**MAYSA SERPA GONÇALVES**

# MOLECULAR EPIDEMIOLOGY AND ANTIMICROBIAL SUSCEPTIBILITY OF *Staphylococcus aureus* AND *Escherichia coli* ISOLATED FROM BOVINE MASTITIS

**LAVRAS – MG**

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Dissertação apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Ciências Veterinárias, área de concentração em Sanidade Animal e Saúde Coletiva, para a obtenção do título de Mestre.

Dr. Alessandro de Sá Guimarães
Orientador
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APROVADA em 29 de Janeiro de 2021.

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## DEDICATÓRIA

*À Santíssima Virgem Maria.*

*Dedico*

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## RESUMO

O Brasil é o terceiro maior produtor de leite do mundo e doenças infecciosas, como a mastite bovina, trazem grandes prejuízos para o setor lácteo. Além disso, alguns patógenos envolvidos no desenvolvimento da doença, também podem causar enfermidades em seres humanos, sendo um problema de saúde pública. Assim, estudos moleculares que avaliam a dinâmica de evolução desses patógenos, bem como sua distribuição espacial e fatores de risco associados são fundamentais para propor medidas de controle. Essas informações permitem monitorar a interface entre cepas bacterianas de origem humana e de animais. Diante disso, para melhor compreender a epidemiologia e a evolução de dois importantes patógenos causadores de mastite bovina e potencialmente "zoonóticos" (*Staphylococcus aureus* e *Escherichia coli*), foram realizados dois estudos com cepas isoladas de bovinos criados em fazendas leiteiras em Minas Gerais, Brasil. O primeiro objetivou avaliar a diversidade genética de isolados de *S. aureus* provenientes de vacas leiteiras utilizando *Multi-locus sequence typing* (MLST) e o perfil de susceptibilidade antimicrobiana. O segundo, por sua vez, teve como objetivo comparar o perfil de virulência e os genótipos (utilizando REP-PCR) de cepas de *E. coli* isoladas de bovinos com mastite subclínica e mastite clínica, e do ambiente de fazendas leiteiras, além de identificar os fatores de virulência e os genótipos potencialmente associados à persistência subclínica da bactéria no úbere. Os resultados mostraram uma alta diversidade entre os isolados de *S. aureus* e um alto número de novos *Sequence Types* (STs) . Também foi observada proximidade genética entre *S. aureus* de origem humana e animal, bem como alta resistência a penicilina, tetraciclinas e isolados resistentes à meticilina (MRSA). Em relação aos resultados do estudo com à *E. coli*, observou-se que o flagelo foi um fator de virulência frequente e pode ser um importante fator para o desenvolvimento de infecções subclínicas e persistentes de *E. coli* na glândula mamária bovina. Os resultados da tipagem molecular por REP-PCR sugerem menor diversidade genética dos microrganismos isolados de *E.coli* de mastite subclínica do que os de mastite clínica e os do ambiente de fazendas leiteiras, embora não tenha sido possível determinar um genótipo específico associado à mastite por *E. coli*.



**Palavras-chave:** MLST. REP-PCR. Zoonose. Multirresistência. Produção de leite.

## ABSTRACT

Brazil is the third largest milk producer in the world and infectious diseases, like bovine mastitis, are extremally important, since causes several economic losses to dairy industry. Besides that, some pathogens involved in mastitis pathogeny can also cause illness in humans, being a public health issue. Therefore, molecular studies that evaluate the dynamic of evolution of these pathogens, as well as distribution and risk factors, are critical to propose control measures and to monitor the interface between human and animal strains. In other to better understand the epidemiology and evolution of two important and zoonotic pathogens of bovine mastitis (*Staphylococcus aureus* and *Escherichia coli*) two studies were conducted with strains isolated from dairy farms in Minas Gerais state, Brazil. The first aimed to evaluate the genetic diversity of *S. aureus* isolated from dairy cows, using Multi-locus sequence typing (MLST), and the antimicrobial susceptibility profiles of these isolates. The second, in turn, aimed to compare the virulence profile and REP-PCR genotypes of *E. coli* isolated from subclinical mastitis, clinical mastitis isolates and dairy farm environment, and to access the virulence factors and genotypes potentially associated with the subclinical persistence into udder. Results showed a high diversity among bovine mastitis *S. aureus* and a great number of new STs were found. Proximity between *S. aureus* isolates from human and animal origin was also observed, as well as high resistance to penicillin and tetracyclines and isolates resistant to methicillin (MRSA). Regarding *E. coli*, it was observed that flagella seems to be a determinant virulence factor in subclinical and persistent infections by this pathogen in bovine mammary gland. Results of molecular typing by REP-PCR suggest that subclinical mastitis isolates are less genetically diverse than clinical mastitis and dairy farm environmental isolates, although it was not possible to determine a specific genotype associated with subclinical and persistent *E. coli* mastitis (MPEC).



**Key words:** MLST. Zoonotic. Multi-drug resistance. Dairy Industry. REP-PCR.

## SUMMARY

# FIRST SECTION

## 1. INTRODUCTION

Brazil is the third largest milk producer in the world (FAO, 2019), with about 1,1 millions of dairy farms (IBGE, 2017). Thus, infectious diseases like bovine mastitis are extremally important to Brazilian dairy industry, resulting in several economic losses (GUIMARÃES *et al*., 2017). The losses are mainly related to milk production decrease, costs with diagnosis and treatment, veterinary service expenses, discarded milk, future milk production loss, reproduction failures and premature culling, and replacement of cows (HEIKKILÄ *et al.*, 2018).

Traditionally, the disease is classified as "contagious mastitis" or "environmental mastitis", according to the microbial agent involved, primary reservoir and mode of transmission (KULKARNI; KALIWAL, 2013). Pathogens classified as contagious are transmitted during milking process and normally cause infection without clinical signs, but increase in somatic cells contain (SCC) is observed. Environmental mastitis pathogens, on the other hand, are present in the environment of dairy farms and the disease usually has intense clinical signs, which might result in the animal to death (RUEGG, 2012).

Among contagious pathogens, *Staphylococcus aureus* stands out as one of the most important, being responsible for milk production losses of 2.3 kg/day (HEIKKILÄ *et al.*, 2018). Usually, this bacteria is associated with subclinical mastitis cases high somatic cells counts (CCS) in milk (RAINARD *et al.*, 2017). *Escherichia coli*, another pathogen of mastitis, is normally an environmental and opportunistic agent, associated with severe and acute cases, although persistent infections have been reported (BLUM; HELLER; LEITNER, 2014; BURVENICH *et al.*, 2003; DÖPFER *et al.*, 1999). Both pathogens can also cause disease humans, being relevant public health issues.

With the advent of molecular technologies, a great genetic diversity has been observed among both contagious and environmental bacterial strains, evidencing that mastitis epidemiology is more complex than proposed by "classic classification" (RUEGG, 2012). In views of that, molecular epidemiological studies are critical to understand the microevolution of the microorganisms, dynamic of transmission of the disease, potential reservoirs and to propose control measures (TIBAYRENC, 2009). In addition, due to the zoonotic potential of these pathogens, it is also possible to evaluate the interface between strains associated with human and animal infections.

There are several molecular typing techniques with different discriminatory powers that can be used according to the objective of the study. Multi-locus sequence typing (MLST) is a sequencing-based genotyping method that assess the polymorphisms in seven housekeeping genes, providing unique allelic profiles, known as sequence types (STs). In *S. aureus*, the level of discrimination (resolution) of MLST allows the assessment of a detailed picture of the global dissemination of this pathogen, supporting insights into its origin and evolution (SAUNDERS; HOLMES, 2007).

REP-PCR is a molecular typing technique based on the Polymerase Chain Reaction (PCR) that use primers that anneal in intergenic repetitive elements described in several bacterial species, mainly enterobacteria such as *E. coli*. These sequences are palindromic and repeated throughout the DNA, generating PCR fragments with different sizes and visualization of specific band patterns (fingerprints) in agarose gel electrophoresis (VERSALOVIC *et al*., 1994). Different linages of bacteria have variation in the quantity and position of these sequences, presenting different patterns, which allows to obtain epidemiological insights (DOMBEK *et al.*, 2000).

To better understand epidemiology of mastitis, evolution of microorganisms involved in and interface with human health, this work contains two articles about two important bovine mastitis pathogens. The first aimed to evaluate the genetic diversity of *S. aureus* isolated from dairy cows in Minas Gerais state, Brazil, using MLST, and the determination of antimicrobial susceptibility profiles of these isolates. The second article aimed to compare the virulence profile and REP-PCR genotypes of *E. coli* isolated from bovine with subclinical and clinical mastitis isolates, and from dairy farm environment in Minas Gerais state, Brazil, and to identify the virulence factors and genotypes potentially associated with *E. coli* subclinical persistence into the udder.

## 2. CONCLUSION

Bovine mastitis is a dynamic and complex disease and the pathogens associated are in constant evolution to adapt to the mammary gland. In this study it was observed a high diversity among bovine mastitis *S. aureus* strains isolated from bovines from dairy farms in Minas Gerais state, Brazil, and a great number of new STs were found. Moreover, proximity between *S. aureus* strains from human and animal origin was also observed, as well as high resistance to penicillin and tetracyclines and de detection of MRSA isolates. These findings highlight the importance of epidemiological and molecular studies about this pathogen, mainly to the human and animal interface. Regarding *E. coli* study, flagella seem to be a determinant virulence factor in subclinical and persistent infections in bovine mammary gland by this pathogen. Results of molecular typing using REP-PCR in *E. coli* strains from bovines and environment of farms in Minas Gerais state, Brazil, suggest that subclinical mastitis strains are less genetically diverse than clinical mastitis and environmental isolates, although it was not possible to determine a specific genotype associated with subclinical and persistent *E. coli* mastitis.

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## SECOND SECTION - ARTICLES

**ARTICLE 1**

Journal: Brazilian Journal of Microbiology

# Genetic diversity and antimicrobial susceptibility of *Staphylococcus aureus* isolated from bovine mastitis in Minas Gerais, Brazil

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## Abstract

The aims of this study were to evaluate the genetic diversity and antimicrobial susceptibility of *Staphylococcus aureus* strains isolated from dairy cows in Minas Gerais, Brazil. Thirty-seven strains isolated from eight herds from five cities were used and susceptibility to 12 antimicrobial agents was tested using disk-diffusion method. All strains were genotyped using multi-locus sequence typing (MLST). High resistance rates for ampicillin [70.27% (26/37)], penicillin [75.68% (28/37)], and tetracycline [70.27% (26/37)] were detected. Multidrug resistance was observed in seven [18.92% (7/37)]

isolates, and two were classified as Methicillin-resistant *Staphylococcus aureus* (MRSA). MLST identified thirty-three novel STs and two known STs (ST126 and ST746). The clonal complexes more frequently observed were: CC97 [78.38%; (29/37)], CC1 [8.11%; (3/37)], CC5 [5.40%; (2/37)]. Minimum-spanning tree (MST) analysis according to data from municipalities, herds, and resistance patterns for all isolates did not show any clustering pattern. However, the MST using ST data from all Brazilian *S. aureus* deposited in PubMLST database depicted an association between the genotype and strain origin of isolation. Isolates from this study that belong to CC97 showed similarity with database strains isolated from milk and dairy products, while those that belong to CC1 and CC5 were similar to database strains isolated from human sources and environment of dairy farm or industry. In conclusion, our results showed a high rate of resistance to penicillins and tetracyclines and a great genetic diversity among the *S. aureus* strains from bovine mastitis genotyped in the present study.

**Keywords:** staphylococci; MLST; multidrug resistance; dairy industry; zoonosis

## 1. Introduction

*Staphylococcus aureus* is a pathogen of humans and animals, which has ability to become resistant to antimicrobials and is considered an important public and animal health issue [1–4]. Due to its high capacity of adaptation to the host and genetic diversity, specific linages evolved to infect particular mammalians species, although transmission between species, including zoonotic transmission, have been reported [5].

In animal health, *S. aureus* stands out as one of the most important pathogen of dairy cattle [6, 7], responsible for causing bovine mastitis. *S. aureus* mastitis causes milk production losses of 2.3 kg/day, in addition to costs with discarded milk, diagnosis and treatment, future milk production loss, premature culling and replacement of cows, among others [9]. In Brazil, the agent is highly prevalent in dairy cattle herds and in the state of Minas Gerais, the prevalence in herdsranging from 28 to 93% have been reported [8, 10, 11].

*S. aureus* is mainly associated with subclinical mastitis cases, causing high somatic cells counts (CCS) detection in milk [3]. The infection among animals is primarily transmitted during the milking process [3, 12], and the bacteria then spread furtively within the herds. To understand the dynamic of the disease transmission, reservoirs, of infections and to propose more effective control measures, it is critical to perform classical and molecular epidemiological studies, which allow the assessment of the frequency, distribution and risk factors associated with staphylococcal mastitis, as well as the characterization of the strains involved [13]. In these studies, it is also possible to evaluate the microevolution of the pathogen and the interface between specific-human and specific-animal strains.

One of the most used epidemiological molecular techniques on *S. aureus* is the multi-locus sequence typing (MLST), which is a sequencing-based genotyping method that assess the polymorphisms in seven housekeeping genes (*arcC, aroE, glpF, gmk, pta, tpi*, and *yqiL*), providing unique allelic profiles known as sequence types (STs). The level of discrimination (resolution) of MLST allows the assessment of a detailed picture of the global dissemination of this pathogen, supporting insights into its origin, pathogenicity and evolution [14]. Thus, the aims of the present study were to evaluate (*i*) the genetic diversity of *S. aureus* isolated from dairy cows located in Minas Gerais state, Brazil, using MLST, (*ii*)to determine the antimicrobial susceptibility profiles of these isolates, and (*iii*) the possible association between these variables and epidemiological data of the isolates.

## 2. Material and Methods

### 2.1.Strains

Thirty-seven strains of *S. aureus* previously isolated from milk samples of dairy cows with mastitis and *S. aureus* ATCC 25923$^{T}$ were used in the present study. Isolation and microbiological characterization of the strains were performed according to described by Brito and Brito [15]. The strains belong to the Collection of Microorganisms of Interest to the Milk Agribusiness (Embrapa Gado de Leite, Brazil), and were isolated between 2009 and 2011from eight herds localized in five municipalities in Minas Gerais state. The municipalities were: Bias Fortes (Herd A n=3 isolates); Bicas (Herd H, n=4); Lima Duarte (Herd D, n=10), Rio Preto (Herd E, n=5; Herd F, n=8; Herd G, n=2), and Santa Rita do Ibitipoca, (Herd B, n=3; Herd C, n=2).

### 2.2.Antimicrobial susceptibility test

Twelve antimicrobial agents were used to assess antimicrobial susceptibility of the isolates using the disk-diffusion method, according to VET01-A4 manual from

Clinical and Laboratory Standards Institute (CLSI 2018) [16]. To classify the isolates as resistant, intermediate, or sensitive to the antimicrobials tested, the CLSI manual VET08 was used (CLSI, 2018) [17]. Multidrug resistance (MDR) was defined as resistance to three or more antimicrobial groups [18]. The antimicrobial groups were as follows: penicillins (ampicillin, oxacillin, and penicillin G); cephems (cephalothin and ceftiofur); lincosamides (clindamycin); macrolides (erythromycin); quinolones (enrofloxacin); aminoglycosides (gentamicin); folate pathway inhibitors (sulfonamide and trimethoprim/sulfamethoxazole); and tetracyclines (tetracycline). Oxacillin resistant *S. aureus* strains were classified as methicillin-resistant *S. aureus* (MRSA) [19].

### 2.3.MLST

MLST was performed based on the DNA sequences of seven conserved housekeeping genes, *arcC* (carbamate kinase), *aroE* (shikimate dehydrogenase), *glpF* (glycerol kinase), *gmk* (guanylate kinase), *pta* (phosphate acetyltransferase), *tpi* (triosephosphate isomerase) and *yqiL* (acetyl coenzyme A acetyltransferase), which were amplified using specific primers as described by Enright et al. (2000) [20]. DNA fragments were sequenced using DYEnamic ET dye terminator cycle sequencing kit and the automatic sequencer DNA MegaBACETM 1000 (GE Healthcare). The quality of sequence was evaluated using Phred software (reliability index > 20) [21] and the consensus sequences were determined using the program CAP3 [22].

Alleles and STs were determined comparing the sequences obtained with those deposited in PubMLST online database (https://pubmlst.org/). Alleles sequences were aligned using MEGA-X version 10.1.8 (Tamura, Stecher, Kumar, 2020) to assess the polymorphisms observed. Isolates that shared four or more identical alleles were grouped in the same clonal complex (CC), according to PubMLST database

(https://pubmlst.org/organisms/staphylococcus-aureus/clonal-complexes). The Hunter and Gaston Diversity Index (HGDI) was calculated to each *locus* and to MLST technique (Hunter & Gaston, 1988) (http://insilico.ehu.eus/mini_tools/discriminatory_power/index.php).

To evaluate population structure and patterns of evolution, genetic comparisons among the isolates were performed using goeBurst algorithm (https://online.phyloviz.net/index) [23]. The same software was used to build the minimum-spanning tree (MST) and to assess possible clustering patterns of the isolates, considering the antimicrobial susceptibility profiles, herds, MDR, and municipalities. Isolates were also compared with all 336 MLST sequence types from Brazilian *S. aureus* strains deposited in PubMLST database (access on 19$^{th}$ September 2020).

**Statistical analysis**

Descriptive analyses were performed with MLST results, antimicrobial susceptibility profile, municipalities and herds of the 37 using Microsoft Excel® (Microsoft Corporation, Redmond, Washington, EUA).

## 3. Results

### 3.1.Antimicrobial susceptibility

The percentages of isolates classified as resistant, intermediate, or susceptible for each antimicrobial tested are shown in Table 1. Resistance was observed mainly to penicillin [75.68% (28/37)], ampicillin [70.27% (26/37)], and tetracycline [70.27% (26/37)].

Susceptibility profiles were constructed based on the antimicrobial groups and eleven profiles were classified (Fig. 1). Multidrug resistance was observed in seven [18.92% (7/37)] isolates and two isolates [2/37; (5.40%)] were classified as MRSA.

### 3.2.MLST

Thirty-five STs were identified among the 37 genotyped isolates, being two previously described (ST126; n = 3; ST746; n = 1) in PubMLST database (pubmlst.org/saureus/) and 33 classified as novel STs. These novel STs were identified based on either the presence of a novel allele not described in the PubMLST database [32/33, (96.97%)] or a unique combination of known alleles [(1/33, 3.03%)]. The number of alleles or STs, the alleles or ST most frequents and the HGDI values are shown in Table 2. The number of alleles per *locus* varied from 7 (*pta*) to 22 (*yqiL*) and the number of novel alleles per *locus* varied from 4 (*pta*) to 15 (*yqiL*). The novel alleles were characterized mostly by nonsynonymous point mutations [41/52 (79%)] (Fig. 2).

Three clonal complexes were observed: CC97 [78.38%; (29/37)], CC1 [8.11%; (3/37)], CC5 [5.40%; (2/37)]; whereas three (5.40%) isolates could not be classified in a clonal complex (Table 5). Different STs and CCs were found in the same herd (Table 3).

The isolates from this study were compared with each other (Fig. 3a) and with all 336 *S. aureus* isolates from Brazil available in MLST database (Fig. 3b) using MSTs. These MLST data were obtained from Brazilian strains isolated between 1997 and 2017 and distributed in all Brazilian regions: Southeast [233/336 (36.35%)], Midwest [61/336 (18.15%)], Northeast [19/336 (5.65%)], South [10/336 (2,98%)], and North [3/336 (0.89%)]. For ten (2.98%) strains information on geographical origin was not available. These strains were isolated from: milk or dairy products (cow, goat, sheep or buffalo) [101/336 (30.06%)], environmental of dairy farm or dairy industry [23/336 (6.85%)], animal [3/336 (0.89%)], other sources (mainly human disease cases) [24/336 (24.40%)] and unknowing sources [127/336 (37.80%)]. Among the isolates it was observed 115 different STs.

MST analysis according to municipality, herd, and antimicrobial resistance profiles considering only the isolates from this study did not show any clustering pattern. On the other hand, MST performed using the isolates from this study and the other Brazilian *S. aureus* isolates deposited in PubMLST showed a clustering pattern for the isolate source of isolation. Most of the isolates from this study were close to Brazilian strains previously isolated from milk and dairy products (CC97), although some isolates exhibited ST similar to isolates from other sources, such as human staphylococcal diseases (CC5) or dairy farms and industry environment (CC1).

## Discussion

Genetic characterization of pathogens bovine associated with mastitis is fundamental to understand the epidemiology of the disease, routes of transmission, reservoirs and to trace control measures. For this, it is necessary to use typing techniques with high discriminatory power and good epidemiological concordance [13]. In this study, using the MLST technique, a high genetic diversity was found among *S. aureus* strains isolated from bovine mastitis, and of the 35 STs identified, 33 (94.28%) STs has not being previously described in Brazil (Table 2).

Many of these new STs are characterized by point mutations in known alleles, which suggests a microevolution of this pathogen to adapt to bovine mammary gland. In fact, a study conducted by Feil *et al*. [24] conclude that point mutations give rise to new alleles at least 15-fold more frequently than does recombination and that these mutations are mainly nonsynonymous, as observed in our study (Fig. 3). In Brazil and especially at Minas Gerais state, which is characterized mainly by small properties, this microevolution of *S. aureus* strains may be even more accelerate, considering the milk production profile with an intense animal trade between herds [25]. This intense trade

combined with inefficient mastitis control measures in the farms favors the constant transmission of strains among cows and herds, which may intensify the selection pressure on the pathogen and thereby the emergence of new STs, as observed in the present study.

On the other hand, it is also important to consider that these new STs found in the present study may be quite common in Brazilian herds (mainly in Minas Gerais state), however had not yet been described, since very few MLST profiles are available at PubMLST database. Indeed, there is only 336 (distributed in 115 different STs) Brazilian *S. aureus* strains deposited in PubMLST of 35,737 *S. aureus* MLST total records. Moreover, of these 336 ST of Brazilian origin deposited, only 47 were identified from bovine mastitis strains (24 STs). This highlights the great scientific contribution of this study to understand the genetic diversity and epidemiology of mastitis caused by *S. aureus* in Brazil.

In contrast, the great number of new alleles and ST observed also resulted in a high genetic diversity among the typed strains, which precluded the observation of clustering patterns that could indicate transmission routes or sources of infection among herds and municipalities. However, it was possible to observe that most of the isolates typed belong toto ST126 and ST746 or were very close to them (Figure 4b). These STs are poorly distributed linages of *S. aureus* around the world and are associated with mastitis in ruminates [2] but mainly described in studies realized in Brazil [2, 26, 27], although ST126 strains have been described causing mastitis in United States [28] and ST746 in Argentina [29]. All *S. aureus* ST746 and most of ST126 strains deposited in PubMLST are isolated from bovine mastitis in Brazil, suggesting that these strains may be adapted to Brazilian dairy herds (milk production system) and thereby easily spread among the properties, as mentioned above.

ST126 is a triple *locus* variant (TLV) and ST746 is single *locus* variant (SLV) of ST97, which is the central genotype of CC97, considered a bovine-specific linage [26, 30, 31]. Although transmission of CC97 strains between cattle and humans are considered relatively rare, reports on human infections caused by strains of this linage are increasing [32]. Because of that, CC97 *S. aureus* has been considered an emerging cause of human infections and the cows a potential reservoir for the emergence of new clones with the capacity for pandemic spread [32], although the epidemiological link is still unclear [5, 32, 33]. The large number of isolates with MLST profiles genetically close to CC97 are of particular concern to Brazil, since CC97 was already detected in strains isolated from samples of fresh Minas cheese (artisanal Brazilian cheese made using raw milk) [34], a potential source for human disease caused by *S. aureus*. Furthermore, another transmission form that cannot be overlooked is from cows to farmers workers, who have constant and direct contact with potentially infected animals [5].

Other relevant issue about CC97 is the emergence of MRSA strains among the isolates that belong to this clonal complex [32, 35, 36], which is a great public health problem concerning zoonotic transmission. In fact, the two MRSA strains identified in the present study, both from Lima Duarte municipality, belonged to CC97. In addition, these two MRSA strains and other isolates also exhibited resistance to other penicillins [28/37 (75.68%)], most of CC97 profile [24/29 (82.76%)], which highlight the concern of zoonotic infections by strains of this CC.

Despite the ccow-to-cow transmission be the most commonly source of infections in bovine mastitis by *S. aureus*,the frequent occurrence of multiple strains with low prevalence or incidence in infected herds suggests that this is not the only route of infection [3]. In this study, in addition to CC97, two other clonal complexes were observed, CC1 [3/37 (8.11%] and CC5 [2/37 (5.40%)]. The two clonal complex are

common and widespread, usually detected in human infections caused by *S. aureus*, but also described in mastitis cases worldwide [27, 37–42].

The evidence of cattle infected with *S. aureus* linages commonly associated with human diseases draws attention to the role of human-to-bovine transmission in bovine mastitis. In fact, according to Boss et al. (2016) [31], *S. aureus* strains of animal origin evolved from human-adapted strains. In the MST analysis, comparing all Brazilian *S. aureus* isolates deposited in PubMLST database and those from the present study (Fig. 4b), it is observed a great similarity among some strains from milk and dairy products and isolates from human infections and environment, most belonging to CC1 and CC5, which also reinforce this epidemiological link. These findings underline the difficult to control and eradicate *S. aureus* from dairy production system, since the farm workers may constitute a stable source of this pathogen. In this sense, specific-human and specific-bovine linages were observed at the same herd, suggesting that different reservoirs can be found in these farms, including humans (Table 5).

The isolates showed high resistance to penicillins and tetracycline (Table 1), which are commonly antimicrobials used for treatment of mastitis in Brazilian dairy cattle, although we could not access the records of commercialization of veterinary drugs available. About other antimicrobial classes tested, most of the isolates were susceptible, suggesting that these classes are not used as much to treat mastitis in this region as penicillins and tetracyclines. On the other hand, as MDR was observed in 18.92% of the isolates, it is possible suppose that other antimicrobial bases have been used as alternatives to penicillins and tetracyclines, which increases selective pressure and favors the emergence of multidrug resistant strains and hinders mastitis control. This emergence is also a public health issue since *S. aureus* may cause disease in humans.

In conclusion, a high genetic diversity and a great number of new STs and alleles were observed among *S. aureus* isolated from dairy cows in Minas Gerais, Brazil, suggesting a dynamic of evolution of this pathogen and a proximity between *S. aureus* isolates from human and animal origin. Moreover, our results also showed high resistance to penicillin and tetracyclines, as well as MRSA isolates, a potential threat to both animal and human health.

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**Conflicts of interest**

Not applicable.

**Ethics approval**

Not applicable.

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411 **Table 1**. Antimicrobial susceptibility of *Staphylococcus aureus* isolated from bovine mastitis in Minas Gerais, Brazil, between 2009 and 2011.

| Antimicrobials | Disk concentration[1] | Resistant | Intermediate | Susceptible |
|---|---|---|---|---|
| Ampicillin | 10 μg | 70.27% (26/37) | 0.00% | 29.73% (11/37) |
| Oxacillin | 1 μg | 5.41% (2/37) | 0.00% | 94,59% (36/37) |
| Penicillin G | 10 U | 75.68% (28/37) | 0.00% | 24.32% (9/37) |
| Cephalothin | 30 μg | 0.00% | 0.00% | 100% (37/37) |
| Ceftiofur | 30 μg | 0.00% | 0.00% | 100% (37/37) |
| Clindamycin | 2 μg | 8.11% (3/37) | 0.00% | 91.89% (34/37) |
| Erythromycin | 15 μg | 8.11% (3/37) | 2.70% (1/37) | 89.19% (33/37) |
| Enrofloxacin | 5 μg | 0.00% | 0.00% | 100% (37/37) |
| Gentamycin | 10 μg | 5.41% (2/37) | 0.00% | 94,59% (36/37) |
| Sulfa + Trimethoprim | 1.25/23.75 μg | 0.00% | 0.00% | 100% (37/37) |
| Sulfonamide | 300 μg | 13.51% (5/37) | 10.81% (4/37) | 75,68% (28/37) |
| Tetracycline | 30 μg | 70.27% (26/37) | 0.00% | 29.73% (11/37) |

412 [1] all purchased from Oxoid, UK

413

414 **Table 2**. Number of different alleles by MLST-*locus* and MLST profiles observed
415 *Staphylococcus aureus* strains isolated from bovine mastitis in Minas Gerais, Brazil,
416 2009-2011.

| ***Locus*/ST** | **Number of alleles/STs** | **Novel alleles/STs (%)** | **Mode[1]** | **HGDI[2]** |
|---|---|---|---|---|
| *arcC* | 8 | 5 (62.5%) | 3 (70.27%) | 0.5030 |
| *aroE* | 14 | 8 (57.1%) | 1 and 68 (32.43% each) | 0.8003 |
| *glpF* | 9 | 6 (66.7%) | 1 (75.68%) | 0.4309 |
| *gmk* | 9 | 3 (33.3%) | 1 and 4 (40.54% each) | 0.6847 |
| *pta* | 7 | 4 (57.1%) | 1 (81.08%) | 0.3453 |
| *tpi* | 13 | 10 (76.9%) | 5 (59.46%) | 0.6441 |
| *yqiL* | 22 | 15 (68.2%) | 92 (21.62%) | 0.9309 |
| MLST | 35 | 33 (94.3%) | 126 (8.11%) | 0.9955 |

417 [1]Most frequent allele or ST. [2]Hunter and Gaston Diversity Index (HGDI)

418 **Table 3.** Number of *Staphylococcus aureus* isolated from bovine mastitis according to
419 clonal complexes (CC) and municipalities, Minas Gerais, Brazil, 2009-2011.

| **City** | **Herd** | **Number of isolates** | **Clonal Complex (CC)** | | | |
|---|---|---|---|---|---|---|
| | | | **CC1** | **CC5** | **CC97** | **UD*** |
| Bias Fortes | A | 3 | 2 | – | – | 1 |
| Santa Rita do Ibitipoca | B | 3 | – | – | 2 | 1 |
| | C | 2 | – | 1 | 1 | – |
| Lima Duarte | D | 10 | – | 1 | 9 | – |
| Rio Preto | E | 5 | – | – | 4 | 1 |
| | F | 8 | – | – | 8 | – |
| | G | 2 | 1 | – | 1 | – |
| Bicas | H | 4 | – | – | 4 | – |
| **Total** | – | 37 | 3 | 2 | 29 | 3 |

420 *UD = undefined.

421

## Figure captions

**Fig. 1.** Antimicrobial susceptibility profiles of *Staphylococcus aureus* isolated from cows with mastitis, in Minas Gerais, Brazil, 2009-2011 Penicillins (PEN), Cephalosporins (CEF), Quinolones (QUI), Tetracyclines (TET), Macrolides (MAC), Lincosamides (LIN), and Folate Pathway Inhibitors (FPI).

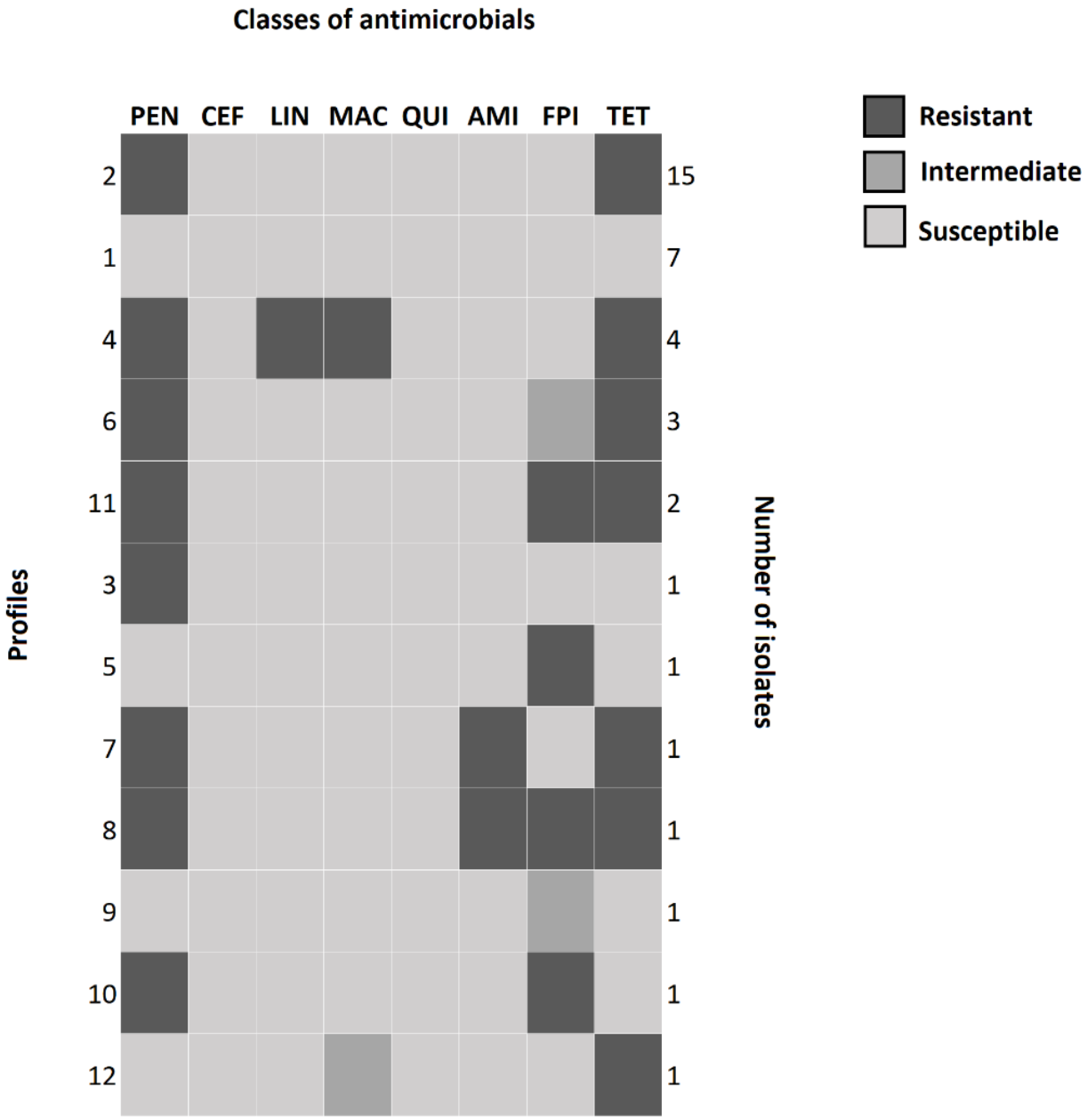
**Fig. 2.** Mutation type by locus of *Staphylococcus aureus* MLST performed in isolates from bovine mastitis, Minas Gerais, Brazil, 2009-2011.

**Fig. 3. (a)** Minimum-spanning trees (MST) of the 37 isolates of *Staphylococcus aureus* isolated from cows with mastitis in dairy herds in Minas Gerais state, 2009-2011, and compared with epidemiological data of municipalities and herds. **(b)** MST generated with MLST data of all Brazilian entries of *Staphylococcus aureus* available in PubMLST (https://pubmlst.org/) and isolates of this study, associated to isolates sources. Circles represent clonal complexes found in this study. Only isolates from this study are identified by numbers. Both MSTs presented was performed using goeBURST algorithm disponible online (https://online.phyloviz.net/index).

438 **ATTACHMENT 1**

439

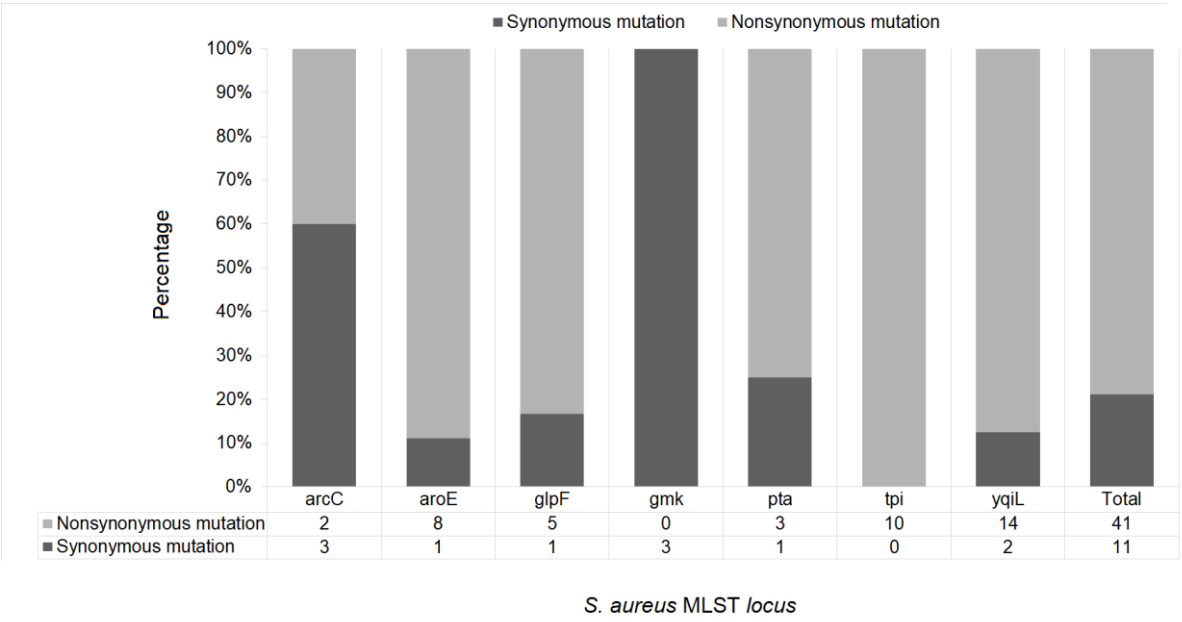
440 **Fig. 1:**




441

442

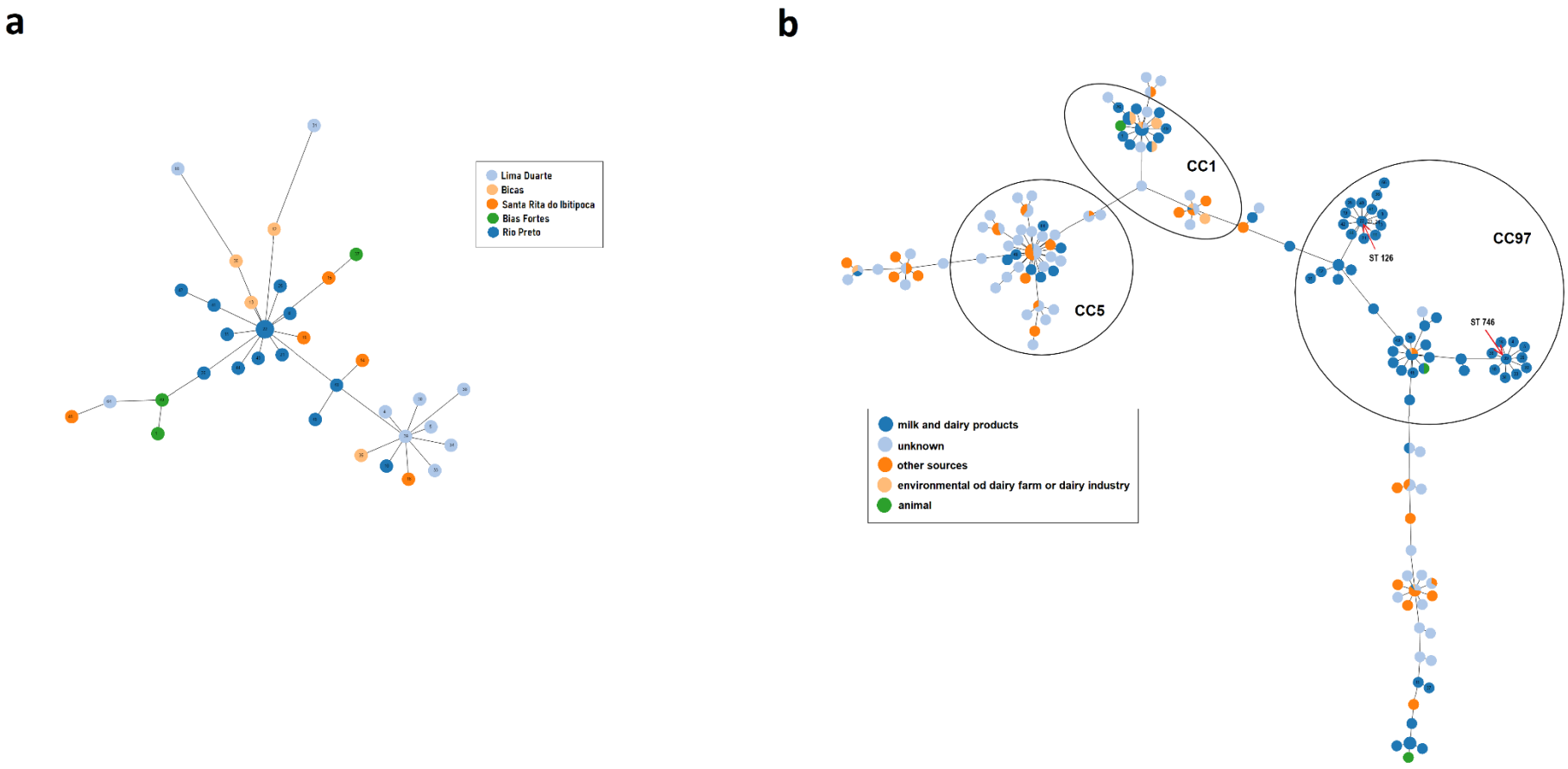
443 **Fig. 2**




444

445

446 **Fig. 3**


a
Lima Duarte
Bicas
Santa Rita do Ibitipoca
Bias Fortes
Rio Preto
b
CC1
CC5
CC97
ST 126
ST 746
milk and dairy products
unknown
other sources
environmental od dairy farm or dairy industry
animal

447

448

1 **ARTICLE 2**
2

3 Journal: Journal of Dairy Research

4 **Flagella are an important virulence factor to subclinical persistence of *Escherichia***
5 ***coli* in bovine mammary gland**

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22

**Summary**

This Research Communication aimed to genotype using REP-PCR and evaluate the virulence profile of 93 *Escherichia. coli* strains isolated from clinical (37) and subclinical mastitis (35), and strains obtained from farm environment (31), in order to assess patterns that are potentially associated with the subclinical persistence of mammary pathogenic *E. coli* - MPEC into de udder. The virulence profile was obtained by prospection of five virulence genes: *lpf*A, *fli*C, F17, *icm*, and *esc*N. Subclinical mastitis showed mainly only *fli*C (profile 3) [12/35 (34.28%)] and *fli*C + *esc*N genes [10/35 (28.57%)], whereas clinical mastitis isolates exhibited mainly *fli*C + *esc*N genes [12/27 (44.44%)] and dairy farm environment isolates the *lpf*A + *esc*N genes [13/31 (41.93%)]. Strains isolated from subclinical mastitis demonstrated 7-fold more chances to be positive to *fli*C than dairy farm environment isolates (DFEI) ($p < 0.05$). Thirty-four genotypes were observed in REP-PCR dendrogram, and clinical mastitis isolates showed more genetic proximity to dairy farm environment isolates than subclinical mastitis isolates. Similar clustering was observed in the minimum-spanning tree (MST). The results suggest that flagella are an important virulence factor for MPEC and that the subclinical persistence of *E. coli* in mammary gland is not related with a specific REP-PCR genotype.

**Key words:** MPEC**,** mammary pathogenic *Escherichia coli*, bovine mastitis, REP-PCR, intramammary infection

## Introduction

Bovine mastitis is the most important and defiant disease in dairy industry, resulting in several economic losses (Ruegg, 2012). The losses are mainly related to milk production decrease, costs with diagnosis and treatment, veterinary service expenses, discarded milk, future milk production loss, reproductive failure and premature culling, and replacement of cows (Heikkilä *et al.*, 2018).

Traditionally, the disease is classified as "contagious mastitis" or "environmental mastitis", according to the microbial agent involved, primary reservoir and mode of transmission (Ruegg, 2012; Kulkarni & Kaliwal, 2013). Pathogens classified as contagious are transmitted during milking process and normally cause infection without clinical signs but with increase in somatic cells contain (SCC) in milk. Environmental mastitis pathogens are present in the environment of dairy farms and the disease caused by them usually has intense clinical signs, which can lead to the animal death (Ruegg, 2012).

In this context, *Escherichia coli* is one of the main pathogen causing mastitis in cows (Bradley, 2002), decreasing milk production of approximately 3.5 kg/day (Heikkilä *et al.* 2018). As *E. coli* is easily found in dairy farm's environment (such as bedding of housed cows, feces, and soil), this agent is classified as an environmental pathogen and is usually associated with severe and acute cases (Burvenich *et al.* 2003). However, cases of persistent and subclinical mastitis have been reported and may represent 4.8% of mastitis cases caused by *E. coli* (Döpfer *et al.* 1999; Blum *et al.* 2014).

Some *E. coli* strains have acquired abilities that allow chronic permanence in the mammary gland and, therefore, the transmission to other animals during milking process, a characteristic of contagious pathogens (Döpfer *et al.* 1999; Shpigel *et al.* 2008). In fact, *E. coli* is a very versatile microorganism and strains adapted to specific niches and species have been reported and classified in pathotypes (Sousa, 2006; Coura *et al*. 2014; Robins-Browne *et al.* 2016). Nowadays, there is an initiative to describe a pathotype adapted to bovine mammary gland as mammary pathogenic *E. coli* (MPEC) (Shpigel *et al.* 2008).

The main characteristic associated with MPEC is high capacity of adherence and invasion of bovine epithelial mammary gland cells (Döpfer *et al.* 2000; Almeida *et al.* 2011; Zhou *et al.* 2019). However, it is still not clear whether exist a specific genotype that exhibits

this ability or whether the MPEC strains share a common set of virulence factors that allows the persistent infections (Blum *et al.* 2015).

Hence, the objectives of this study were (i) to compare the virulence profile and REP-PCR genotypes of subclinical mastitis isolates (SMI), clinical mastitis isolates (CMI) and dairy farm environment isolates (DFEI) of *E. coli* from dairy farms in Minas Gerais State, Brazil, and (ii) to determine the virulence factors and genotypes potentially associated with the subclinical persistence into udder.

## Material & Methods

*Bacterial strains and culture conditions*

Ninety-three *E. coli* strains isolated from milk samples of dairy cows showing clinical (n = 27) and subclinical (n = 35) mastitis, and from dairy farm environment (feces) (n = 31) were used in this study. All strains were isolated from dairy farms localized in Minas Gerais state, Brazil, between 2004 and 2017.

*E. coli* strains were isolated from bovine milk samples sent to the Bacteriology Laboratory to diagnosis. All *E. coli* strains isolated from mastitis cases available in the collection were used in this study. Environmental strains were isolated from dairy cow feces collected in ten dairy farms localized in South of Minas Gerais State. The samples were collected directly on fresh feces of lactating cows using sterile swabs. Around three samples were collected in each farm and one *E. coli* strain was isolated in each fecal sample.

Milk samples were plated in tryptic soy agar (Sigma-Aldrich Corporation, Saint Louis, MO, USA) enriched with 5% equine blood and incubated for 24 hours at 37º C. Feces were plated onto MacConkey agar incubated for 24 hours at 37º C (Sigma-Aldrich Corporation, Saint Louis, MO, USA). Suggestive Gram-negative colonies were tested using KOH (potassium hydroxide) and oxidase tests. The isolates presumptively identified as *E. coli* were submitted to identification by phenotypic tests according to Chair et al., 2004. The strains were maintained frozen at - 80 ºC in Brain Heart Infusion broth (Sigma-Aldrich Corporation, Saint Louis, MO, USA) with 20% glycerol.

*DNA extraction*

DNA extraction was performed using Wizard® Genomic DNA Purification Axygen kit (Promega Corporation, Madison, WI, USA), according to the fabricant recommendations. DNA quality and concentration were determined using NanoVue Plus™ spectrophotometer (GE Healthcare, Chicago, IL, USA).

*Species-specific PCR*

To confirm the isolates as *E. coli*, all strains were submitted to PCR assays using primers derived from the nucleotide sequences flanking the gene encoding the universal stress protein (*usp*A), according to described by Chen & Griffiths (1998), with adaptations on the thermocycling: 5 min initial denaturation at 95 ºC, 35 cycles of 1 min at 95 ºC, 1 min at 66.4 ºC and 1 min at 72 ºC, followed by 7 min final extension at 72 ºC. The primers used, and the size of the fragments are presented in Supplementary material (Table S1). *E. coli* strain ATCC 25922 was used as positive control and PCR mix without DNA template was used as negative control in all assays. Amplicons were separated by electrophoresis in 1.2% agarose gels (w/v) and visualized using 0.5 x Gelred® (Biotium, Inc., Fremont, CA, USA).

*Detection of virulence genes*

PCR for five *E. coli* virulence factors were performed: *lpf*A (long polar fimbriae) according to Blum & Leitner (2013); flagella *fli*C (flagella) according to Dego *et al* (2012); F17 (fimbriae) according to Cid *et al* (1999); *icm* (type VI secretion system) according to Ma *et al* (2013); *esc*N (type III secretion system) according to Kyaw *et al* (2003). The primers used, and the size of the fragments are presented in Supplementary material (Table S1). Strains of the laboratory collection that were identified as positive previously to each gene were used as positive and negative control, respectively. DNA template was used as negative control in all assays. PCR mix without DNA template was also used as negative control in all assays. Amplicons were separated by electrophoresis in 1.2% agarose gels (w/v) and visualized using 0.5 x Gelred® (Biotium, Inc., Fremont, CA, USA).

*REP-PCR*

REP-PCR reactions were performed using PCR Ludwig® kit (Ludwig Biotecnologia Ltda., Alvorada, RS, Brazil) in a final volume of 25 according to Mohapatra et al. (2007). PCR conditions were: 5 min initial denaturation at 95 ºC, 30 cycles of 30 s at 95 ºC, 1 min at 40 ºC, 8 min at 65 ºC, followed by a 16 min final extension at 65 ºC. Amplicons were separated by electrophoresis 1% (w/v) agarose gels and visualized by ethidium bromide staining (0.5 mg/mL) (Ludwig Biotecnologia Ltda, Brazil).

Fingerprints were analyzed using the software BioNumerics® 7.5 (Applied Maths, Sint-Martens-Latem, Belgium) and dendrograms analyzes were performed using Dice coefficient and the unweighted pair group method with arithmetic mean (UPGMA). The minimum-spanning tree (MST) was generated using the same software and compared with data for presence of virulence genes, source (milk or dairy farm environment) and mastitis clinical presentation (clinical or subclinical) to assess clustering patterns of the strains. MST was performed using the UPGMA to calculate the distance matrix Prim's algorithm associated with the priority rule and the permutation resampling. The tree with highest reliability score was presented.

*Statistical analyzes*

Descriptive analyzes to compare presence of virulence genes and the source of the isolate (SMI, CMI or DFEI) were performed using Microsoft Excel® (Microsoft Corporation, Redmond, Washington, EUA). Chi-square test and the *odds ratio* were calculated using the EpiInfo™ software 7.2.2.6 (Centers for Disease Control and Prevention-CDC, Atlanta, Georgia, USA) to analyze possible association between these variables.

## Results

The occurrence of five tested virulence genes according to source of isolation and the association between these variables are shown in Table 1.

All isolates were negative to *F17* and *icm* genes. All DFEI were positive to *esc*N and CMI exhibited 3.5 times more chance to be positive to *esc*N gene compared with SMI. On the other hand, SMI showed 7.3 times more chance to harbor the *fli*C gene than DFEI.

Regarding the *lpf*A gene, DFEI showed 4.7 times more chance to exhibit this gene compared with SMI.

Eight virulence profiles were constructed from the results of the five genes analyzed (Supplementary material, Figure S1). SMI exhibited mainly only the *fli*C gene (profile 3) [12/35 (34.28%)] and *fli*C + *esc*N (profile 5) genes [10/35 (28.57%)]. CMI exhibited mainly *fli*C + *esc*N genes (profile 5) [12/27 (44.44%)] and DFEI *lpf*A + *esc*N genes (profile 7) [13/31 (41.93%)].

Thirty-four genotypic profiles were observed in the dendrogram among the isolates studied (Figure 1a). The dendrogram and the MST (Figure 1b) showed genetic proximity between CMI and DFEI and segregation of some SMI. Similar clustering could also be seen in the MST.

## Discussion

The dynamic and interaction among parasite, host and environmental in infectious diseases are very complex, being hard to define one of these links as determinant to the clinical presentation of bovine mastitis. Because of that, studies about MPEC have been drawing conflicting conclusions (Shpigel *et al.* 2008; Blum *et al.* 2015; Leimbach *et al.* 2017). Our results demonstrated a clustering of SMI using REP-PCR results (Figure 1), suggesting that these strains are less genetically diverse than CMI and DFEI. Blum & Leitner (2013) observed less genetic diversity in MPEC using multi-locus sequence typing (MLST) and pulsed-field gel electrophoresis (PFGE) when compared with environmental *E. coli* strains. However, these evidences are not enough to support the existence of an *E. coli* genotype adapted to the mammary gland causing subclinical disease. On the other hand, our results strongly suggest that flagella are an important factor in subclinical infections caused by *E. coli*. Thus, it is possible to speculate that MPEC may be determined by a set of virulence factors, including flagella, the allows the persistence in the mammary gland (Blum *et al.* 2008) and not by a specific genetic profile determined by fingerprint typing methods or house-keeping genes.

Flagellum is The flagella are an important virulence factor for *E. coli*, which mainly allows mobility in liquid environments, but is also related with adhesion and invasion of host cells, including mammary gland cells (Zhou *et al.* 2015). Compared to DFEI, SMI

exhibited 7-fold more chances to be positive to *fli*C gene, which encodes bacterial flagellin protein. In this sense, according to a study performed by Almeida *et al* (2011) comparing chronic and acute *E. coli* mastitis strains, the chronic strains were more capable to adhere, invade and multiplicate in epithelial mammary gland cells. In agreement with these results, adhesion and invasion abilities of MPEC were also reported in other studies (Döpfer *et al.* 2000; Dogan *et al.* 2006). Altogether, these and our findings, strongly suggest that flagella are a key virulence factor in infections caused by MPEC, probably by giving great mobility in milk, which allows to reach mammary gland cells more quickly, besides collaborating to adhesion and invasion of these cells. Additionally, the low expression of TLR-5 in bovine mammary gland (Porcherie *et al.*, 2012) can explain the absense of strong immune response and clinical signals infections caused by MPEC, which was expected at first sight. Moreover, bacterial flagellin is not also recognized by bovine mammary epithelial cells (Porcherie *et al.* 2012), allowing subclinical and chronic permanence of the pathogen inside the udder without being detected.

Beyond flagella, long polar fimbriae (*lpf*A) is an *E. coli* virulence factor related with adhesion and invasion of host cells and have been mentioned as a key virulence factor in infections by MPEC (Dogan *et al.* 2012; Blum & Leitner, 2013; Zhou *et al.* 2019). Nonetheless, curiously, this gene was poorly found in SMI, possibly indicating that MPEC needs a virulence factor that allows the adhesion e invasion in epithelial mammary cells, but this factor does not necessarily have to be the long polar fimbriae and may be the flagella. Likewise, type VI secretion system (SST6) is a virulence factor previously related with MPEC (Richards *et al.* 2015) that it was not identified in our study. Another secretion system – type III, prospected by PCR to *esc*N gene – was found in about half of SMI, albeit it was more frequent in CMI and DFEI. These results suggest that secretion system is a common virulence factor in pathogenic *E. coli*, as already described in literature (Buttner, 2012), but is not exclusively associated with in infections caused byMPEC, since most of the subclinical isolates did not harbor neither the two systems prospected.

Persistent *E. coli* strains probably pass to stages of subclinical disease between the clinical episodes (Döpffer *et al.* 1999), then is probably that some strains causing subclinical mastitis can be MPECs. However, although CMI showed more genetic proximity to DFEI when compared with SMI (Figure 1) in REP-PCR results – evidencing the environmental

route of transmission of these pathogens – we cannot state that all CMI strains are opportunistic pathogens and strictly related with acute and transient cases. Actually, regarding virulence profile, CMI were more similar to SMI than to DFEI and 59.3% of the isolates showed the flagella gene. Then, assuming that MPEC is defined by a set of virulence factors and strains present in the environmental could have these factors (Blum *et al.* 2008), these strains also may adapt to mammary gland and cause persistent and contagious infections (Ruegg, 2012). This fact highlights the role of environmental as a source of MPEC to mammary gland, although less important that cow-to-cow transmission.

This epidemiological link (environmental of dairy farm as source of mastitis clinical and subclinical isolates) may explain the high genetic proximity among most of the isolates (> 90% of similarity) (Figure 1) in REP-PCR, although it is possible to observe greater proximity between CMI and DFEI and a segregation of some SMI. On the other hand, it is important to consider that even highly genetically similar strains can cause disease with variable degrees of severity and clinical signs, according to capacity of immune response and other factors attributed to the cows infected (Burvenich *et al.* 2003). Other issue that can explain the high silimilarity among the isolates is the REP-PCR lower power of discrimination when compared to others molecular techniques, as PFGE (Bae *et al.* 2014). This explanation is less likely, since REP-PCR techniques was already used to molecular typing of *E. coli* from different sources (Dombek et al. 2000; Mohapatra et al. 2007; Chapaval et al. 2010).

## Conclusion

Flagella seems to be a determinant virulence factor in subclinical and persistent infections by *E. coli*. Results of molecular typing by REP-PCR realized with *E. coli* from farms localized in Minas Gerais state, Brazil, suggest that subclinical mastitis isolates are less genetically diverse than clinical mastitis and dairy farm environmental isolates, but it was not possible to determine a specific genotype associated with subclinical and persistent *E. coli* mastitis (MPEC).

## Acknowledgments

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## Tables

**Table 1**. Frequency of virulence genes by source in *Escherichia coli* isolated from cows with subclinical and clinical mastitis and from dairy farm environment, Minas Gerais, Brazil, 2004-2017.

| Gene | Source | Positive | Negative | OR | CI (95%) | P-value |
|---|---|---|---|---|---|---|
| **F17** | SMI | 0/35 | 35/35 | - | - | - |
| | CMI | 0/27 | 27/27 | - | - | - |
| | DFEI | 0/31 | 31/31 | - | - | - |
| ***lpf*A** | SMI | 8/35 (22.9%) | 27/35 (77.1%) | Base category | - | - |
| | CMI | 3/27 (11.1%) | 24/27 (88.9%) | 0.4219 | 0.1003 - 1.7741 | 0.2230 |
| | DFEI | 18/31 (58.1%) | 13/31 (41.9%) | 4.6731 | 1.6131 - 13.5379 | 0.0035 |
| **ICM SST6** | SMI | 0/35 | 35/35 | - | - | - |
| | CMI | 0/27 | 27/27 | - | - | - |
| | DFEI | 0/31 | 31/31 | - | - | - |
| ***fli*C** | DFEI | 11/31 (35.5%) | 20/31 (64.5%) | Base category | - | - |
| | SMI | 28/35 (80%) | 7/35 (20%) | 7.2727 | 2.4020 - 22.0205 | 0.0002 |
| | CMI | 16/27 (59.3%) | 11/27 (40.7%) | 2.6446 | 0.9132 - 7.6588 | 0.0702 |
| ***esc*N** | SMI | 14/35 (40%) | 21/35 (60%) | Base category | - | - |
| | CMI | 19/27 (70.4%) | 8/27 (29.6%) | 3.5625 | 1.2249 - 10.3609 | 0.0175 |
| | DFEI | 31/31 (100%) | 0/31 | - | - | - |

SMI = subclinical mastitis; CMI = clinical mastitis; DFEI = dairy farm environment; OR = Odds Ratio; CI = confidence interval.

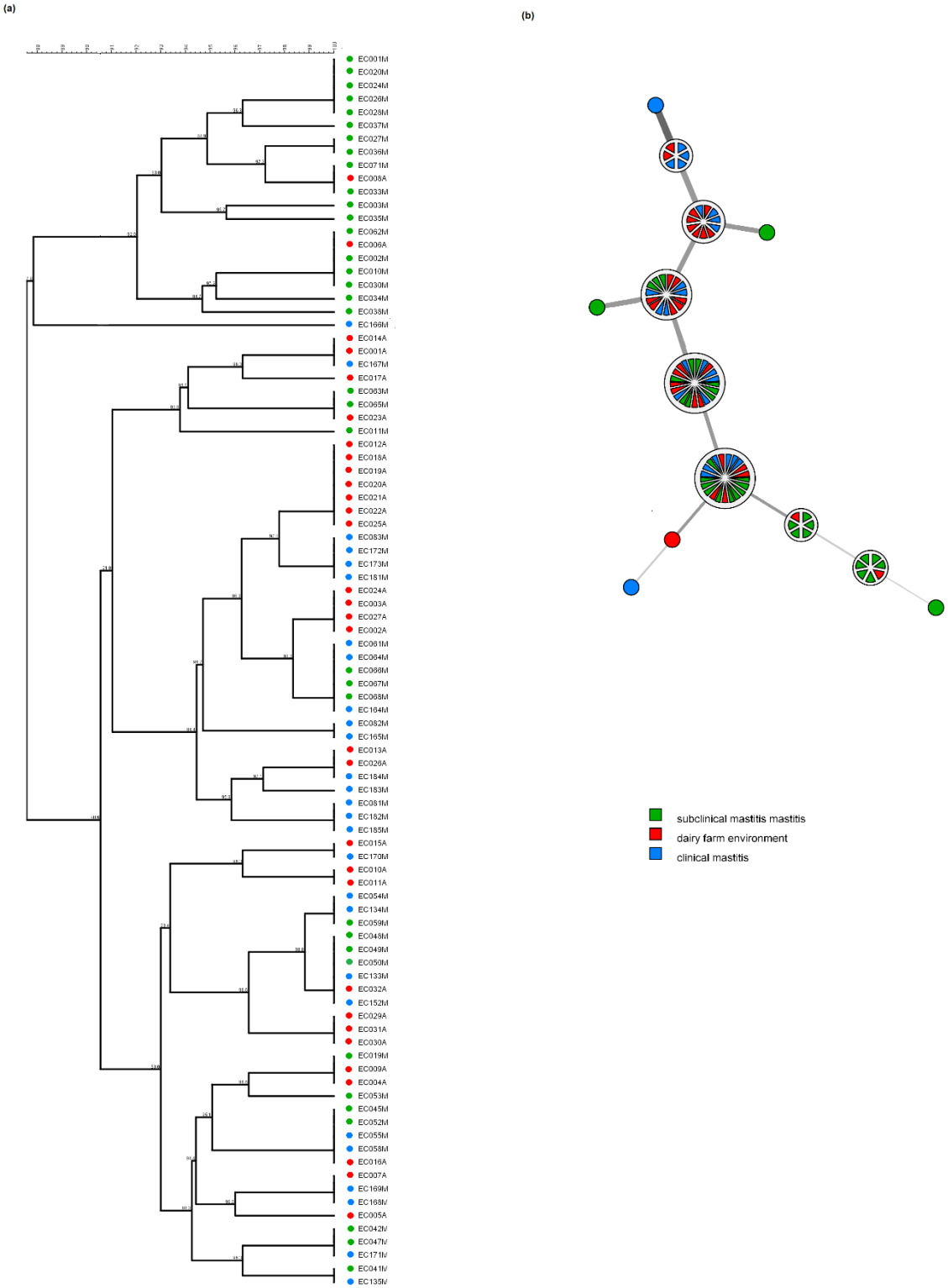
## Figures legends

**Figure 1.**

Dendrogram (a) and minimum-spanning tree (MST) (b) performed using REP-PCR fingerprints of *Escherichia coli* isolated from cows showing subclinical and clinical mastitis and from dairy farm environment, Minas Gerais, Brazil, 2004-2017.

# Figures

## Figure 1:



## Supplementary material

**Table S1.** Fragment sizes, primers sequences and methodology references used to prospect virulence genes in *Escherichia coli* isolated from cows with mastitis and from dairy farm environment.

| Gene | Primers sequences | Methodology references |
|---|---|---|
| *lpfA* (879bp) | F -5'GGACATCCTGTTACAGCGCGCA<br>R -5'TCGCCACCAATCACAGCCGAAC | (Blum and Leitner, 2013) |
| *fliC* (146bp) | F - 5' CCGGTGGTGATAACGATGGG<br>R - 5' CAGGTGTACCGCCTGAAGTG | (Dego *et al.* 2012) |
| *F17* (254bp) | F - 5' TATCCTTGGAATACTGGCGG<br>R - 5' CCAGTGGTGTAATCCGTGTT | (Cid *et al.* 1999) |
| *icm* (495bp) | F - 5'AGAAACCTCCTGACTGAGTTGG<br>R - 5'TTTCATTCCGTTATCCACTTTAAG | (Ma *et al.* 2013) |
| *escN* (815bp) | F - 5'CGCCTTTTACAAGATAGAAC<br>R -5'CATCAAGAATAGAGCGGAC | (Kyaw *et al.* 2003) |

**Figure S1.** Virulence profiles obtained prospecting five virulence genes (*lpf*A, *fli*C, F17, *icm* and *esc*N) in *Escherichia coli* isolated from cows with subclinical and clinical mastitis and from dairy farm environment, Minas Gerais, Brazil, 2004-2017.