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Cultural and metagenomic based identification of a microbiome from subclinical mastitis in cows

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ABSTRACT

Metagenomic and traditional microbial culture based analyses of milk samples from cows harbouring subclinical mastitis pathogens were carried out to identify the microbial community structure of milk. A total of 77 Triple cross (TP), Kankrej and Gir lactating cows and 301 quarters were screened for subclinical mastitis. A total of 106 isolates belonging to five different microbial genera were recovered from 91 quarters of 41 cows, including 15 quarters having mixed bacterial infections by cultural examination. Pyrosequencing readings obtained from the breed wise pooled DNA of subclinical mastitis milk samples were analyzed using the SEED subsystem database of Meta Genome Rapid Annotation with Subsystem Technology (MG-RAST). Among the five genera, *Staphylococcus, Streptococcus, Micrococcus, Bacillus* and *Escherichia,* detected in the subclinical mastitis milk samples by culture based methods, four genera, *Staphylococcus, Streptococcus, Bacillus* and *Escherichia,* were identified in the corresponding pyrosequencing data, while *Micrococcus* was not found. In contrast, the pyrosequencing yielded 28 bacterial species, of which only two species, *S. aureus* and *E. coli,* were identified by the cultural method. *S. agalactiae, t*he third species identified by cultural method, was not found in the pyrosequencing data. Metagenomic analysis additionally identified 19 genera and 26 species in comparison with the routine cultural methods. Many of the fastidious / anaerobic bacterial organisms, which are difficult to cultivate by routine methods, were identified by metagenomic analyses.

Key words: subclinical mastitis, metagenomic, cows

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Introduction

Subclinical mastitis occurs without visible changes in the appearance of the milk and/or the udder, but milk production decreases by 10 to 20 per cent, with an undesirable effect on its constituents and nutritional value, rendering it low quality and unfit for processing (HOLDWAY, 1992). The annual economic loss due to mastitis in India has been estimated to be 7165 crores (BASAL and GUPTA, 2010). Mastitis is a multietiological disease, of which about 95 per cent of the reported cases are caused by *Streptococcus, Staphylococcus* and *E. coli* (JAIN et al., 2012; KANDEMIR et al., 2013).

Metagenomics is presently entering a new phase of development as the implementation of the massively parallel throughput afforded by second-generation sequencing approaches becomes more widespread and applied to an increasing number of environments. Traditional dideoxy termination (Sanger) based DNA sequencing in metagenomics historically relied on large insert libraries, propogated in an *E. coli* host. Genes that may have been otherwise unstable or toxic in the cloning vector host cell can be accessed without cloning bias in the sequencing profile. In addition to circumventing the need for cloning, the throughput afforded by second-generation sequencing technology enables a new approach to comparative metagenomics (MARGULIES et al., 2005). Sequence representation (abundance) can now be used to contextualize datasets for driving pattern recognition and uncovering unique properties within natural microbial communities.

Pyrosequencing is a relatively new molecular technique with an incredible potential for metagenomic analysis. It is based upon what is known as a "sequencing-by-synthesis" method, utilizing specific enzymes to record each nucleotide inserted into a complementary DNA strand (AHMADIAN et al., 2006). The pyrosequencing technique has been used successfully to evaluate the microbial diversity of soil samples, detect medically significant pathogens, and distinguish different species of *Mycobacteria (*JONASSON et al., 2002; ROESCH et al., 2007; TUOHY et al., 2005). Recently, work related to milk microbiome signatures of subclinical mastitis-affected cattle has been reported (BHATT et al., 2012), and it highlighted the usefulness of the metagenomic approach in understanding the possible role of the microbiome pool in subclinical mastitis in a comprehensive way. Although the concept behind pyrosequencing was developed in the 1980s, the actual procedure was not presented before the mid-1990s by a group of researchers at the Royal Institute of Technology in Stockholm (AHMADIAN et al., 2006; RONAGHI et al., 1996).

Only 0.001-0.1 per cent of the total microbes in sea water, 0.25 per cent in fresh water, 0.25 per cent in sediments and only 0.3 per cent soil microorganisms are cultivable *in vitro* (AMANN et al., 1995). Further, milk culture may yield no bacteria from truly sub-clinically infected glands due to the presence of very low numbers of pathogens when samples are collected (PHUEKTES et al., 2001). The "no-growth" samples have remained

problematic for mastitis laboratories, veterinarians, and dairy producers, and studies have reported failure of growth of bacteria in up to 30 per cent of milk samples from clinical and subclinical bovine mastitis, even after 48 h of conventional culture (SHARMA et al., 2009). A number of workers carried out metagenomic studies using different samples (ANDERSSON et al., 2008; BHAYA et al., 2007; TURNBAUGH et al., 2009). Looking to the economic importance of subclinical mastitis, the multiple etiological factors and paucity of documented research on metagenomic analysis of subclinical mastitis samples, the present research was carried to determine the complex microbial diversity in mixed populations causing subclinical mastitis in cows.

Materials and **methods**

Samples. A total of 77 lactating cows were included, comprising: 31 Triple cross (TP) (Kankrej × Jersey × Holstein Friesian), 29 Kankrej and 17 Gir affiliated with the farms of the College of Veterinary Science and Animal Husbandry, Anand Agricultural University, Anand, India. A total of 301 quarters were screened for subclinical mastitis, following the guidelines of the International Dairy Federation (1987). Seven quarters from five cows could not be included, because two cows had two nonfunctional quarters each and three cows each had one quarter nonfunctional.

Isolation and *identification of bacteria from the affected quarters.* About 0.01 mL of thoroughly mixed quarter milk samples were inoculated onto 5 per cent sheep blood agar, for primary bacterial isolation. The plates were incubated at 37 °C for 48 h. Following the incubation, the plates were examined for bacterial growth and the morphological characteristics of the bacterial colonies were recorded. Identification of the bacterial isolates was performed as per the method described by Cowan and Steel (COWAN and STEEL, 1974).

Isolation of genomic DNA from the subclinical mastitis milk samples. Milk samples of TP, Kankrej and Gir cows, which were found positive for subclinical mastitis, were used for DNA extraction and breed wise pooled DNA was used for metagenomic analysis. Before being subjected to DNA extraction, milk was filtered through 3 µ nitro cellulose filters to remove somatic cells and separate microbial agents, and then the DNA template was extracted from the subclinical mastitis milk samples according to CREMONESI et al. (2006). DNA was quantified by ND-1000 spectrophotometer (Nanodrop Technologies, Inc USA).

Pyrosequencing and *Sequence Analysis.* The breed wise pooled DNA of all three breeds of cows were subjected to a single pyrosequencing run, using a 454 Life Sciences technology based high throughput sequencer (GS FLX 454 Life Sciences). In brief, the samples were mobilized to generate smaller fragments 600-800 bp in size, and the fragments were processed as described by the manufacturer, to apply adaptors at the ends, emulsion PCR and pyrosequencing. Sequencing was carried out for 200 cycles with the flow of A, T, G and C nucleotides sequentially and image capture. Capture images were processed by image processing software.

Bioinformatics and *statistical analysis.* Data generated after the sequencing were transferred to the High throughput cluster work station and then analysed using the inbuilt software GS Run Browser provided by Roche, for image processing and signal processing. The generated data output was in the form of FASTA reads. The reads obtained in FASTA format of TP, Kankrej and Gir cows were uploaded into MG-RAST server version 2.0 (http://metagenomics.nmpdr.org) (MEYER et al., 2008; OVERBEEK et al., 2005) for further taxonomic information on the number of significant hits of microorganisms present in the pooled DNA of subclinical mastitis milk samples of TP, Kankrej and Gir cows, and to compare our data sets against the database of other microorganisms available in the SEED subsystem of MG-RAST, which is referred to as the SEED database (MEYER et al., 2008). The IDs given by MG-RAST in the present study were: 4448440.3, 4448441.3 and 4448442.3 for TP, Kankrej and Gir cows respectively.

Results

Isolation and *identification of bacteria from the affected quarters.* A total of 106 isolates of five different microbial genera viz.: *Staphylococcus, Streptococcus, Micrococcus, Escherichia* and *Bacillus,* were recovered from 91 quarters of 41 cows, including 15 quarters with mixed bacterial infections on cultural examination. Amongst the isolates, *Staphylococci* were the most predominant bacterial species, followed by *Str. agalactiae, Micrococci, E. coli* and *Bacillus species.*

Metagenomic analysis of subclinical mastitis milk samples. In the present study, the pyrosequencing reads obtained from the breed wise pooled DNA of subclinical mastitis milk samples of TP, Kankrej and Gir cows were analyzed using the SEED subsystem database of MG-RAST. The MG-RAST server was designed for analyzing complete, or near-complete, archaeal and bacterial genomes and the set of organisms present in the sample, and it makes this available via the phylogenetic profile. In the present study, the Eukaryote group was not further analyzed and was eliminated because of it matches the mammalian genome using the SEED subsystem database of the MG-RAST.

TP cows. The TP MASTITIS READS data set contained 1,960 contigs totaling 274,190 basepairs (bp) with an average fragment length of 139.89 bp. The longest sequence length was 560 bp and shortest sequence length 40 bp. A total of 54 sequences (2.76%) could be matched to proteins in the SEED subsystem (using an E-value of 1×10^{-5}). The metagenome received 122 hits against the SEED protein non-redundant database (6.22 % of the fragments) and, on the zero hits against the ribosomal RNA database Greengenes (0.00%) using an E-value of 1×10^{-5} and a minimum alignment length of 50 bp.

Classification of microbial communities based on SEED subsystem. Only 2.90 per cent (57/1960) of the sequences could be phylogenetically identified to the domain level in SEED database using an E-value of 1×10^{-5} and 49.12 per cent (28) of these were

Bacteria with Eukaryote accounting for 50.88 per cent (29). In the bacterial domain, the different hierarchies of bacterial classification identified were phylum (4), class (6), subclass/order (11), family (15), genus (18) and species (21). The bacterial phylum identified were Chlamydiae/Verrucomicrobia group 3.57 per cent (1), Actinobacteria 10.71 per cent (3), Proteobacteria 64.29 per cent (18) and Firmicutes 21.43 per cent (6). Six classes of bacteria identified were Actinobacteria (4), Chlamydiae (1), Bacilli (5), Alphaproteobacteria (1), Betaproteobacteria (6) and Gammaproteobacteria (11). Eleven subclasses/order, fifteen families, eighteen bacteria genus and twenty-one bacteria species were identified (Table 1).

Kankrej cows. The KANKREJ MASTITIS data set contained 170 contigs, totaling 17,727 bp, with an average fragment length of 104.28 bp. The longest sequence length was 327 bp and the shortest sequence length 41 bp. A total of 39 sequences (22.94%) could be matched to proteins in the SEED subsystem (using an E-value of 1×10^{-5}), based on 57 hits against the SEED protein non-redundant database (33.53 % of the fragments) and on 2 hits against the ribosomal RNA database Greengenes (1.18%), using an E-value of 1×10^{-5} and a minimum alignment length of 50 bp.

Classification of microbial communities based on the SEED subsystem. Only 7.65 per cent (13/170) of the sequences could be phylogenetically identified to the domain level in the SEED database, using an E-value of 1×10^{-5} and 100 per cent (13) of these were Bacteria. In the bacterial domain, the different hierarchies of bacterial classification identified were phylum (3), class (5), order (6), family (6), genus (7) and species (7). Amongst the bacterial phylum identified were Thermotogae 7.69 per cent (1), Proteobacteria 15.38 per cent (2) and Firmicutes 76.92 per cent (10). Five classes of bacteria identified were Bacilli (9), Mollicutes (1), Alphaproteobacteria (1), Betaproteobacteria (1) and Thermotogae (1). Six orders and families, seven genus and species were identified (Table 2).

Gir cows. The GIR MASTITIS data set contained 301 contigs totaling 42,548 bp with an average fragment length of 141.36 bp. The longest sequence length was 454 bp and the shortest sequence length 40 bp. A total of 12 sequences (3.99%) could be matched to proteins in the SEED subsystem (using an E-value of 1×10^{-5}) based on 21 hits against the SEED protein non-redundant database (6.98 % of the fragments) and on the 0 hit against the ribosomal RNA database Greengenes (0.00%) using an E-value of 1×10^{-5} and a minimum alignment length of 50 bp.

Classification of microbial communities based on the SEED subsystem. Only 4.32 per cent (13/301) of the sequences could be phylogenetically identified to the domain level in the SEED database using an E-value of 1×10^{-5}. Of these, 84.62 per cent (11) were Bacteria, and Eukaryota accounted for 15.38 per cent (2). In the bacterial domain, the different hierarchies of bacterial classification identified were phylum (2), class (2), order (4), family (4), genus (5) and species (6). The bacterial phyla identified were Proteobacteria 90.91 per cent (10) and Firmicutes 9.09 per cent (1). The two classes

Table 1. Identification of bacteria to the species level from subclinical mastitis milk samples of TP cows

Sr. No.	Domain	Phylum	Class	Subclasses/Order	Family / Genus	Species	Hits
1	Bacteria	Actinobacteria	Actinobacteria	Actinobacteridae/ Actinomycetales	Microbacteriaceae / *Leifsonia*	*Leifsonia xyli* subsp. *xyli*	1
2					Propionibacteriaceae / *Propionibacterium*	*Propionibacterium acnes*	1
3					Streptomycetaceae / *Streptomyces*	*Streptomyces coelicolor*	1
4		Chlamydiae/ Verrucomicrobia group Chlamydiales	Chlamydiae	Chlamydiae	Chlamydiaceae / *Chlamydophila*	*Chlamydophila abortus*	1
5		Firmicutes	Bacilli	Bacillales	Bacillaceae / *Bacillus*	*Bacillus* sp.	1
6					Staphylococcaceae / *Staphylococcus*	*Staphylococcus aureus* subsp. *aureus*	1
7						*Staphylococcus epidermidis*	2
8				Lactobacillales	Lactobacillaceae / *Lactobacillus*	*Lactobacillus acidophilus*	1
9					Streptococcaceae / *Streptococcus*	*Streptococcus mitis*	1
10		Proteobacteria	Alphaproteobacteria	Caulobacterales	Caulobacteraceae / *Caulobacter*	*Caulobacter* sp.	1

Table 1. Identification of bacteria to the species level from subclinical mastitis milk samples of TP cows (continued)

Sr. No.	Domain	Phylum	Class	Subclasses/Order	Family / Genus	Species	Hits
11	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Burkholderiaceae / *Burkholderia*	*Burkholderia cenocepacia*	1
12						*Burkholderia cepacia*	1
13					Burkholderiaceae / *Ralstonia*	*Ralstonia solanacearum*	3
14				Nitrosomonadales	Nitrosomonadaceae / *Nitrosomonas*	*Nitrosomonas europaea*	1
15				Alteromonadales	Pseudoalteromonadaceae / *Pseudoalteromonas*	*Pseudoalteromonas atlantica*	1
16				Enterobacteriales	Enterobacteriaceae / *Salmonella*	*Salmonella* Dublin	1
17				Enterobacteriales	Enterobacteriaceae / *Serratia*	*Serratia marcescens*	4
18			Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae / *Azotobacter*	*Azotobacter vinelandii*	1
19					Pseudomonadaceae / *Pseudomonas*	*Pseudomonas aeruginosa*	1
20						*Pseudomonas mendocina*	2
21				Xanthomonadales	Xanthomonadaceae / *Stenotrophomonas*	*Stenotrophomonas maltophilia*	1

Table 2. Identification of bacteria to the species level from subclinical mastitis milk samples of Kankrej cows using pyrosequencing

Sr. no	Domain	Phylum	Class	Subclasses / Order	Family / Genus	Species	Hits
1	Bacteria	Firmicutes	Bacilli	Bacillales	Bacillaceae / *Bacillus*	*Bacillus subtilis* subsp. *subtilis*	7
2					Bacillaceae / Exiguobacterium	*Exiguobacterium* sp.	1
3				Lactobacillales	Lactobacillaceae / *Lactobacillus*	*Lactobacillus delbrueckii* subsp. *bulgaricus*	1
4			Mollicutes	Acholeplasmatales	Acholeplasmataceae / *Phytoplasma*	Aster yellows witches'-broom phytoplasma AYWB	1
5		Proteobacteria	Alphaproteobacteria	Rhizobiales	Phyllobacteriaceae / *Parvibaculum*	*Parvibaculum lavamentivorans*	1
6			Betaproteobacteria	Burkholderiales	Burkholderiaceae / *Ralstonia*	*Ralstonia solanacearum*	1
7		Thermotogae	Thermotogae	Thermotogales	Thermotogaceae / *Thermococcus*	*Thermosipho melanesiensis*	1

Table 3. Identification of bacteria to the species level from subclinical mastitis milk samples of Gir cows using pyrosequencing

Sr. no	Domain	Phylum	Class	Subclasses / Order	Family / Genus	Species	Hits
1	Bacteria	Firmicutes	Bacilli	Bacillales	Bacillaceae / *Bacillus*	*Bacillus* sp.	1
2		Proteobacteria	Gamma -proteobacteria	Aeromonadales	Aeromonadaceae / *Aeromonas*	*Aeromonas hydrophila* subsp. *hydrophila*	1
3				Enterobacteriales	Enterobacteriaceae / *Escherichia*	*Escherichia coli*	1
4					Enterobacteriaceae / *Shigella*	*Shigella boydii*	1
5				Pseudomonadales	Pseudomonadaceae / *Pseudomonas*	*Pseudomonas aeruginosa*	6
6							
7							
8						*Pseudomonas fluorescens*	1

of bacteria identified were Bacilli (1) and Gammaproteobacteria (10). Four orders and families, five genera and six species were identified (Table 3).

Breed wise comparison between cultural and metagenomic based identification

TP cows. In the cultural based methods, five types of the bacteria were identified at genus level (*Staphylococcus, Streptococcus, Micrococcus, Bacillus* and *Escherichia)* and amongst the genera, *S. aureus, Str. agalactiae* and *E. coli* were identified at species level. In the comparison between cultural and metagenomic based identification, the genera *Staphylococcus, Streptococcus, Bacillus* and *Escherichia,* identified by cultural methods, were also identified by pyrosequencing, while *Micrococcus,* identified by the cultural method, was not found by pyrosequencing. Fifteen bacterial genera, viz.: *Leifsonia, Propionibacterium, Streptomyces, Chlamydophila, Lactobacillus, Caulobacter, Burkholderia, Ralstonia, Nitrosomonas, Pseudoalteromonas, Salmonella, Serratia, Azotobacter, Pseudomonas* and *Stenotrophomonas,* identified at genus level in pyrosequencing, were not identified by cultural methods. In the cultural methods, *S. aureus,* identified at species level, was also identified in pyrosequencing. However, *Str. agalactiae* and *E. coli,* identified by cultural methods, were not identified by pyrosequencing. Eighteen species: *Leifsonia xyli, Propionibacterium acnes, Streptomyces coelicolor, Chlamydophila abortus, Staphylococcus epidermidis, Lactobacillus acidophilus, Streptococcus mitis, Burkholderia cenocepacia, Burkholderia cepacia, Ralstonia solanacearum, Nitrosomonas europaea, Pseudoalteromonas atlantica, Salmonella* Dublin, *Serratia marcescens, Azotobacter vinelandii, Pseudomonas aeruginosa, Pseudomonas mendocina* and *Stenotrophomonas maltophilia,* identified in pyrosequencing, were not identified by cultural methods.

Kankrej cows. In the cultural based methods, four genera, *Staphylococcus, Streptococcus, Micrococcus* and *Bacillus,* were identified, and amongst these genera, *S. aureus* and *Str. agalactiae* were identified at species level. In the comparison between cultural and metagenomic methods, the genus *Bacillus,* identified by cultural methods, was also identified by pyrosequencing, while *Staphylococcus, Streptococcus* and *Micrococcus genera,* identified by cultural methods, were not found by the pyrosequencing method. Six bacterial genera: *Exiguobacterium, Lactobacillus, Phytoplasma, Parvibaculum, Ralstonia* and *Thermococcus,* identified at genus level in pyrosequencing, were not found by cultural methods. In the cultural methods, *S. aureus* and *S. agalactiae,* identified at species level, were not identified by pyrosequencing. In pyrosequencing, six bacterial species *Bacillus subtilis, Lactobacillus delbrueckii, Aster yellows witches'-broom phytoplasma, Parvibaculum lavamentivorans, Ralstonia solanacearum* and *Thermosipho melanesiensis* identified, were not found by cultural methods.

Gir cows. In the cultural methods, the genera *Staphylococcus, Streptococcus, Micrococcus* and *Escherichia* were identified. In the comparison of cultural methods with pyrosequencing, the *Escherichia* genus, identified by cultural method, was also found by

the pyrosequencing method. However, *Staphylococcus, Streptococcus* and *Micrococcus* genera were not found in pyrosequencing. The genera *Bacillus, Aeromonas, Shigella* and *Pseudomonas,* identified by the pyrosequencing method, were not cultivated by cultural methods. In the culture based method, the species *Escherichia coli* identified was also found in pyrosequencing, but *S. aureus* and *Str. agalactiae,* cultivated by cultural methods, were not found in the pyrosequencing data. In pyrosequencing, the species *Aeromonas hydrophila, Shigella boydii, Pseudomonas aeruginosa* and *Pseudomonas fluorescens* identified were not observed by cultural methods.

Discussion

It is widely accepted that up to 99% of the microbes in the environment cannot be readily cultivated (KAMAGATA and TAMAKI, 2005; SEKIGUCHI, 2006). To overcome these difficulties and the limitations associated with cultivation techniques, different DNA-based molecular methods have been developed for characterizing microbial species and assemblages, and these have significantly influenced our understanding of microbial diversity and ecology (DELONG, 2005).

Overall comparison between cultural and metagenomic based identification. An evaluation of the data obtained by the culture based and metagenomic approaches shows that these two methods display a staggering disparity in the results obtained by each method. Overall, out of five genera, *Staphylococcus, Streptococcus, Micrococcus, Bacillus* and *Escherichia,* detected in the subclinical mastitis milk samples of TP, Gir and Kankrej breeds by culture based methods, four genera, *Staphylococcus, Streptococcus, Bacillus* and *Escherichia,* were identified in the corresponding pyrosequencing data, while *Micrococcus,* identified by culture based methods, was not found in the pyrosequencing data.

In contrast, pyrosequencing yielded 28 bacterial species, of which only two species, *S. aureus* and *E. coli,* were identified by the cultural methods. *Str. agalactiae,* the third species identified by the cultural method, was not found in the pyrosequencing data. In pyrosequencing, overall 28 bacterial species were identified from all the three breeds of cows, viz: *Leifsonia xyli, Propionibacterium acnes, Streptomyces coelicolor, Chlamydophila abortus, S. aureus, Staphylococcus epidermidis, Lactobacillus acidophilus, Streptococcus mitis, Burkholderia cenocepacia, Burkholderia cepacia, Ralstonia solanacearum, Nitrosomonas europaea, Pseudoalteromonas atlantica, Salmonella* Dublin, *Serratia marcescens, Azotobacter vinelandii, Pseudomonas aeruginosa, Pseudomonas mendocina, Stenotrophomonas maltophilia, Bacillus subtilis, Lactobacillus delbrueckii,* Aster yellows witches'-broom phytoplasma, *Parvibaculum lavamentivorans, Thermosipho melanesiensis, Aeromonas hydrophila, E. coli, Shigella boydii* and *Pseudomonas fluorescens.* Of these, apart from *S. aureus* and *E. coli,* 26 bacterial species were additionally identified and not by the culture based method.

The results revealed that Proteobacteria and Firmicutes were the main phyla in the milk sampled from three breeds. Proteobacteria is a diverse phylum and includes a wide variety of pathogens (MADIGAN and MARTINKO 2005). Proteobacteria are Gram-negative and are considered as environmental mastitis pathogens (HOGAN et al., 1999), while Gram-positive Firmicutes are generally considered as contagious mastitis pathogens (SMITH and HOGAN, 1995).

Public health importance. In the present study, out of 28 bacterial species identified by the metagenomic method, seventeen species are known to cause disease or opportunistic infections in humans. These are: *Propionibacterium acnes, Chlamydophila abortus, S. aureus, Staphylococcus epidermidis, Streptococcus mitis, Burkholderia cenocepacia, Burkholderia cepacia, Salmonella* Dublin, *Serratia marcescens, Pseudomonas aeruginosa, Pseudomonas mendocina, Stenotrophomonas maltophilia, Bacillus subtilis, Aeromonas hydrophila, E. coli, Shigella boydii* and *Pseudomonas fluorescens.* Identification of these organisms may form a useful database in future to plan a strategy to apply public health measures with regards to milk or milk products.

Unidentified sequences in metagenomic analysis. The data read were analyzed by metagenome rapid annotation using subsystem technology, Metagenomics RAST server 2.0 (http://metagenomics.anl.gov) (MEYER et al., 2008). MG-RAST uses BLAT to find sequences in the metagenomic dataset which are homologous to sequences in the M5NR database. The alignments are the comparison of two or more sequences showing the degree of similarity.

In the present findings, a major portion of the sequences obtained in the metagenomic data were not identified in the SEED subsystem of MG-RAST, because of their lower correlation to known organisms. As newly discovered organisms are defined and their 16S sequences added to the MG-RAST databases, these organisms will be positively identified.

Unifying the findings of metagenomic analysis, it can be inferred that the analysis yielded an in-depth picture of the possible bacterial organisms involved in the udder environment. The disparity with the cultural method might be due to a focused attempt to apply the routinely used cultural methods, and this might have resulted in missing a few of the organisms, which could have been obtained by adding more protocols for cultural methods. However, the real scientific output, obtained by the metagenomic analysis, may be with regards to identifying those organisms, which are difficult to cultivate or have never been mentioned as mastitis pathogens / organisms. They also might have been missed by cultural methods because of their very low concentration in subclinical mastitis milk, but were promptly detected by the highly sensitive pyrosequencing approach. The data thus generated may prove to be useful in considering these newly identified organisms in the future course of technical programmes and future approaches to dealing with the problem of subclinical mastitis.

Conclusions

The present study revealed that Metagenomic analysis of subclinical mastitis samples of TP, Kankrej and Gir cows identified bacterial organisms belonging to phyla (5), class (8), Subclass / order (15), Family (19), Genus (23) and species (28). Metagenomic analysis additionally identified 19 genera and 26 species in comparison with the routine cultural methods. Many of the fastidious / anaerobic bacterial organisms, which are difficult to cultivate by routine methods, were identified by metagenomic analyses.

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References

AHMADIAN, A., M. EHN, S. HOBER (2006): Pyrosequencing: history, biochemistry and future. Clinica Chimica Acta 363, 83-94.

AMANN, R. I., W. LUDWIG, K. H. SCHLEIFER (1995): Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. Microbiol. Rev. 59, 143-169.

ANDERSSON, A. F., M. LINDBERG, H. JAKOBSSON, F. BÄCKHED, P. NYRÉN, L. ENGSTRAND (2008): Comparative analysis of human gut microbiota by barcoded pyrosequencing. PLoS ONE 3, e2836.

BASAL, B. K., D. K. GUPTA (2010): Economic analysis of bovine mastitis in India at Punjab - Review. Indian J. Dairy Sci. 62), 337-345.

BHATT, V. D., V. B. AHIR, P. G. KORINGA, S. J. JAKHESARA, D. N. RANK, D. S. NAURIYAL, A. P. KUNJADIA, C. G. JOSHI (2012): Milk microbiome signatures of subclinical mastitis-affected cattle analysed by shotgun sequencing. J. Appl. Microbiol. 112, 639-650.

BHAYA, D., A. R. GROSSMAN, A. S. STEUNOU, N. KHURI, F. M. COHAN, N. HAMAMURA, M. C. MELENDREZ, M. M., BATESON, D. M. WARD, J. F. HEIDELBERG (2007): Population level functional diversity in a microbial community revealed by comparative genomic and metagenomic analyses. ISME 1, 703-713.

COWAN, S.T., K. J. STEEL (1974): Manual for the Identification of Medical Bacteria. Cambridge: Cambridge University Press, pp.140-43.

CREMONESI, P., B. CASTIGLIONI, G. MALFERRARI, I. BIUNNO, C. VIMERCATI, P. MORONI, S. MORANDI, M. LUZZANA (2006): Technical Note: Improved method for rapid DNA extraction of mastitis pathogens directly from milk. J. Dairy Sci. 89, 163-169.

DELONG, E. F. (2005): Microbial community genomics in the ocean. Nature Rev. Microbiol. 3, 459-469.

HOGAN J. S., R. N.GONZALEZ, R. J. HARMON, S. C. NICKERSON, S. P. OLIVER, J. W. PANKEY, K. L. SMITH, (1999): Laboratory Handbook on Bovine Mastitis, National Mastitis Council, Inc., Madison, Wisconsin, USA.

HOLDWAY, R. J. (1992): Bovine mastitis in New Zealand dairy herds. Part III. The cost of mastitis to the New Zealand dairy farmers during the 1991/1992 dairy season. Published report to the livestock improvement corporation, Hamilton.

INTERNATIONAL DAIRY FEDERATION (IDF) (1987): Defination and guidelines for diagnosis of bovine mastitis. Bull. Intl. Dairy Fed., pp. 258.

JAIN, B., A. TEWARI, B. B. BHANDARI, M. K. JHALA (2012): Antibiotic resistance and virulence genes in *Streptococcus agalactiae* isolated from cases of bovine subclinical mastitis. Vet. arhiv 82, 423-432.

JONASSON, J., M. OLOFSSON, H. MONSTEIN (2002): Classification, identification, and subtyping of bacteria based on pyrosequencing and signature matching of 16S rDNA fragments. Acta Pathol. Microbiol. Immunol. Scand. 110, 263-272.

KAMAGATA, Y., H. TAMAKI H (2005): Cultivation of uncultured fastidious microbes. Microbes Environ. 20, 85-91.

KANDEMİR, F. M., M. YÜKSEL, N. OZDEMIR, H. DEVECİ (2013): A different approach to diagnosis of subclinical mastitis: milk arginase activity. Vet. arhiv 83, 603-610.

MADIGAN, M., J. MARTINKO (2005): Brock Biology of Microorganisms (11th ed.). Prentice Hall.

MARGULIES, M., M. EGHOLM, W. E. ALTMAN, S. ATTIYA, J. BADER, L. BEMBEN, J. BERKA, M. BRAVERMAN, Y. J. CHEN, Z. CHEN, S. L. DEWELL, J. M. FIERRO, X. GOMES, B. C. GODWIN, W. HE, S. HELGESEN, C. HO, G. IRZYK, S. JANDO, M. ALENQUER, T. JARVIE, K. JIRAGE, J. B. KIM, J. KNIGHT, J. LANZA, J. LEAMON, S. LEFKOWITZ, M. LEI, J. LI, K. LOHMAN, H. LU, V. MAKHIJANI, K. MCDADE, M. MCKENNA, E. MYERS, E. NICKERSON, J. NOBILE, R. PLANT, B. PUC, M. RONAN, G. ROTH, G. SARKIS, J. SIMONS, J. SIMPSON, M. SRINIVASAN, K. TARTARO, A. TOMASZ, K. VOGT, G. VOLKMER, S. WANG, Y. WANG, M. WEINER, P. YU, R. BEGLEY, J. ROTHBERG (2005): Genome sequencing in microfabricated high-density picolitre reactors. Nature 437, 376-380.

MEYER, F., D. PAARMANN, M. D'SOUZA, R. OLSON, E. M. GLASS, M. KUBAL, T. PACZIAN, A. RODRIGUEZ, R. STEVENS, A. WILKE, J. WILKENING AND R. A. EDWARDS (2008): The metagenomics RAST server-a public resource for the automatic phylogenetic and functional analysis of metagenomes. BMC Bioinf. 9, 386.

OVERBEEK, R., T. BEGLEY, R. M. BUTLER, J. V. CHOUDHURI, H. Y. CHUANG, M. COHOON, V. CRE´CY-LAGARD, N. DIAZ, T. DISZ, R. EDWARDS, M. FONSTEIN, Ed. D. FRANK, S. GERDES, E. M. GLASS, A. GOESMANN, A. HANSON, D. IWATA-REUY, R. JENSEN, N. JAMSHIDI, L. KRAUSE, M. KUBAL, N. LARSEN, B. LINKE, A. C. MCHARDY, F. MEYER, H. NEUWEGER, G. OLSEN, R. OLSON, A. OSTERMAN, V. PORTNOY, G. D. PUSCH, D. A. RODIONOV, C. RUCKERT, J. STEINER, R. STEVENS, I. THIELE, O. VASSIEVA, Y. YE, O. ZAGNITKO, V. VONSTEIN (2005): The subsystem approach to genome annotation and its use in the project to annotate 1000 genomes. Nucleic Acids Res. 33, 5691-5702.

PHUEKTES, P., P. D. MANSELL, G. F. BROWNING (2001): Multiplex polymerase chain reaction assay for simultaneous detection of *Staphylococcus aureus* and streptococcal causes of bovine mastitis. J. Dairy Sci. 84, 1140-1148.

ROESCH, L. F. W., R. R. FULTHORPE, A. RIVA, G. CASELLA, A. K. M. HADWIN, A. D. KENT, S. H. DAROUB, F. A. O. CAMARGO, W. G. FARMERIE, E. W. TRIPLETT (2007): Pyrosequencing enumerates and contrasts soil microbial diversity. ISME 1, 283–290

RONAGHI, M., S. KARAMOHAMED, B. PETTERSSON, M. UHLEN, P. NYREN (1996): Real-time DNA sequencing using detection of pyrophosphate release. Anal. Biochem. 242, 84-89.

SEKIGUCHI, Y. (2006): Yet-to-be cultural microorganisms relevant to methane fermentation processes. Microbes Environ. 21, 1-15.

SHARMA, A., N. SINDHU, V. K. JAIN (2009): 16S-23S rRNA intergenic spacer based molecular detection of *Staphylococcus aureus* directly from mastitic milk of crossbred cows. Indian J. Anim. Health 79, 350-352.

SMITH, K. L., J. S. HOGAN (1995): Epidemiology of mastitis. Proc. 3rd Int. Mastitis Seminar, Tel Aviv, Israel S6, 3-12.

TUOHY, M. J., G. S. HALL, M. SHOLTIS, G. W. PROCOP (2005): Pyrosequencing™ as a tool for the identification of common isolates of *Mycobacterium* sp. Diagn. Microbiol. Infect. Dis. 51, 245-250.

TURNBAUGH, P. J., M. HAMADY, T. YATSUNENKO, B. L. CANTAREL, A. DUNCAN, R. E. LEY, M. L. SOGIN, W. J. JONES, B. A. ROE, J. P. AFFOURTIT, M. EGHOLM, B. HENRISSAT, A. C. HEATH, R. KNIGHT, J. I. GORDON (2009): A core gut microbiome in obese and lean twins. Nature 457, 480-484.

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SAŽETAK

Radi identifikacije mikrobne zajednice u mlijeku provedena je metagenomska i uobičajena kulturelna pretraga uzoraka mlijeka krava sa supkliničkim mastitisom. Ukupno je 77 trostruko križanih Kankrej i Gir mliječnih krava i 301 četvrt vimena bilo pretraženo na supklinički mastitis. Izdvojeno je bilo 106 izolata svrstanih u pet različitih rodova iz 91 četvrti od 41 krave uključujući i 15 četvrti kod kojih je kulturelnom pretragom bila ustanovljena mješovita bakterijska infekcija. Sljedovi mješavine DNA izdvojeni iz uzoraka mlijeka kod supkliničkog mastitisa očitani pirosekvenciranjem bili su analizirani po podsustavu SEED baze podataka „Meta Genome Rapid Annotation with Subsystem Technology (MG-RAST)". Iz pretraženih uzoraka mlijeka bilo je izdvojeno pet rodova: *Staphylococcus, Streptococcus, Micrococcus, Bacillus* i *Escherichia*. Četiri su bila dokazana postupkom pirosekvenciranja: *Staphylococcus, Streptococcus, Bacillus* i *Escherichia*, dok *Micrococcus* nije bio dokazan. S druge strane, pirosekvenciranjem je bilo dokazano 28 bakterijskih vrsta, od kojih su samo dvije, *S. aureus* i *E. coli,* bile dokazane klasičnom kulturelnom pretragom. *S. agalactiae,* treća vrsta identificirana kulturelnom pretragom nije bila dokazana postupkom pirosekvenciranja. Metagenomskom analizom dodatno je bilo dokazano 19 rodova i 26 vrsta u usporedbi s rutinskom kulturelnom pretragom. Mnoge anaerobne bakterije, koje je vrlo teško uzgojiti rutinskim metodama, bile su identificirane metagenomskom analizom.

Ključne riječi: supklinički mastitis, metagenomika, krave