prevalence, distribution of bacteria, and antimicrobial resistance. Journal of Dairy Science 87: 2433 – 2441.

Preethirani PL, Isloor S, Sundareshan S, Nuthanalakshmi V, Deepthikiran K, Sinha AY, Rathnamm D, Nithinpradhu K, Sharada R, Mukkur TK, Hegde NR (2015) Isolation, biochemical and molecular identification and in-vitro antimicrobial resistance patterns of bacteria isolated from Bubaline subclinical mastitis in South India. PLoS One 10: 142717.

Riffon R, Sayasith H, Khalil H, Dubreuil P, Drolet M, Legace J (2001) Development of a rapid and sensitive test for identification of major pathogens in bovine mastitis by PCR. Journal of Clinical Microbiology 39: 2584-2589.

Schukken YH, Wilson DJ, Welcome F, Garrison-Tikofsky L, Gonzalez RN (2003). Monitoring udder health and milk quality using somatic cell counts. Veterinary Research 34: 579–596.

Shome BR, Das Mitra S, Bhuvana M, Krithiga N, Velu D, Shome R, Isloor S, Barbuddhe SB, Rahman H (2011) Multiplex PCR assay for species identification of bovine mastitis pathogens. Journal of Applied Microbiology 111: 1349–1356.

Sundareshan S, Hari Babu Y, Isloor S, Awati B, Hegde NR (2012) Isolation and phenotype-based speciation of coagulase-negative staphylococci (CoNS) isolated from bovine milk samples. Frontier Journal of Animal Science 1: 34–39.

Taponen S, Salmikivi L, Simojoki L, Simojoki H, KoskinenMT, Pyorala S (2009) Real-time polymerase chain reaction-based identification of bacteria in milk samples from bovine clinical mastitis with no growth in conventional culturing. Journal of Dairy Science 92: 2610–2617

Vondrakova L, Pazlarova J, Demnerova K (2014) Detection, identification and quantification of *Campylobacter jejuni*, coli and lari in food matrices all at once using multiplex qPCR. Gut Pathogen 6: 12.