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Denaturing gradient gel electrophoresis (DGGE): An alternative culture independent method for bacterial screening in bovine milk sample

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Bovine mastitis is a disease that has a great impact on Brazilian livestock production. Some bacteria described as mastitis-causing agents are not easily cultivable in conventional media, making their diagnosis difficult. The aim of the present study was to detect bacteria present in milk from mastitic and non-mastitic quarters of dairy cattle in Rio de Janeiro-Brazil, using a culture-independent and culture-dependent method. Milk samples were collected from healthy and mastitic quarters. Blood agar medium was used to isolate bacteria. Polymerase Chain Reaction - Denaturing Gradient Gel Electrophoresis (PCR-DGGE) followed by gene sequencing was used to detect bacterial species. Bacteria were isolated from 12 milk samples and *Staphylococcus aureus* was the only species identified. Bands unique to mastitis were detected in some animals, signaling possible causative agents of the disease in the herd. Species of the genus *Streptococcus* identified in these samples could not be isolated in culture medium. The present study concluded that the DGGE technique proved to be efficient in detecting bacteria that have difficulty growing in culture medium.

Key words: Bovine mastitis, polymerase chain reaction, *Staphylococcus aureus*, *Streptococcus*.

INTRODUCTION

In 2017, Brazil ranked fourth in the world milk production, behind the United States, India and China. However, the number of lactating cows reached the second position, behind only India, revealing the low milk yield of our herd. So Brazilian productivity (1,525 L/cow/year) is far surpassed by the United States, China, the United Kingdom, Turkey, New Zealand, France, Russia and Germany. These data are worrisome since Brazil is a country with large dairy herds but Brazilian cows are considered as low production potential (FAOSTAT, 2017).

Factors related to this reduction in productivity include mastitis, an inflammatory disease with a multifactorial etiology that interferes with the number of somatic cells

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and the physical and chemical characteristics of the milk, leading to a reduction in quality. Mastitis is considered the greatest economic impact disease on milk production, responsible for condemning tons of milk per year in Brazil and responsible for reaching about 72% of the Brazilian herd (Oliveira et al., 2013).

Considering the clinical aspect, it is classified into clinical or subclinical mastitis. In clinical mastitis, there are obvious signs of inflammation, such as edema, temperature increase, hardening and pain in the mammary gland, and/or appearance of lumps, pus or any change in milk characteristics, and systemic symptoms such as depression, dehydration and decrease of food intake. These symptoms can be verified by physical examination of the udder by inspection and palpation, in addition to the collection of the first jets of milk to perform a test of the screened mug to detect alterations in the appearance of milk (Dürr, 2005).

Subclinical mastitis is prevalent in Brazilian herds, responsible for reaching 90% of mastitic cows. There are no evident changes in the udder of the cows, and their spread is related to the moment of milking, due to inadequate hygiene conditions, either by handlers or milking equipment. The diagnosis can be made through the somatic cell count (CCS) in milk, where its increase reflects the increase of leukocytes consequent to mammary infection. Thus, CCS reflects the health status of the mammary gland and measures the risk of non-physiological changes in milk composition, constituting an essential tool in the evaluation and monitoring of udder health (Blowey and Edmondson, 2010). However, the most common test for this diagnosis is the California Mastitis Test (CMT). The CMT is a qualitative test that indicates the presence of somatic cells to a greater or lesser degree in milk.

This consists in collecting milk from the mammary quarters, individually, in a suitable tray, adding a neutral anionic detergent, which acts by breaking the leucocyte membrane, releasing the nucleic material (DNA), which presents a gelatinous mass. Both tests must be confirmed by microbiological diagnosis (Langoni et al., 2009). Mastitis can evolve to spontaneous cure or, in most cases, to a chronic condition, making it necessary to identify the causative agent to adjust the control and treatment measures (Brasil, 2012).

Several bacteria are incriminated in cases of subclinical mastitis in dairy cattle. *Staphylococcus aureus*, *Streptococcus agalactiae*, *Corynebacterium bovis* and *Streptococcus dysgalactiae* are among the main microorganisms involved. In Brazil, *S. aureus* is the main species (Oliveira et al., 2013). In addition to *S. aureus*, some coagulase-negative species are also isolated from mastitis samples as causative agents (Abril et al., 2020).

Microbial culture is still considered standard method to assay the etiology of mastitis. However, some bacteria related as mastitis causing pathogens are not easily cultivable in conventional media which compromises its accuracy. Another issue that must be taken into account when using isolation and identification procedures is that many mastitis-related bacteria are also considered commensal, hindering the precise diagnosis (Schukken et al., 2009). Molecular techniques have been used to characterize bovine mastitis isolates and allowed us to know the genetic profiles of these agents and to cross-reference such information to understand the diversity of the circulating clones in the region studied, an essential factor for the proper development of prevention programs and successful therapies (Marques et al., 2013).

This study was developed to analyze the bacterial diversity presented in the milk of cows with subclinical mastitis. To achieve this goal the bacterial populations from mastitic and non-mastitic mammary glands were compared through culture and identification procedures. Also, the culture-independent technique, Denaturing Gradiente Gel Electrophoresis (PCR-DGGE) followed by sequencing, was used to identify the prevalent bacterial species in the milk samples.

MATERIALS AND METHODS

Sampling

The present study was carried out in a dairy farm located in the South Fluminense area of Rio de Janeiro, Brazil. Before the milking, the California Mastitis Test (CMT) was performed to diagnose subclinical mastitis. The somatic cell count (CCS) was performed by flow cytometry at ESALQ-USP/Piracicaba, SP. Milk presenting a ≤ 200,000 $SC.ml^{-1}$ counting was considered normal (Langoni et al., 2011). The results of the present study were compared to the Brazilian Normative Instruction 62 which recommended a maximum value of 500,000 $cells.ml^{-1}$ (Brasil, 2011). Ten animals presenting both healthy and mastitic mammary quarters were selected. Two samples of each animal comprising healthy and ill mammary quarters were collected, totaling 20 samples. The collections were performed in the morning, by manual and individual milking (Fonseca and Santos, 2000).

Cultivation dependent methodology

Milk samples were previously incubated at 37°C for 6 h and subsequently inoculated onto Blood agar (HiMedia® base agar with 5% ram blood) for the primary isolation. After 37°C incubation for 24 h, the resulting colonies were submitted to Gram staining. According to the characteristics, the isolates were processed for phenotypic identification (Koneman et al., 2008).

To confirm phenotypic identification, the isolates were submitted to MALDI-TOF MS technique. The spectra of each sample were generated in a mass spectrometer (MALDI-TOF LT MicroflexBruker, Bruker, Billerica, MA) equipped with a 337 nm nitrogen laser in the linear mode controlled by the FlexControl 3.3 program (Bruker Daltonics). The spectra were collected in the mass range between 2,000 and 20,000 $m.s^{-1}$ and later analyzed by the MALDI Biotyper 2.0 (Bruker) program, with the standardized configurations for bacterial identification. It was considered as acceptable the identification that presented values equal to or greater than two in a scale ranging from zero to three (Motta et al., 2014).

Cultivation independent methodology

Bacterial total DNA extraction was performed according to Tiago et al. (2015). The first PCR used the primers 27f (Suzuki and Giovannoni, 1996) and 1512r (Kane et al., 1993). The products of this reaction were used as template for the second PCR using primers that amplify the V3 region of 16S rDNA, GC-338f and 518r (Ovreâs et al., 1997). For PCR reactions, *S. aureus* controls from clinical samples belonging to the genetic material bank of the Veterinary Bacteriology Laboratory were used. The products of the second PCR reaction were evaluated on an 8% polyacrylamide gel and a concentration gradient between 40 and 70% defined from the mixture of urea and deionized formamide solutions. In this step, the PCR products are separated by induced denaturation, adopting different positions along the gel according to the different types of gene sequence and molecular weights of the microorganisms. Electrophoresis was performed at 70 V and 60°C for 18 h in a DcodeTM "Universal Mutation Detection System" (BIO-Rad, Richmond, USA). The gels were photographed and the images analyzed with the Bionumerics software (AppliedMaths, Saint-Martens-Latem).

DGGE bands were excised from the gel and transferred to microtubes containing 5 µl of water and subsequently incubated at 4°C for 12 h. Two microliters of eluted DNA were submitted to a PCR reaction using 338f and 518r primers. The PCR products were purified using the Exo-Sap Kit (USB Corporation, Cleveland, Ohio) as recommended by the manufacturer. Both strands were sequenced on Applied Biosystems ABI 3130xl sequencer at Helixxa Bases for Life (Campinas/SP). The sequences were edited in the DNA Sequence Assembler v4 program (2013), and then compared with other sequences in the NCBI GenBank database (www.ncbi.nlm.nih.gov/) using the BLAST algorithm for species inference (Altschul et al., 1997).

RESULTS

Of the 20 milk samples collected, 12 presented microbial growth when inoculated onto blood agar medium (Table 1). The only species detected was *S. aureus*. It was not possible to detect bacterial growth in eight samples from four animals (3, 6, 9, 10), even in the milk samples collected from quarters with positive California Mastitis Test (CMT).

By means of DGGE gel analysis it was possible to notice differences in bacterial population between milk samples from healthy and mastitic quarters of the same animal. This difference considered not only the presence or absence of the band, called the Operational Taxonomic Unit (OTU), but also the intensity of the bands (Figure 1).

There is no relationship of CCS with the number of bands in the gel or with the intensity of its bands, but exclusive bands were detected in mastitic milk samples of the following animals: 3 (band 4 - *Enterococcus faecium*), 4 (band 6 - *Bacillus* species), 6 (band 13 - *S. agalactiae*) and 7 (band 17 - *Streptococcus uberis*) as shown in Figure 1 indicated with red arrows and shown in Table 2. Other exclusive bands are present in non-mastitic milk samples of the following animals: 4 (band 8 - *Bacillus* spp.), 5 (band 11 - *Amphibacillus* species), 6 (band 14 and 15 - both *S. aureus*) and 7 (band 18 - *S. aureus*) (Table 2).

Bands were detected in both mastitic and non-mastitic milk samples. Some bands have higher intensity in mastitic milk samples as 1, 2, 3, 10, 12 (*S. aureus*, *S. aureus, Enterococcus faecalis, S. aureus, S. aureus,* respectively). Otherwise, some have higher intensity in non-mastitic milk samples as 5, 7, 16, 19, 21, 22 and 23 (*S. aureus, Staphylococcus haemolyticus, S. aureus, S. aureus, S. aureus, Bacillus* spp.) (Figure 1 and Table 2).

DISCUSSION

The use of independent of cultivation methods techniques based on nucleic acids such as Denaturing Gradient Gel Electrophoresis (DGGE) allows the evaluation of a larger number of samples at the same time and a broader and faster detection of the potential pathogens presented in the samples, increasing the expected results. It was noticeable in this study that culture-dependent methods did not allow for a more comprehensive assessment of the organisms presented in both healthy and mastitic milk samples, and that even spending a higher cost for individual and specific evaluations of these micro-organisms was not so sensitive in detecting the bacterial diversity.

The only species isolated by culture-dependent methodology with blood agar medium was *S. aureus*, which is predictable since it is widely disseminated in dairy environment and consequently considered the main agent related to bovine mastitis (Lazzari et al., 2014). It is important to consider that the presence of an agent is not a sufficient criterion to attribute the etiology of a disease (Viana et al., 2014). Bacteria such as *S. aureus* are also considered part of the microbiota of the cows. Other criteria should be taken into account such as, agent concentration, pathogenicity of the strain and immune status of the animal (Schukken et al., 2009).

Four samples presented no microbial growth in blood culture medium, reinforcing the difficulties in the adoption of culture-dependent protocols, since it is not possible to state that the absence of growth is due to specific bacterial requirements or even to some inhibitory agent, biotic or abiotic, interfering in the growth of species present in the sample, or even if the inflammatory process detected by CMT was caused by bacterial agent. It is important to point out that the protocol used for bacterial isolation is standardized for type-of-sample analysis (Blagitz et al., 2011).

The evaluation of the intensity of the bands is a semi-quantitative analysis that suggests an increased DNA concentration in the sample. This technique had already been used to characterize the surface microbiota of mammary gland in a study of mastitis bacterial diversity (Braem et al., 2012) and also in the diagnosis of bacteria

Table 1. Distribution of bacterial species isolated from milk samples in blood agar medium.

Animal	CCS (SC.ml^{-1})	CMT/quarter	Sample	Isolates
1	1,429,000	Positive	1P	*Staphylococcus aureus*
		Negative	1N	*Staphylococcus aureus*
2	897,000	Positive	2P	*Staphylococcus aureus*
		Negative	2N	*Staphylococcus aureus*
3	1,071,000	Positive	3P	NBG
		Negative	3N	NBG
4	9,999,000	Positive	4P	*Staphylococcus aureus*
		Negative	4N	*Staphylococcus aureus*
5	1,618,000	Positive	5P	*Staphylococcus aureus*
		Negative	5N	*Staphylococcus aureus*
6	100,000	Positive	6P	NBG
		Negative	6N	NBG
7	273,000	Positive	7P	*Staphylococcus aureus*
		Negative	7N	*Staphylococcus aureus*
8	346,000	Positive	8P	*Staphylococcus aureus*
		Negative	8N	*Staphylococcus aureus*
9	269,000	Positive	9P	NBG
		Negative	9N	NBG
10	793,000	Positive	10P	NBG
		Negative	10N	NBG

CCS = Counting somatic cells; SC = somatic cells; CMT = California mastitis test; NBG= no bacterial growth.
Source: Authors

that present difficulties to be cultured (Kuang et al., 2009). The most bands present in both milk samples are from *Staphylococcus* genus. It is worth noting that the lack of *S. aureus* growth in some samples may be due to the possibility of intermittent secretion of this agent (Abril et al., 2020). *S. aureus* may be considered as the most important species within the study, being detected in most samples. In a general sense it is possible to assume that the greater its presence in the mammary glands, the greater the probability of occurrence of the disease, the risk to health and the loss in production.

It was possible to detect the presence of exclusive OTU obtained from non-mastitic milk samples in three animals. *Amphybacillus* species were detected only in healthy samples and may represent part of commensal microbiota. This kind of microorganism is very important in balancing the effect of pathogenic bacteria on the mammary gland. Samples presenting these bacteria showed no signs of disease or pathogenic bacteria. Similarly, *Bacillus* spp. can be considered a commensal organism and in this study was identified in both samples' types.

A very important finding was the detection of *Streptococcus* spp. only by DGGE technique in samples where there was no microbial growth in the culture medium. Bacterial culture in blood agar has for many years been the standard method for identification of mastitis pathogens. Depending on the culture medium used, the inoculum volumes applied and the specific analyses, the sensitivity of *Streptococcus* detection ranges from 20.5 to 78% (Keefe, 1997). The samples containing *Streptococcus* spp. were the same where *S. aureus* could not be cultured by the conventional method. This suggests a possible interference mechanism acting on these bacteria.

Streptococcus spp. and *Enterococcus faecium* were

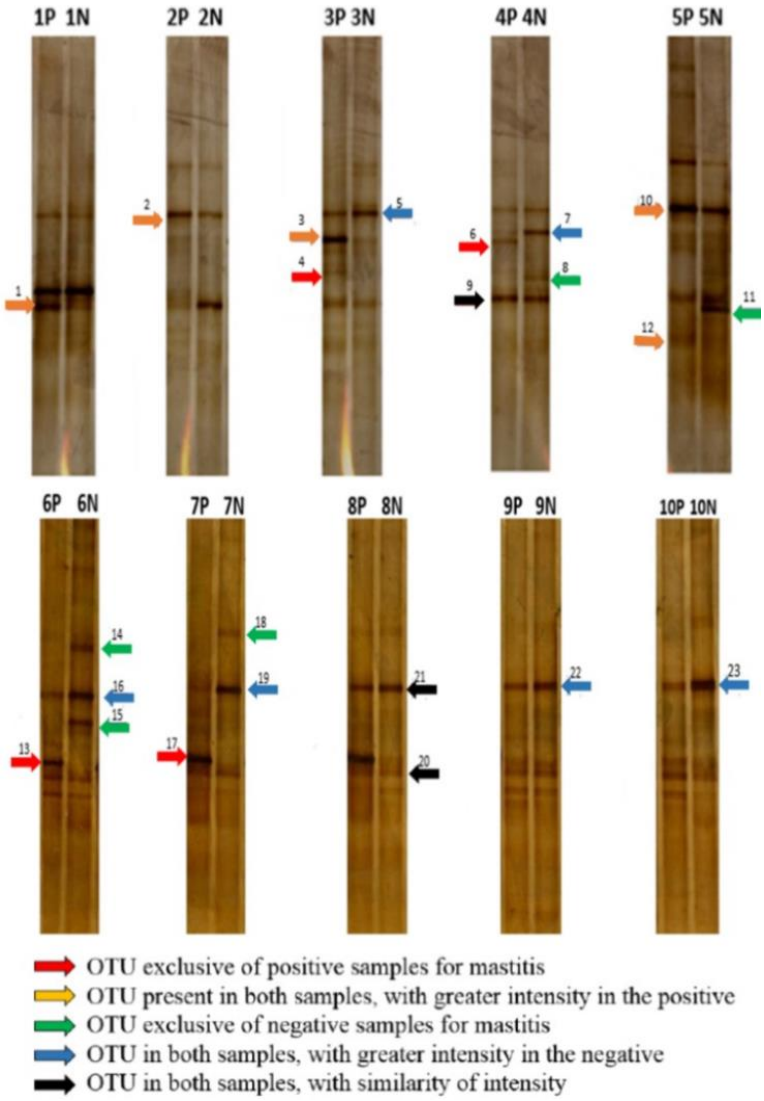

Figure 1. Denaturing Gradient Gel Electrophoresis of 16S rDNA gene of bacteria from quarter-milk samples with positive (P) and negative (N) California Mastitis Test. P = California Mastitis Test (CMT) positive, N = CMT negative.
Source: Authors

detected only in mastitic quarters, and could represent a correlation with the disease since they were not found in healthy quarters. *Enterococcus faecalis* was found in both samples, but with more intensity in the positive samples, suggesting a pathogenic potential. Although the study focused on subclinical mastitis, another interesting result was the detection of *Streptococcus uberis* in samples of mastitic milk once this bacterium is known to be implicated in clinical mastitis (Abureema et al., 2014).

The DGGE gel observation indicated differences between the bacterial microbiota in both milk samples. The OTU sequencing from DGGE is a tool that could differentiate the commensal from the potential pathogenic microbiota. As a matter of fact, it is relevant to point out that the control of this disease is related to the implementation of milking good practices, sanitary management and the comprehension of mastitis impact on production. When this concept is fully established the consequent economic problems and the prevalence of these bacteria in the herds tend to diminish.

Conclusion

The DGGE technique proved to be efficient in detecting bacteria that have difficulty growing in the culture medium

Table 2. Distribution of bacterial species in milk samples from mastitic and non-mastitic quarters by culture-independent methodology.

Bands no.	Animal	Sample*	Bands characteristics	Specie inference
1	1	1P	OTU in both samples, with higher intensity in positive mastitic quarters	*Staphylococcus aureus*
2	2	2P	OTU in both samples, with higher intensity in positive mastitic quarters	*Staphylococcus aureus*
3	3	3P	OTU in both samples, with higher intensity in positive mastitic quarters	*Enterococcus faecalis*
4	3	3P	OTU in both samples, with higher intensity in positive mastitic quarters	*Enterococcus faecium*
5	3	3P	OTU in both samples, with higher intensity in negative mastitic quarters	*Staphylococcus aureus*
6	4	4P	Exclusive OTU from positive mastitic quarters	*Bacillus* spp.
7	4	4N	OTU in both samples, with higher intensity in negative mastitic quarters	*Staphylococcus haemolyticus*
8	4	4N	Exclusive OTU from negative mastitic quarters	*Bacillus* spp.
9	4	4P	OTU in both samples, with similar intensity	*Staphylococcus aureus*
10	5	5P	OTU in both samples, with higher intensity in positive mastitic quarters	*Staphylococcus aureus*
11	5	5N	Exclusive OTU from negative mastitic quarters	*Amphibacillus* spp.
12	5	5P	OTU in both samples, with higher intensity in positive mastitic quarters	*Staphylococcus aureus*
13	6	6P	Exclusive OTU from positive mastitic quarters	*Streptococcus agalactiae*
14	6	6N	Exclusive OTU from negative mastitic quarters	*Staphylococcus aureus*
15	6	6N	Exclusive OTU from negative mastitic quarters	*Staphylococcus aureus*
16	6	6P	OTU in both samples, with higher intensity in negative mastitic quarters	*Staphylococcus aureus*
17	7	7P	Exclusive OTU from positive mastitic quarters	*Streptococcus uberis*
18	7	7N	Exclusive OTU from negative mastitic quarters	*Staphylococcus aureus*
19	7	7N	OTU in both samples, with higher intensity in negative mastitic quarters	*Staphylococcus aureus*
20	8	8P	OTU in both samples, with similar intensity	*Enterococcus faecalis*
21	8	8P	OTU in both samples, with higher intensity in negative mastitic quarters	*Staphylococcus aureus*
22	9	9P	OTU in both samples, with higher intensity in negative mastitic quarters	*Bacillus* spp.
23	10	10P	OTU in both samples, with higher intensity in negative mastitic quarters	*Staphylococcus aureus*

*P = California Mastitis Test (CMT) positive, N = CMT negative.
Source: Authors

and presented itself as a diagnostic alternative for mastitis control in milk productions.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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