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Molecular determination of the etiological agent of bovine mastitis from Andean production units

Determinación molecular del agente etiológico de la mastitis bovina de muestras provenientes de unidades productoras andinas

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Abstract

Bovine mastitis is a disease that affects the farms of small and medium producers in the cantons of Cayambe and Pedro Moncayo, Pichincha Province-Ecuador. Treating this disease is not easy due to the different microorganisms that cause it. This study focused on the molecular determination by means of polymerase chain reaction (PCR) of the etiological agents of mastitis, having multiple advantages when recognizing family, gender and species of microorganisms. It is a method capable of detecting resistance genes of antibiotics, an important analysis when diagnosing and treating diseases. The aim of this research is to identify bacteria causing bovine mastitis by using biochemical and molecular tests. Biochemical tests such as: Gram staining, Catalase, Coagulase, and Mannitol Salt Agar were efficient to obtain pure strains and determine the gender of some bacteria. Specific primers (RNA16S) were used for the molecular identification of 9 etiological agents causing the disease in the productive units. The microorganisms found were *Staphylococcus pasteuri, Staphylococcus warneri, Staphylococcus sp., Staphylococcus epidermidis, Staphylococcus aureus, Staphylococcus saprophyticus, Sphingomonas sp., Streptococcus dysgalactiae, Streptococcus uberis*, mostly present in clinic mastitis. To detect resistance genes, specific primers were used, of which 7 samples presented the gene for resistance to blaTEM (β-lactam) and 6 samples presented the gene for resistance to tetA (tetracyclines). Multi-resistance was identified in the species *Staphylococcus pasteuri, Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus uberis, Sphingomonas sp*.

Keywords: Mastitis, PCR, sequencing, biochemistry.

Resumen

La mastitis bovina es una enfermedad que afecta a las ganaderías de pequeños y medianos productores de los cantones Cayambe y Pedro Moncayo, Provincia de Pichincha-Ecuador. El tratamiento de esta enfermedad resulta complicado debido a la variedad de microorganismos que la provocan. El presente estudio se enfocó en la determinación molecular por medio de reacción en cadena de la polimerasa (PCR) de los agentes etiológicos de la mastitis. Esta técnica presenta múltiples ventajas al reconocer familia, género y especie de microorganismos, y es un método capaz de detectar genes de resistencia de antibióticos, lo que resulta importante al momento de diagnosticar y tratar enfermedades. El objetivo de esta investigación se centró en la identificación de bacterias causantes de la mastitis bovina, utilizando pruebas bioquímicas y moleculares. Las pruebas bioquímicas como tinción Gram, Catalasa, Coagulasa, y Agar Manitol Sal fueron eficientes para obtener cepas puras y determinar el género de algunas bacterias. Se utilizaron primers específicos (RNA16S) para la identificación molecular de 9 agentes etiológicos causantes de la enfermedad en las unidades productivas. Los microorganismos encontrados fueron; *Staphylococcus pasteuri, Staphylococcus warneri, Staphylococcus sp., Staphylococcus epidermidis, Staphylococcus aureus, Staphylococcus saprophyticus, Sphingomonas sp., Streptococcus dysgalactiae, Streptococcus uberis*, la mayoría presentes en mastitis clínica. Para detectar genes de resistencia se utilizaron primers específicos, de los cuales 7 muestras presentaron el gen para resistencia a blaTEM (β-lactámicos) y 6 muestras presentaron el gen para resistencia a tetA (tetraciclinas). Se identificó multirresistencia en las especies: *Staphylococcus pasteuri, Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus uberis, Sphingomonas sp*.

Palabras clave: Mastitis, PCR, secuenciación, bioquímica.

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1 Introduction

According to INEC and ESPAC (2016), 6,202,408 liters of milk are produced daily in Ecuador, in the Sierra region 4,810,551 liters (75% of national production), and in the province of Pichincha 873,272 liters. Torres (2018) estimates that Cayambe parish produces about 425,000 liters of milk daily, of which 51,000 liters are for consumption, 106,250 liters for artisanal production and 267,750 liters for bulk sale. Similarly, Céspedes Pachacama (2012), state that there is a daily milk production of 13,905 liters daily in Pedro Moncayo parish.

Bonifaz and Conlago (2016) state that mastitis causes great discomfort to farmers, causing a decrease in production and quality of milk, being affected in its chemical, physical and bacteriological composition, presenting a lower percentage of total solids, protein, fat and calcium. Mastitis is known as inflammation and irritation of the mammary gland caused by various pathogens, presenting increased somatic cells and causing discomfort in the animal; as a side effect of this infection, the texture of milk changes its organoleptic characteristics (Bhattarai et al., 2018).

According to Martínez (2012), the clinical symptoms are increased number of leukocytes, altered composition and appearance (lumps), fever, red, swollen and warm breast quarters. The occurrence of mastitis depends on the hygienic conditions of milking rooms (Zhang et al., 2018), age, breed, deliveries, lactation period, milk production and milking equipment conditions. If milking equipment is not used, the health and hygiene of the person in charge of milking the cows should be monitored (Gil Ruiz et al., 2016). Disease also occurs when environmental and animal management factors interact in such a way that the udder is exposed to pathogenic microorganisms. There are three essential factors that promote mastitis, the host, the infectious agent and the environment (Ruiz Gil et al., 2016).

Disease also occurs when environmental and animal management factors interact in such a way that the udder is exposed to pathogenic microorganisms. There are three essential factors that promote mastitis, the host, the infectious agent and the environment (Owens and Nickerson, 2011).

Studies have been conducted to identify the etiological agents that cause mastitis (Cervantes et al., 2017). The use of selective culture media has allowed isolate *Streptococcus agalactiae, Streptococcus uberis, Staphylococcus aureus, E. coli, Klebsiella, Pseudomona, Candida albicans* and *Proteus mirabilis*. Hernández et al. (2015) when identifying the etiological agents determined that *Staphylococcus aureus* is the most important microorganism with a 26% prevalence on the total of identified microorganisms in Boyacá parish.

The standard method for identifying pathogens consists of isolates in selective culture media, however, using this method will only be diagnosed up to the level of genus of the bacteria (Peña et al., 2012).

The prevalence of this disease in Cayambe and Pedro Moncayo is a problem, and the pathogenic microorganisms responsible continuously change their ecological dynamics by the mutations suffered by the etiological agents. For this reason, measures have been taken to identify the pathogens causing mastitis through biochemical tests and molecular techniques that allow establishing the pathogens and resistance to multiple antibiotics by their misuse when treating this disease (Bonifaz and Conlago, 2016).

Hence, the aim of this research is to determine by molecular techniques the etiological agents of bovine mastitis from samples coming from farms in Cayambe and Pedro Moncayo, as well as to identify the bacteria of the disease through 3 biochemical tests, to detect the resistance blaTEM genes for β-lactams and tetA for tetracyclines, in bacteria isolated in this research by PCR technique and finally to perform the molecular identification of the isolated microorganisms through sequencing of the ribosomal region RNA 16S.

2 Materials and methods

2.1 Milk sampling

Milk samples were taken from livestock farms located in Cayambe (coordinates 0°02′38″N 78°09′22″W) and Pedro Moncayo (coordinates 0°02′37″N 78°20′57″W) which had been previously identified as positive for mastitis by California Mastitis Test (CMT).

A total of 24 milk samples were collected following protocol LCL 001 of the milk quality laboratory of the Salesian Polytechnic University, which indicates that 40 mL of milk should be taken per container for its analysis in the FOSSOMATIC somatic cells (SCC) equipment by flow cytometry. Samples exceeding 500,000 CCS/dL as determined by Nieto et al. (2012) were selected for this study.

2.2 Microbial isolation

Milk samples were sown by swab in Triptein Soya Agar (TSA) medium, recommended for detecting and counting a wide range of bacteria, and incubation at 37°C for 48 hours (Britania Tripteína Soya Agar, 2015). Subsequently, several subcultures were performed to obtain pure colonies using the planting by exhaustion technique in TSA medium (Milián et al., 2014). Pure bacterial colonies were confirmed by Gram stain.

2.3 Identification by biochemical tests

Four biochemical tests of bacterial identification were performed: Gram stain to differentiate bacilli and cocci Gram (+) and Gram (-); Catalase using 30% hydrogen peroxide expecting to visualize presence or absence of bubbling (Wanger et al., 2017); Coagulase test in human plasma, where pure strains and incubated at 37°C for 24 hours were inoculated to analyze presence or absence of coagulation (American Society for Microbiology, 2016); and Agar Mannitol Salt (AMS) where sowing of strains were performed, isolated and incubated at 37°C for 48 hours in order to differentiate bacteria of the genus *Staphylococcus* (Vila et al., 2004). The purpose of these tests was to prevent molecular analyzes from being performed on repeated bacterial species.

2.4 DNA extraction and 16S rRNA amplification

The pure strains were massified in Tryptic Soya Broth (TSB) medium until reaching a concentration of 12×10^8 (CFU/mL) which was determined by comparison with turbidity patterns of the McFarland scale. In 1.5 mL tubes, 1 mL of bacterial culture of each massified strain was placed and the sample was centrifuged to form a pellet of bacteria that was used for DNA extraction based on Becton et al. (2005). Total DNA extraction was performed using the Köchl et al. (2005) DNA extraction protocol. The presence or absence of DNA was confirmed by 1% agarose gel electrophoresis (González de Buitrago, 2010). The DNA obtained was preserved in a solution of Tris-EDTA at -20°C (Tan and Yiap, 2009).

The amplification of the 16S rRNA region was performed using the first 27F (5′TCCTACGGGAGGCAGCAGT3′) and 1492R (5′GGACTACCAGGGTATCTAATCCTGTT3′) designed by Marchesi et al. (1998). The PCR technique and Labnet Multigene branded thermo-cycler were used under the following conditions: initial denaturation at 95°C for 5 minutes, 25 denaturation cycles at 95°C for 1 minute, annealing at 60°C for 2 minutes, initial extension for 1 minute at 72°C and final extension for 7 minutes at 72°C followed by maintenance at 4°C (Kang et al., 2015). The presence of amplicons was determined by 1% agarose gel electrophoresis.

2.5 Sequencing and sequence analysis

Amplicons were sequenced using the Sanger technique in MACROGEN, South Korea. The sequences obtained were compared with existing sequences in the GeneBank of the National Center for Biotechnological Information (NCBI). Subsequently, the sequences were aligned with the Muscle software tool Mega 6 to obtain a phylogenetic tree using the *neighbor joining* tool of Mega 6 to make a correct taxonomic classification of the isolated etiological agents (Fuertes and Cerna, 2018).

2.6 Antibiotic resistance gene amplification blaTEM (β-lactam) and tatA (tetracycline)

The presence or absence of antibiotic resistance genes was determined using the following specific primers described by Tao et al. (2014), for blaTEM: Forward (5′GCA CGA GTG GGT TAC ATC GA 3′) and Reverse (5′GGT CCT CCG ATC GTT GTC AG 3′). The approximate size of the resistance gene is 300 bp; for tetA: Forward (5′GCT ACA TCC TGC TTG CCT TC 3′) and Reverse (5′CAT AGA TCG CCG TGA GG 3′). According to Belding and Boopathy (2018), the approximate size of the resistance

gene is between 250 and 300 bp. The results obtained were verified in 1% agarose gel electrophoresis.

3 Results and discussion

3.1 Identification by biochemical tests

A total of 17 bacterial strains were isolated. The Gram stain resulted in 15 Gram-positive and 2 Gram-negative species as shown in Table 1.

After having applied catalase tests, 11 catalase positive and 6 catalase negative species were identified. Coagulase tests identified 1 coagulase-positive species and 16 coagulase-negative species. Chaneton (2010) states that coagulase negative *Staphylococcus* are the most prevalent pathogens, being identified according to the type of infection 47% in latent infection and 28% in subclinical mastitis.

Planting Agar Mannitol Salt (AMS) allowed to classify 4 species as positive to mannitol fermentation, 8 species negative to mannitol fermentation and 5 bacterial species that did not give a determined result.

Table 1. Results obtained from the Biochemical tests.

Code of the sample	Application of Biochemical tests			
	Gram Staining	Catalase	Coagulase	Agar Mannitol Sal
835	+	+	-	+
A2r	+	+	-	+
D2r	+	+	-	****
I2r	+	+	-	+
A3r	+	+	-	+
809	+	+	-	-
798	+	+	+	****
799c1	+	+	-	-
H3R	+	+	-	-
11	+	-	-	****
11^a	+	-	-	****
1C	-	+	-	-
2C	-	+	-	****
1B	+	-	-	-
E1	+	-	-	-
99	+	-	-	-
799	+	-	-	-

Classification result by biochemical tests of bacterial isolates; (+) = Positive result, (-) = Negative result, (****) = Unfinished result.

3.2 Identification by molecular methods

The total DNA obtained was approximately 7000 bp, based on the molecular classification of the Thermo Scientific 2× Phire Plant Direct kit (Figure 1).

Amplification of the 16S rRNA region resulted in bands of approximately 1300 bp (Figures 2 and 3). The validity of the obtained sequences was analyzed using FinchTV software, which discarded 3 sequences due to lack of adequate quality. With the remaining sequences the identity of 15 species corresponding to the Bacteria domain was obtained.

The sequences obtained in this research were compared with sequences present in the database at the NCBI GeneBank. The identity percentages exceeded 97% in all cases except for strains coded as 1C, 2C and E1. Sol-Church and Frenck (2014), suggest accepting identity percentages higher than 95%. Sequences of the species cataloged as D2r, 798 and 2C did not have a taxonomic record in the NCBI database being cataloged as non-cultured bacteria.

To make a correct identification of the isolated etiological agents, a phylogenetic tree was developed using the 15 obtained sequences and 4 external reference sequences downloaded from the NCBI database (Figure 4).

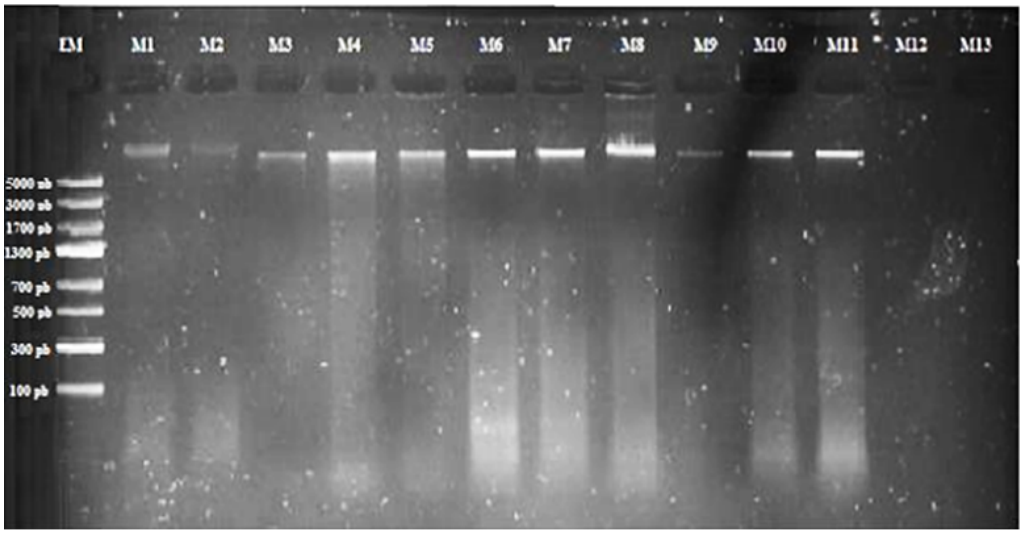

Figure 1. Total DNA in electrophoresis gel.

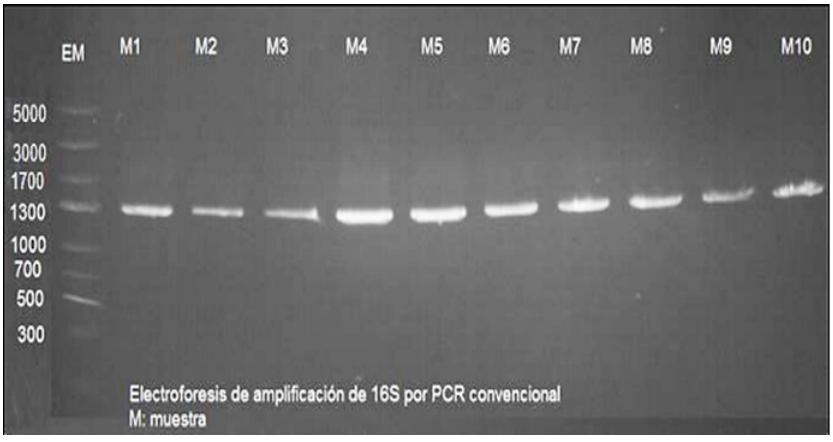

Figure 2. Amplification of 16S electrophoresis by conventional PCR.

Isolated species were identified as etiological agents causing mastitis in dairy cattle in the study area. Based on the information obtained with the phylogenetic tree, three clades were established:

- Clade (I): The isolates identified as *Staphylococcus* sp. (A3r), emphS. pasteuri (835, A2r), *S. Warneri* (I2r), *S. epidermidis* (809) and *S. saprophyticus* (799c1), are found in this clade. There were species that could not be identified at taxonomic level as is the case of Bacterium strain 2BL_4 (D2r) which was aligned with *S. warneri* and an uncultured bacteria clone ncd2254e01c2 (798), species that were grouped to the reference sequence of *Staphylococcus aureus*, used as external species, determining that these may belong to the same genus but with a different strain.

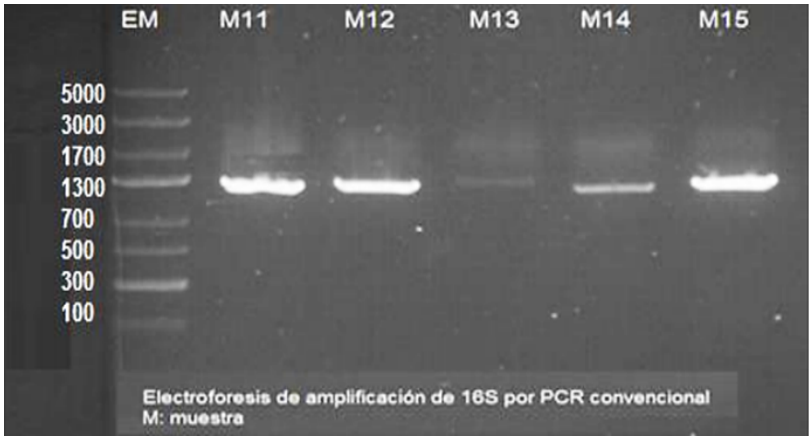

Figure 3. Amplification of 16S electrophoresis by conventional PCR.

- Clade (II): They include *Sphingomonas* sp. (C1) and Bacterium strain 4BL_5 (2C) closely grouped with the reference strain type of ATCC *Sphingomonas paucimobilis*, relationship confirmed by Clavijo et al. (2012), since there are genera that are within the same group, share characteristics and also coincide with molecular identification.

- Clade (III): All isolates in clade III coincide to be of the same *Streptococcus* genus when grouped in the following species *S. uberis* (99, 799), *S. dysgalactiae* (B1) which was grouped with *Streptococcus* sp. (E1).

In Table 2, the genera of pathogens identified in this research are *Staphylococcus, Sphingomonas* and *Streptococcus*. Bacteria including *Sphingomonas* and *Streptococcus* bacteria are normal components of skin microbiota (Chen et al., 2018).

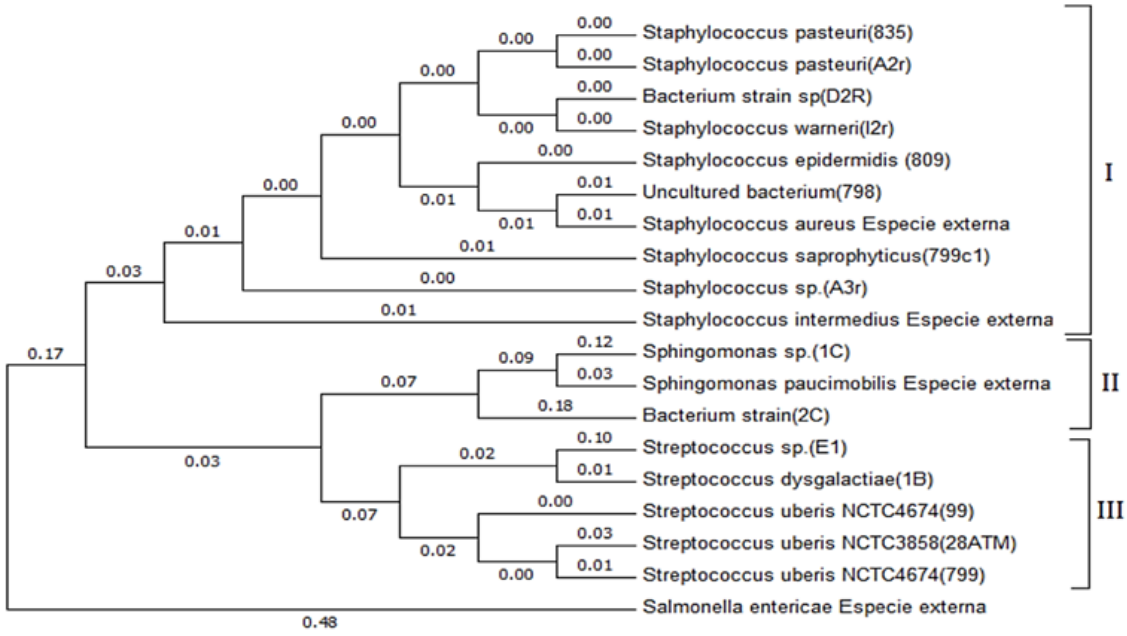

Figure 4. Phylogenetic tree elaborated from the sequencing of the 16S rRNA region of the isolated species.

According to Fariña et al. (2013), *Sthaphylococcus* is one of the most frequently isolated genera in mastitis prevalence studies. Lange et al. (2015) say that species belonging to the *Streptococcus* genus are linked to bovine mastitis as opportunistic bacteria. Ruiz et al. (2011) in their study on subclinical mastitis associated with manual and mechanical milking. *Staphylococcus* sp. is the main pathogen with 61% predominance followed by *Streptococcus* sp. with 29.6% incidence. Braga et al. (2018) when performing an identification of bovine mastitis pathogens by MALDI-TOF mass spectrophotometry determined that approximately 80% of the cases of mastitis were caused by the group of bacteria *Staphylococcus aureus, Streptococcus uberis* and *Streptococcus dysgalactiae*.

Table 2. Taxonomic classification of etiological agents causing bovine mastitis isolated in Cayambe and Pedro Moncayo, Pichincha province.

Code	Species	Query cover (%)	Identity	Access
835	*Staphylococcus pasteuri*	99	97	gi|388894673|JQ726643.1
A2r	*Staphylococcus pasteuri*	100	99	gi|762210140|LN623633.1
D2r	*Staphylococcus* sp.	98	99	gi|1193828147|MF112182.1
I2r	*Staphylococcus warneri*	99	99	gi|1409189597|MF662223.1
A3r	*Staphylococcus* sp. *strain CLC-F26*	100	99	gi|1408623646|MH518208.1
809	*Staphylococcus epidermidis strain TWSL_20*	96	99	gi|846351151|KT184900.1
798	*Staphylococcus* sp.	91	98	gi|322180115|JF194710.1
799c1	*Staphylococcus saprophyticus strain WWi167*	99	99	gi|1393269660|MH396756.1
1C	*Sphingomonas* sp. *strain Ap02E*	78	89	gi|1080032407|KX990222.1
2C	*Sphingomonas* sp.	97	91	gi|1193828182|MF112217.1
1B	*Streptococcus dysgalactiae strain JZ R-75*	34	99	MH119693.1
E1	*Streptococcus* sp. *XJ150-1212-NJR1*	95	90	gi|564983989|KF828882.1
99	*Streptococcus uberis strain NCTC4674*	99	99	gi|1403452574|LS483408.1
799	*Streptococcus uberis strain NCTC3858*	99	97	gi|1403426722|LS483397.1

Source: NCBI(2019).

Davies et al. (2016) mention that 63% of potential transmission events and 38% of incidence of clinical mastitis are caused by *S. uberis* in dairy herds. Nam et al. (2009) mention that about 40% of clinical mastitis cases are caused by Gram (-) bacteria linked to mastitis, the most common non-coliform pathogen associated with intramammary infections is *Sphingomonas* sp.

3.3 Antibiotic resistance assay

Amplification of the blaTEM resistance gene for the β-lactam family resulted in bands of approximately 300 bp, data corroborated by Tao et al. (2014). The

resistance gene was found in *Staphylococcus pasteuri, Staphylococcus warneri, Staphylococcus epidermidis, Staphylococcus saprophyticus, Streptococcus uberis, Sphingomonas* sp. and *Staphylococcus aureus* species (Figure 5).

On the other hand, amplification of the tetA resistance gene for the tetracycline family resulted in bands of approximately 250 bp. According to Belding and Boopathy (2018), the resistance gene for the tetracycline antibiotic family has a size of between 210 and 300 bp. The resistance gene was found in *Staphylococcus pasteuri, Staphylococcus epidermidis, Streptococcus uberis, Streptococcus dysgalactiae, Staphylococcus aureus* and *Sphingomonas* sp. (Figure 6).

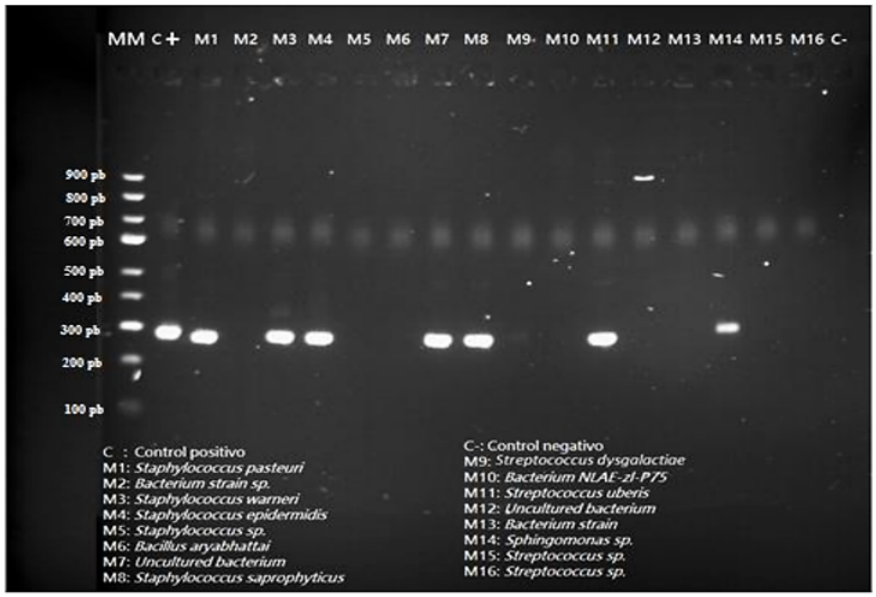

Figure 5. Electrophoresis gel showing presence/absence of blaTEM resistance gene for β-lactam antibiotics.

Corroborating the results obtained in this study (Table 3), further antibiotic resistance studies in mastitis-related pathogens have determined that β-lactam-resistant species are *Staphylococcus warneri, Streptococcus uberis* (Bonetto, 2014), *Staphylococcus epidermidis* (Fariña et al., 2013), *Staphylococcus saprophyticus, Sphingomonas* sp., *S. aureus* (Ramírez et al., 2018) and *Staphylococcus pasteuri* (Arrepia, 2008). Likewise, according to studies carried out by other authors, *S. epidermidis* (Ramírez et al., 2018), *S. uberis, S. dysgalactiae* (Florentin, 2007) and *S. pasteuri* (Savini et al., 2009) have resistance to tetracyclines, the latter showing resistance to different antibiotic compounds such as methicillin/ oxacillin, macrolides, lincosamides, streptogramins and tetracyclines.

Table 3. Species resistant to β-lactam and tetracyclines.

Species with blaTEM resistance	Species with tetA resistance
Staphylococcus pasteuri	*Staphylococcus pasteuri*
Staphylococcus aureus	*Staphylococcus aureus*
Staphylococcus epidermidis	*Staphylococcus epidermidis*
Streptococcus uberis	*Streptococcus uberis*
Sphingomonas sp.	*Sphingomonas* sp.
Staphylococcus warneri	*Streptococcus dysgalactiae*
Staphylococcus saprophyticus	

4 Conclusions

The use of the molecular technique allowed to identify genus and species in most of the isolated microorganisms, concluding that the molecular identification of the etiological agent is important in the study of bovine mastitis as it is a more specific test

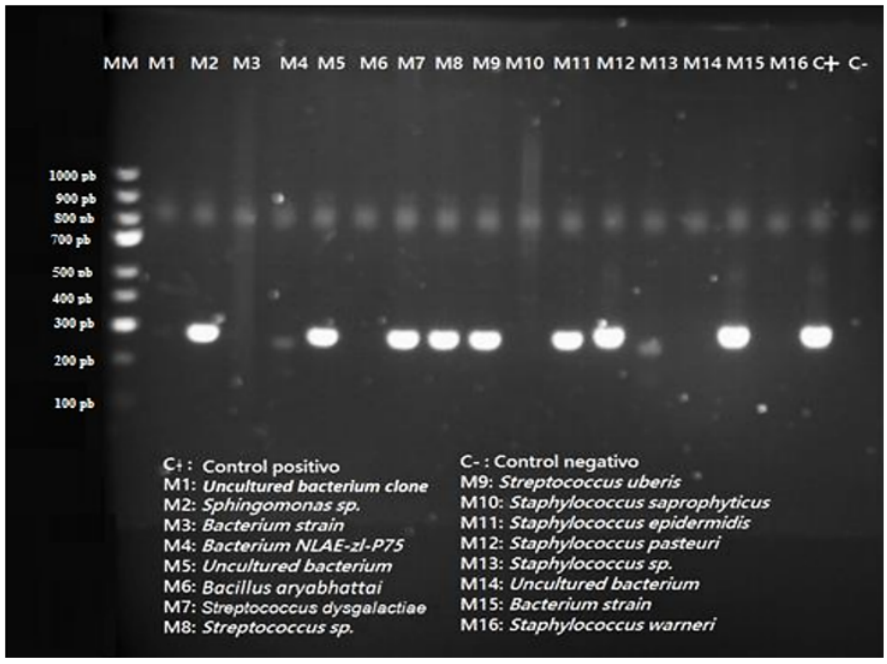

Figure 6. Electrophoresis gel showing presence/absence of tetA resistance gene for antibiotics of the tetracycline family.

than biochemical.

It could be determined that the etiological agents with more prevalence of bovine mastitis in Cayambe and Pedro Moncayo are the microorganisms belonging to the genera *Staphylococcus* and *Streptococcus*, being the most common *Staphylococcus pasteuri, Staphylococcus warneri, Staphylococcus* sp., *Staphylococcus epidermidis, Staphylococcus aureus, Staphylococcus saprophyticus, Sphingomonas* sp., *Streptococcus dysgalactiae, Streptococcus uberis*.

The molecular technique also allowed to identify in the bacteria causing bovine mastitis the presence of resistance genes to two families of antibiotics. The bacteria resistant to β-lactam were *Staphylococcus pasteuri, Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus uberis* and *Sphingomonas* sp; and bacteria determined as resistant to tetracyclines were *Staphylococcus pasteuri, Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus uberis, Sphingomonas* sp., *Streptococcus dysgalactiae*.

The molecular identification of different genera and bacterial species that cause bovine mastitis together with the study of antibiotic resistance genes allows a safer diagnosis to establish an effective treatment against this multifactorial disease that affect lactating cows in most dairy production units in Ecuador.

Author Contributions

NFBG; Conceptualization, Project Administration, Research methodology, Responsibility, Visualization, Original draft writing, review and editing. XAGJ; Methodology, field and laboratory research, milk sampling, microbiology, data processing at the molecular level. BGFF; Methodology, laboratory research, microbiology data processing at the molecular level. NJBG; Conceptualization, data processing, formal analysis, data curation.

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