QUANTIFICATION OF \textit{Staphylococcus aureus} AND \textit{Escherichia coli} FROM BOVINE SUBCLINICAL MILK SAMPLES BY CONVENTIONAL PCR

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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 \textit{Staphylococcus aureus} and \textit{Escherichia coli} for assessing bacterial load in milk samples from mastitis cases. The \textit{nuc} and \textit{uidA} genes produced the lowest detection limit for \textit{Staph. aureus} and \textit{E. coli} respectively. The present findings estimated that, as few as 60 and 89 genome copies or organisms of \textit{Staph. aureus} and \textit{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 \textit{E. coli} alone, nine (10 \%) were found to be positive for \textit{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 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 uidA 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 (Accesison 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.

| Gene   | Primer sequence 5’ - 3’ (Forward, Reverse) | Cycling conditions | Amplicon (bp) | Reference       |
|--------|--------------------------------------------|-------------------|---------------|----------------|
| nuc    | GTGCTGGCATAATGATGGAATGGT                   | 94°C for 10min    | 181           | Hegde et al., 2013 |
|        | TACGCCCTATCGTITTTGATGTC                   | 94°C / 30 sec     |               |                 |
|        |                                             | 57°C / 30 sec     |               |                 |
| uidA   | ATACACCCTGTGAGCATGCTGTGC                  | 72°C / 30 sec     | 486           | Chern et al., 2011 |
|        | CACCAGATGCCATGTTCATCTGCC                  | 72°C for 10min    |               |                 |

Table 1 Primers and cycling conditions used to amplify the single copy genes of Staph. aureus and E. coli for duplex PCR
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, Rachennahalli, 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 (uidA) 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)

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

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 for SCM. 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 Staph. aureus and E. coli. Expected amplicon sizes of 181 bp and 468 bp were observed for nuc and uidA 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
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| 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 (Organisms per ml) |
|---------|------------|----------|----------------------------------------|---------------------------------|-------------------------------------------------|
| 1       | 2          | 29,00,000| *Staph. aureus*                         | 2.7 × 10⁶                         | 19.6 (3.9 × 10⁸)                                 |
| 2       | 4          | 35,50,000| *Staph. aureus*                         | 3 × 10⁶                          | 21.7 (4.4 × 10⁸)                                |
| 3       | 5          | 5,50,000 | *E. coli*                               | 3 × 10⁹                          | 7.8 (0.8 × 10⁴)                                 |
| 4       | 7          | 9,50,000 | Negative                                | Negative                         | Negative                                        |
| 5       | 10         | 39,50,000| Mixed                                  | E - 5 × 10⁷                      | 26.2 (2.9 × 10⁴)                                |
| 6       | 12         | 5,50,000 | Negative                                | Negative                         | Negative                                        |
| 7       | 14         | 19,50,000| *E. coli*                               | 8 × 10⁶                          | 14.1 (1.6 × 10⁴)                                |
| 8       | 16         | 23,00,000| *Staph. aureus*                         | 3.7 × 10⁷                         | 17.9 (3.6 × 10⁴)                                |
| 9       | 18         | 53,50,000| Mixed                                  | E - 4 × 10⁹                      | 27.3 (3.1 × 10⁴)                                |
| 10      | 20         | 6,00,000 | Negative                                | Negative                         | Negative                                        |
| 11      | 22         | 7,00,000 | Negative                                | Negative                         | Negative                                        |
| 12      | 23         | 6,50,000 | *Staph. aureus*                         | 15 × 10⁴                         | 11.7 (2.37 × 10⁵)                               |
| 13      | 25         | 41,00,000| Mixed                                  | E - 7 × 10⁸                      | 25.9 (2.9 × 10⁸)                                |
| 14      | 27         | 50,00,000| *E. coli*                               | 1.3 × 10⁹                         | 27.5 (3.1 × 10⁴)                                |
| 15      | 28         | 9,00,000 | Negative                                | Negative                         | Negative                                        |
| 16      | 30         | 48,00,000| Mixed                                  | E - 6 × 10⁸                      | 21.6 (2.4 × 10⁸)                                |
| 17      | 31         | 39,50,000| *E. coli*                               | 5 × 10⁶                          | 20.7 (2.3 × 10⁸)                                |
| 18      | 33         | 5,00,000 | *E. coli*                               | 6 × 10⁵                          | 11.8 (1.3 × 10⁸)                                |
| 19      | 36         | 22,50,000| *Staph. aureus*                         | 2.7 × 10⁷                         | 16.4 (3.3 × 10⁶)                                |
| 20      | 38         | 5,50,000 | Negative                                | Negative                         | Negative                                        |
| 21      | 39         | 34,50,000| Mixed                                  | E - 7 × 10⁸                      | 22.9 (2.6 × 10⁷)                                |
| 22      | 41         | 16,50,000| *E. coli*                               | 8 × 10⁶                          | 16.2 (1.8 × 10⁷)                                |
| 23      | 44         | 5,00,000 | Negative                                | Negative                         | Negative                                        |
| 24      | 46         | 29,00,000| *Staph. aureus*                         | S - 6 × 10⁶                      | 20.3 (4.1 × 10⁸)                                |
| 25      | 48         | 16,00,000| *Staph. aureus*                         | S - 2 × 10⁵                      | 13.8 (2.8 × 10⁸)                                |
| 26      | 50         | 5,50,000 | Negative                                | Negative                         | Negative                                        |
| 27      | 53         | 36,00,000| Mixed                                  | E - 7 × 10⁹                      | 20.5 (2.3 × 10⁹)                                |
| 28      | 54         | 4,50,000 | *Staph. aureus*                         | 8 × 10⁴                          | 7.2 (1.4 × 10⁴)                                 |
| 29      | 58         | 16,50,000| *E. coli*                               | 5 × 10⁶                          | 16.7 (1.9 × 10⁷)                                |
| 30      | 61         | 6,00,000 | Negative                                | Negative                         | Negative                                        |
| 31      | 63         | 23,00,000| *Staph. aureus*                         | 1.4 × 10⁸                        | 18.9 (3.8 × 10⁸)                                |
| 32      | 67         | 40,00,000| *E. coli*                               | 1 × 10⁶                          | 14.8 (1.6 × 10⁸)                                |
| 33      | 69         | 50,00,000| Mixed                                  | E - 9 × 10⁹                      | 27.1 (3 × 10⁸)                                  |
| 34      | 71         | 11,50,000| Negative                                | Negative                         | Negative                                        |
| 35      | 73         | 7,50,000 | *E. coli*                               | 6 × 10⁵                          | 12.3 (1.4 × 10⁸)                                |
| 36      | 76         | 40,00,000| Mixed                                  | E - 8 × 10⁹                      | 23.4 (2.6 × 10⁸)                                |
| 37      | 79         | 24,00,000| *E. coli*                               | 7 × 10⁵                          | 19.3 (2.2 × 10⁸)                                |
| 38      | 83         | 7,50,000 | Negative                                | Negative                         | Negative                                        |
| 39      | 87         | 30,00,000| *E. coli*                               | 8 × 10⁶                          | 21.7 (2.4 × 10⁸)                                |
| 40      | 90         | 23,00,000| *E. coli*                               | 1 × 10⁵                          | 19.8 (2.2 × 10⁸)                                |
Figure 1 Standardization of duplex PCR for *nuc* (Staph. aureus) and *uidA* (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: $18.92 \times 10^6$ CFUs / 5 µl; Lane D1: $2.28 \times 10^6$ CFUs / 5 µl; Lane D2: $0.45 \times 10^6$ 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: $22.4 \times 10^6$ CFUs / 5 µl; Lane D1: $1.46 \times 10^6$ CFUs / 5 µl; Lane D2: $0.81 \times 10^6$ CFUs / 5 µl; Lane Pos: Positive control)

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

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