



# Prevalence and etiology of mastitis in dairy cattle in El Oro Province, Ecuador

Said AMER<sup>1,2)\*</sup>, Fernando Lenin Aguilar GÁLVEZ<sup>1)</sup>, Yasuhiro FUKUDA<sup>3)</sup>, Chika TADA<sup>3)</sup>, Ivan Ludeña JIMENEZ<sup>1)</sup>, Wunster Favian Maza VALLE<sup>1)</sup> and Yutaka NAKAI<sup>3)\*</sup>

<sup>1)</sup>Departamento de Medicina Veterinaria y Zootecnia, Facultad de Ciencias Agropecuarias, Universidad Técnica de Machala (UTMACH), Ecuador

<sup>2)</sup>Department of Zoology, Faculty of Science, Kafr El Sheikh University, Kafr El Sheikh 33516, Egypt

<sup>3)</sup>Laboratory of Sustainable Environmental Biology, Graduate School of Agricultural Science, Tohoku University, 232-3 Yomogida, Naruko-onsen, Osaki, Miyagi 989-6711, Japan

**ABSTRACT.** This study described the occurrence of clinical and subclinical forms of mastitis in 250 cattle from 5 dairy farms around the cities of Santa Rosa and Machala, El Oro Province, Ecuador. Clinical mastitis (CM) was determined based on obvious changes in milk (mild), signs of inflammation in the udder (moderate), and/or generalized clinical symptoms (severe). Subclinical mastitis (SCM) was assessed using the California mastitis test. CM and SCM were detected in 30 (12.0%) and 150 (60%) of the 250 tested cattle, respectively. Prevalence at the udder quarter level was 57.7% (577/1,000), which was higher among forequarters (369/577; 63.9%) than hindquarters. Of the 577 mastitic milk samples subjected to microbiological analysis, 35 were excluded due to contamination and 20 tested negative. Identification of bacterial isolates revealed that 33.3% of the 93 CM samples contained coliforms, 25.8% coagulase-positive staphylococci, 20.4% coagulase-negative staphylococci (CNS), 9.7% streptococci, 7.5% *Bacillus* spp., and 3.2% *Klebsiella* spp. Bacterial profiling of the 429 SCM milk samples showed that 55.4% contained CNS, 22.1% *Bacillus* spp., 9.3% streptococci, and 6.1% coagulase-positive staphylococci. *In vitro* antibiotic susceptibility testing of the obtained isolates indicated that all were susceptible to amoxicillin, ampicillin, cefotaxime, enrofloxacin, sulfamethoxazole-trimethoprim, gentamicin, and neomycin. No multidrug-resistant strains were observed.

**KEY WORDS:** *Bacillus* spp., Ecuador, mastitis, staphylococci, streptococci

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Mastitis in livestock originates as a reaction of udder tissues to microbial infection or chemical, thermal, or mechanical injury. This disease negatively affects the animal's condition and decreases productivity and milk quality [49], in addition to potential public health challenge. Mastitis results in substantial economic losses, either directly due to low production and early culling of affected animals, or indirectly owing to the cost of treatment and/or veterinary consultations [20, 21]. Furthermore, clinical mastitis (CM) is a serious animal welfare issue, since it is associated with pain and reduced well-being [30]. Subclinical mastitis (SCM) is the main form of this disease in dairy herds worldwide [1, 29, 38, 43, 47], and results in increased numbers of somatic cells in the produced milk and changes in its physical and chemical qualities [32].

A vast range of microorganisms can cause mastitis in cattle, with the major pathogens historically responsible for the majority of cases being *Staphylococcus* (*Sta.*) *aureus*, *Streptococcus* (*Str.*) *agalactiae*, *Str. uberis*, *Str. dysgalactiae* and *Escherichia* (*E.*) *coli* [45]. However, the 'minor' pathogens, including coagulase-negative staphylococci (CNS) and various bacilli have attracted increasing attention [41]. It is well established that CNS and a great variety of *Bacillus* spp. are present in different environmental compartments [40], resulting in the coining of the term 'environmental' mastitis, opposing the 'contagious' form, mainly caused by the major mastitis pathogens, particularly *Sta. aureus* and *Str. agalactiae*. A change in mastitis etiology, from contagious to environmental, is apparent in many countries, accompanied by reductions in the efficiency of traditional mastitis control programs [42]. The distribution of mastitis pathogen strains differs within individual animals in a herd, and between herds, countries, and host species.

Antimicrobial therapy is the most reliable approach to the treatment of mastitis in dairy cattle and the maintenance of animal health and welfare. Effective treatment of bovine mastitis depends on the antimicrobial susceptibility of the causative agent, the disease's clinical manifestation, the cattle breed, and the treatment regimen [3]. The emergence of drug resistance is a serious challenge for mastitis control. As resistance profiles are often herd specific [51], the choice of treatment protocol should be based

\*Correspondence to: Nakai, Y.: yutaka.nakai.d1@tohoku.ac.jp, Amer, S.: mssamer5@yahoo.com

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**Table 1.** Distribution of farms and animals sampled in the present study

Farm	Location	Breed	Herd size	No. of sampled animals	CM <sup>c</sup> (%)	SCM <sup>d</sup> (%)
1	Santa Ines	BS/HF <sup>a</sup>	50	27	2 (7.4)	8 (29.6)
2	Santa Ines	BS/HF	50	31	7 (22.6)	20 (64.5)
3	Santa Rosa	BS/HF, J/B <sup>b</sup>	165	105	14 (13.3)	60 (57.1)
4	Santa Rosa	BS/HF	100	58	5 (8.6)	45 (77.6)
5	Santa Rosa	BS/HF	50	29	2 (6.9)	17 (58.6)

a) BS/HF=Brown Swiss × Holstein, b) J/B=Jersey × Brahman, c) CM=Clinical mastitis, d) SCM=Subclinical mastitis.

on knowledge of the antimicrobial sensitivity of the strain(s) implicated. Combining more than one of synergistic antimicrobial agents may be more effective than using a single drug, and can achieve a high cure rate [27]. Immunotherapy using beads carrying antibodies directed against the causative agent(s), facilitating microbial clearance via phagocytosis, is a newly developed and increasingly adopted approach [28].

Ecuador is an upper middle-income country that promotes animal farming, especially that involving small to medium herds. Importantly, small and medium dairy herds can sustain the development process in low- and middle-income countries [18]. However, very little is known on the incidence and etiology of bovine mastitis in Ecuador. Therefore, the present study was designed to determine prevalence of mastitis in dairy cattle in El Oro Province, Ecuador, and the identity and antimicrobial susceptibility of the causative bacteria.

## MATERIALS AND METHODS

### Ethical statement

This study was approved by the Institutional Committee of Research of the Universidad Tecnica de Machala (UTMACH), Ecuador. In addition, prior written consent was given by the farm owners before sampling. No experiments were conducted on the animals tested.

### Study farms

A total of 250 dairy cattle from 5 farms located around the cities of Santa Rosa and Machala, El Oro Province, Ecuador, were included in the present study (Table 1). The animals were a mixed breed (Holstein Friesian and Brown Swiss). All lactating cows, except those having received antibiotics in the 3 days before sampling, were included in the study. The cows in the farms under investigation differed greatly in age, number of milking days, number of calves, and milk yield.

### Collection of samples

Cases of CM were determined based on obvious changes in milk (mild), signs of inflammation in the udder (moderate), and/or generalized clinical symptoms (severe). Signs of udder inflammation included hotness, redness, hardness of one or more quarters, and a strong pain reaction upon palpation. Generalized clinical symptoms comprised severe udder inflammation accompanied by high temperature and loss of appetite. SCM cases were defined as those without obvious clinical signs and with a high somatic cell count, as determined using the California mastitis test (CMT) [4]. CMT reagent (Milktest, Arthur Schopf Hygiene GmbH & Co. KG, Neubeuern, Germany) was mixed with an equal volume (2 ml) of milk in a four-well paddle for 10 sec, and the results were recorded within 20 sec. CMT results were interpreted using a scoring system ranging from 0 to 4:0 for no reaction, 1 for trace, 2 for weakly positive, 3 for distinctly positive, and 4 for strongly positive. Milk samples from individual mastitic quarters were aseptically collected for bacteriological assays from CM and SCM (CMT score of 3 or 4) cases, as described previously [22, 23]. Briefly, the udder was washed clean and dried using disposable tissue. After discarding the first 5 to 7 streams of milk, the teat ends were disinfected with cotton swabs soaked in 70% alcohol, allowed to dry and 5–10 ml of milk were collected in sterile numbered screw-cap tubes. The milk samples were transported in an ice box at 4°C to the microbiology laboratory at UTMACH.

### Culture and isolation of bacteria

Milk samples from CM and SCM cases were analyzed microbiologically using standard laboratory methods [22]. A loopful of milk was inoculated onto blood agar (agar-based medium enriched with 5% sterile sheep blood) and MacConkey agar plates (Difco, Detroit, MI, U.S.A.), and incubated aerobically at 37°C for 24 to 48 hr. Culture plates on which 3 or more colony types grew were considered contaminated and excluded. Subcultures were made to obtain pure isolates for morphological, biochemical, and molecular identification [48]. Gram-positive cocci were identified by tests of  $\alpha$ - and  $\beta$ -hemolysis, growth on mannitol salt agar (Difco), catalase activity, and coagulase production (positive or negative) using rabbit plasma [6]. Identification of *Bacillus* spp. was confirmed by Gram staining [39]. Gram-negative bacteria were identified based on growth on MacConkey agar, motility, and indole and oxidase tests.

For molecular identification, subcultures of a number of representatives of each bacterial type were grown in lysogeny broth at 37°C for 24 hr, centrifuged at 3,000 rpm for 5 min, washed three times with cold sterile phosphate-buffered saline (PBS) (pH 7.2), and fixed in 95% ethanol.

### DNA extraction and polymerase chain reaction (PCR) analysis

Individual bacterial pellets fixed in ethanol were washed thoroughly with PBS, and genomic DNA was extracted using a NucleoSpin® Tissue kit (MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany). The 16S rRNA gene was amplified as described by Lane [26], using the primer set BACT8f (5'-AGAGTTTGATCCTGGCTCAG-3') and BACT1492r (5'-ACGGTTACCTTGTTACGACTT-3'). The volume of each PCR was 25  $\mu$ l, including 12.5  $\mu$ l of EmeraldAmp® PCR Master Mix (2 $\times$  premix) [Takara Biotechnology (Dalian) Co., Ltd., Dalian, China], 1  $\mu$ l of genomic DNA, 0.75  $\mu$ l (0.3  $\mu$ M final concentration) of each primer, and 10  $\mu$ l of sterile molecular biology-grade H<sub>2</sub>O. PCR conditions consisted of an initial denaturation step at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, and extension at 72°C for 2 min, before a final extension step at 72°C for 7 min. Aliquots (5  $\mu$ l) of the PCR products were electrophoresed on 1% agarose gels and stained with ethidium bromide for visualization.

### DNA sequence analyses

PCR products were directly sequenced using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, U.S.A.) and an ABI 3130xl Genetic Analyzer (Applied Biosystems). Sequences were assembled using ChromasPro software (version 1.5) (<http://www.techneylsium.com.au/ChromasPro.html>), before being aligned with each other and reference sequences from database (NCBI) using ClustalX (<http://www.clustal.org/>). The phylogenetic tree was constructed using the maximum likelihood method, as implemented in MEGA6.06 (<http://www.megasoftware.net>). The robustness of the tree was assessed with 1,000 bootstrap replicates. Unique sequences were submitted to GenBank/the DNA Data Bank of Japan under accession numbers LC317286-LC317315.

### In vitro antibiotic susceptibility test

Antimicrobial susceptibility was evaluated using the Kirby-Bauer method. Briefly, representative strains (75 CNS, 75 *Bacillus* spp., 25 coagulase-positive staphylococci, 25 streptococci and 25 coliforms) of the isolated bacteria were spread on Mueller-Hinton agar plates (Difco), and their susceptibility to the following antibiotics (Oxoid, Basingstoke, U.K.) was tested: amoxicillin (10  $\mu$ g/disc), amoxicillin/clavulanic acid (2:1) (AMC; 30  $\mu$ g/disc), ampicillin (AMP; 10  $\mu$ g/disc), cefotaxime (CTX; 30  $\mu$ g/disc), enrofloxacin (ENR; 5  $\mu$ g/disc), gentamicin (GEN; 30  $\mu$ g/disc), neomycin (NEO; 30  $\mu$ g/disc), penicillin G (PEN; 10 units/disc), streptomycin (STR; 10  $\mu$ g/disc), sulfamethoxazole/trimethoprim (SXT; 25  $\mu$ g/disc) and tetracycline (TET; 30  $\mu$ g/disc). Zones of inhibition (in mm) were measured after approximately 18 hr of incubation at 37°C, and the results were interpreted following Clinical and Laboratory Standards Institute [13] tables. The results are expressed in terms of susceptibility and resistance, with the number of susceptible isolates out of the total number tested being given.

## RESULTS

### Prevalence of mastitis

Cattle with CM and SCM were observed at all of the farms included in this study. The prevalence of moderate CM was found to be 11.6% (29/250). The milk samples taken from the affected udder quarters of these animals consisted of serous fluid or contained blood. Severe CM was seen in one animal (0.4%), which had a gangrenous form and died within one week of detection. SCM was detected in 150/250 (60%) cattle. Prevalence at the level of udder quarters was 57.7% (577/1,000), and was higher among forequarters (369/577; 63.9%) than hindquarters.

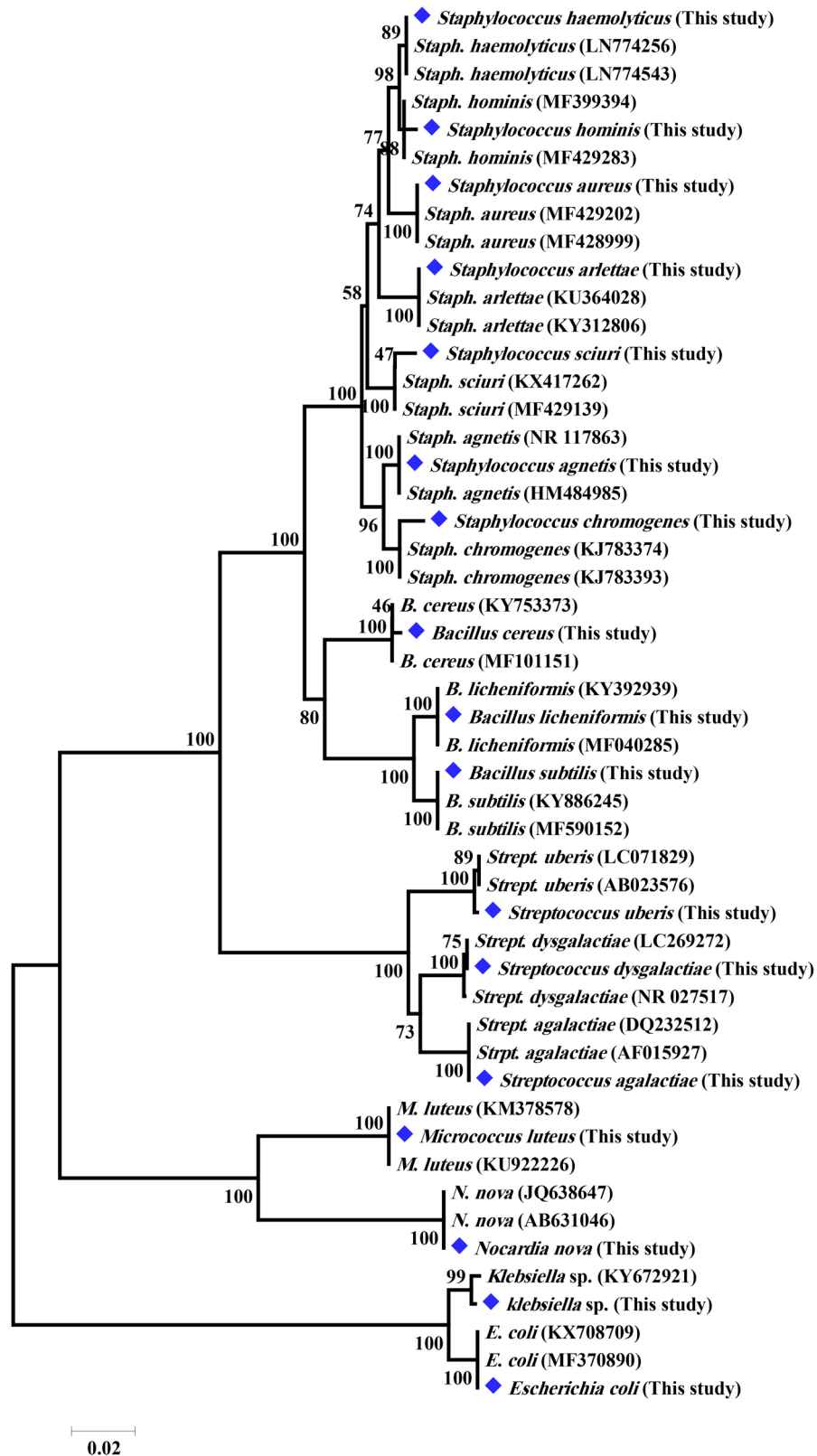
### Microbiological results

A total of 577 milk samples from mastitic quarters were subjected to microbiological analysis. Of these samples, 35 were excluded due to contamination and 20 resulted in no growth. Identification of bacteria isolated from the 93 CM samples indicated the presence of coliforms (33.3%, 31/93), coagulase-positive staphylococci (25.8%, 24/93), CNS (20.4%, 19/93), streptococci (9.7%, 9/93), *Bacillus* spp. (7.5%, 7/93) and *Klebsiella* spp. (3.2%, 3/93).

Bacteria in SCM samples were identified as CNS (55.4%, 238/429), *Bacillus* spp. (22.1%, 95/429), streptococci (9.3%, 40/429) and coagulase-positive staphylococci (6.1%, 26/429). *Micrococcus* spp., coliforms, and *Nocardia nova* were detected in a small number of cases (14/429, 3.3%; 11/429, 2.6%; and 5/429, 1.2%, respectively).

### Genotyping

Analysis of the PCR-amplified 16S rRNA gene sequences from representative CM samples identified the coliform bacteria present as *E. coli*, the coagulase-positive staphylococci as *Sta. aureus*, the CNS as *Sta. epidermidis*, *Sta. haemolyticus*, and *Sta. chromogenes*, the streptococci as *Str. uberis* and *Str. dysgalactiae*, and the *Bacillus* isolates as *B. cereus*. Analysis of bacteria from the SCM samples indicated that the CNS species present included *Sta. epidermidis*, *Sta. haemolyticus*, *Sta. sciuri*, *Sta. arlettae*, *Sta. hominis*, *Sta. agnetis*, and *Sta. chromogenes*. Moreover, the *Bacillus* species were identified as *B. cereus*, *B. licheniformis*, and *B. subtilis*, the coagulase-positive staphylococci as *Sta. aureus*, and the coliforms as *E. coli*. In addition, the streptococci were found to include *Str. uberis*, *Str. agalactiae*, and *Str. dysgalactiae*. The *Micrococcus* isolates were classified as *M. luteus*. In the phylogeny generated, the sequences of the isolated bacteria clustered with those of corresponding taxa from the database (Fig. 1).



**Fig. 1.** Phylogenetic relationships among bacterial species isolated from mastitic cattle based on partial 16S rRNA gene sequences. The evolutionary relationships among 51 taxa were inferred using the neighbor-joining method and Saitou and Nei distances. Numbers at the nodes indicate percentage bootstrap values from 1,000 replicates.

### Antimicrobial susceptibility assay

*In vitro* antibiotic susceptibility tests of the isolated strains indicated that they were susceptible to AMC, AMP, CTX, ENR, SXT, GEN and NEO. However, PEN was ineffective against all *Bacillus* spp. and coliform isolates, and effective against 23/75 CNS and 8/25 coagulase-positive staphylococci. Concerning STR, 53/75 CNS, 22/75 *Bacillus* spp., 16/25 coagulase-positive staphylococci, 25/25 streptococci, and 8/25 coliforms were susceptible. TET susceptibility was noted in 68/75 CNS, 52/75 *Bacillus* spp., 17/25 coagulase-positive staphylococci, and 25/25 streptococci and coliforms. All the bacterial isolates were susceptible to one or more antibiotics; thus, no multidrug-resistant strains were isolated in this study. In addition, no difference was evident between CM and SCM cases in terms of the antimicrobial susceptibility patterns of the isolated bacteria.

## DISCUSSION

Small and medium dairy herds predominate in the livestock industries of low- and middle-income countries [18], representing an important contribution to their economies. In the present investigation of cattle in El Oro Province, Ecuador, both CM and SCM were observed at each of the 5 dairy farms involved. The overall prevalence of CM was found to be 12.0% (30/250). Similar figures have been reported for CM among dairy cattle in India (11.5%) [7] and Ethiopia (12.5%) [60], although its prevalence among dairy cattle in China is estimated to be lower (3.3%) [19]. The frequency of bacterial species isolated from the CM cases indicated the occurrence of environmental (*E. coli*, CNS species including *St. epidermidis*, *St. haemolyticus*, and *St. chromogenes*, streptococci including *Str. uberis* and *Str. dysgalactiae*, and *B. cereus*) and contagious (*St. aureus*) forms. However, pathogens associated with environmental mastitis were more dominant. Consistent with this, the most common pathogens detected in a survey of CM cases in China were *E. coli*, *Klebsiella* spp., CNS, *Str. dysgalactiae*, and *St. aureus* [19], and environmental bacteria including CNS, *Str. uberis*, and *Str. agalactiae* have been found to be common pathogens in dairy cattle in Brazil [36]. Moreover, CNS species such as *St. chromogenes*, *St. epidermidis*, and *St. haemolyticus* were frequently isolated in a study of CM among Canadian cows [15]. In partial agreement with the present results, *E. coli* was identified as the most prevalent pathogen associated with CM in Wisconsin, U.S.A., followed by environmental streptococci, *Klebsiella* spp. and CNS [37]. Furthermore, a survey of dairy farms in England and Wales revealed strains of *Str. uberis* and *E. coli* to be the most common bacteria encountered in cases of CM [5]. However, it has been reported that *St. aureus* predominates in clinical cases in India and Ethiopia [7, 50].

In the present investigation, 60% (150/250) of the cattle tested had SCM. In general agreement with this finding, the prevalence of SCM has been estimated to be 36.7% in Poland [54], 46.4% in Brazil [12], 48.8% in Tanzania [53] and 50.4% in Rwanda [34]. The prevalence of intramammary infection tends to differ greatly from farm to farm and country to country [10, 11, 14, 29, 56]. Discrepancies in the incidence of mastitis may be attributed to differences in animal breeds, management systems, and/or husbandry. Identification of the bacteria isolated from SCM cases indicated that the main pathogenic species were CNS (55.4%). This group of versatile bacteria included *St. epidermidis*, *St. haemolyticus*, *St. sciuri*, *St. arlettae*, *St. hominis*, *St. agnetis*, and *St. chromogenes*. In keeping with these findings, CNS have been implicated in the majority of subclinical udder infections in dairy cattle in various geographical regions, including Mexico [29], Finland [41, 57], Poland [54], Uganda [9], Ethiopia [60], Rwanda [34] and China [19, 59]. Similar CNS species profiles have been documented in intramammary infections in Canadian dairy cattle [15]. In contrast, Bjork *et al.* [9] reported *St. epidermidis* and *St. haemolyticus* to be the only CNS species recovered from mastitic cows in Uganda.

*Bacillus* spp., identified as *B. cereus*, *B. licheniformis*, and *B. subtilis*, were present in 22.1% of cases in the current work. *Bacillus* spp. has also been identified as important pathogens in both CM and SCM in previous investigations [33, 35]. Nonetheless, these results contradict other studies showing that mastitis caused by *Bacillus* spp. is rare in dairy cows [2, 52]. Here, streptococci including *Str. uberis*, *Str. agalactiae*, and *Str. dysgalactiae* were responsible for 9.3% of the SCM cases detected, whereas *St. aureus* was relatively infrequently isolated (6.1%). Similarly, it has been reported that contagious mastitis caused by the pathogens *Str. agalactiae* and *St. aureus* remains a problem in Canada, Rwanda and Poland [31, 34, 54], despite the predominance of CNS. In addition, mastitis is commonly associated with *Str. uberis*, *Str. dysgalactiae* and *St. aureus* in Finland [55]. In contrast to the results of the present investigation, *St. aureus* was found to be the most common of the bacteria isolated from cases of SCM in Uruguay and Tanzania and CNS the rarest [20, 53].

An event common to all the farms under investigation was the lying down of animals after milking, which might promote the colonization of the opened teat canals by environmental pathogens. Notably, CNS, streptococci such as *Str. agalactiae*, and *Bacillus* spp. are known to be prevalent throughout the dairy environment, including on teat skin, milkers' skin and gloves, and farm floors, which represent reservoirs of bacteria associated with intramammary infections [16, 24, 40]. However, the persistence and virulence of the different CNS species that cause such infections is subject of much debate [58].

*In vitro* testing of the isolated strains against various antibiotics revealed that they were susceptible to AMC, AMP, CTX, ENR, SXT, GEN and NEO. However, PEN was only moderately effective against CNS (23/75) and coagulase-positive staphylococci (8/25), and ineffective against all *Bacillus* spp. and coliform isolates. STR was active against streptococci, but varied in effectiveness with respect to other bacteria. The streptococci and coliform strains tested were highly sensitive to TET, high susceptibility to which was also noted among other bacterial species. The results obtained are comparable to those reported previously for bacteria isolated from mastitis cases involving different animal species in a number of regions [8, 17, 44, 46]. However, considering the low number of isolates tested, it is very difficult to reach a firm conclusion concerning antibiotic susceptibility patterns, especially those of coliforms, coagulase-positive staphylococci, and streptococci. Contrary to the results outlined here, Kaczorek *et al.* [25] reported that *Streptococcus* spp. are more resistant to GEN, kanamycin, and TET, but highly susceptible to PEN, ENR and marbofloxacin.

Interestingly, no multidrug-resistant strains were isolated during the course of this study.

**Conclusion:** The results of the present study indicated that clinical (12.0%) and subclinical (60%) forms of mastitis are highly prevalent among dairy cattle in El Oro Province, Ecuador. Bacteria associated with environmental mastitis were most often implicated in the CM (*E. coli*, *Sta. epidermidis*, *Sta. haemolyticus*, *Sta. chromogenes*, *Str. uberis*, *Str. dysgalactiae* and *B. cereus*) and SCM (*Sta. epidermidis*, *Sta. haemolyticus*, *Sta. sciuri*, *Sta. arlettae*, *Sta. hominis*, *Sta. agnetis*, *Sta. chromogenes*, *B. cereus*, *B. licheniformis*, *B. subtilis* and *Str. dysgalactiae*) cases examined. However, contagious mastitis pathogens, including *Sta. aureus*, *Str. uberis*, and *Str. agalactiae*, were also responsible for a considerable proportion of cases. All of the bacterial isolates tested were susceptible to one or more antibiotics; therefore, no multidrug-resistant strains were detected in the present study.

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