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

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Research Article

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Abstract

We compared the virulence profile and REP-PCR genotypes of *Escherichia coli* strains isolated from subclinical and clinical mastitis cases and dairy farm environments in Minas Gerais State, Brazil, to determine virulence factors and genotypes potentially associated with subclinical persistence in the udder. The virulence profile was obtained by the search for three virulence genes: *lpf*A (long polar fimbriae), *fli*C (flagella), and *esc*N (type III secretion system). Subclinical isolates exhibited mainly the *fliC* gene (33.33%) and *fliC* + *escN* genes (30.30%). Clinical isolates exhibited mainly *fliC* + *escN* genes (50%) and environmental isolates the *lpfA* + *escN* genes (58.04%). Strains isolated from subclinical mastitis showed 6.75 times more positivity to *fli*C than environmental isolates. Thirty-four genotypes were observed in the REP-PCR analysis, and clinical mastitis isolates indicated more genetic proximity to dairy farm environment isolates than subclinical mastitis isolates. In conclusion, the results suggested that flagella may be an important virulence factor for mammary persistent *E. coli* infection in cattle, however, none of the *E. coli* REP-PCR genotypes were associated with subclinical infection.

Escherichia coli is one of the main pathogens causing infection in the bovine mammary gland worldwide (Bradley, 2002). Bovine mastitis is the most challenging disease in the dairy industry, resulting in several economic losses (Ruegg, 2012). Due to its widespread presence in dairy farm environments, such as cow bedding, faeces and soil, *E. coli* is classified as an environmental and opportunistic pathogen in the bovine mammary gland and the infections are usually associated with severe and acute mastitis cases (Burvenich *et al*., 2003). However, persistent and subclinical mastitis have also been reported and may represent 4.8% of all *E. coli* mastitis cases (Döpfer *et al*., 1999; Blum *et al*., 2014).

E. coli is known to be a very versatile microorganism, with pathotypes adapted to specific niches and species (Sousa, 2006; Coura *et al*., 2014; Robins-Browne *et al*., 2016). Indeed, some *E. coli* strains have acquired abilities that allow chronic permanence in the mammary gland and transmission to other animals during the milking process (Döpfer *et al*., 1999; Shpigel *et al*., 2008). In this context, there is an initiative to describe an *E. coli* pathotype adapted to the bovine mammary gland, named mammary pathogenic *E. coli* (MPEC) (Shpigel *et al*., 2008). Among the virulence factors associated with *E. coli* pathogenicity, the flagellum has been highlighted as more than a motility organelle. This structure is also related to biofilm formation, protein export, host immune system modulation and adherence, which is one of the most important mechanisms for the invasion and colonization of epithelial cells (Duan *et al*., 2012; Haiko and Westerlund-Wikström, 2013). Likewise, adherence has been reported as an important mechanism in *E. coli* infections of bovine mammary gland cells (Döpfer *et al*., 2000; Almeida *et al*., 2011; Zhou *et al*., 2019).

Hence, we aimed to compare the virulence profile and REP-PCR genotypes of *Escherichia coli* strains isolated from subclinical and clinical mastitis cases and the dairy farm environment in Minas Gerais State, Brazil, to determine virulence factors and genotypes potentially associated with subclinical persistence in the udder.

Material and methods

Bacterial strains and culture conditions

Eighty-eight *E. coli* strains isolated from milk samples of dairy cows showing clinical ($n = 24$) and subclinical ($n = 33$) mastitis and from dairy farm environment (faeces) ($n = 31$) were used

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in this study. All strains were isolated from dairy farms located in Minas Gerais state, Brazil, between 2004 and 2017. All the *E. coli* isolates are part of the microorganisms collection of the Laboratório de Bacteriologia, Universidade Federal de Lavras, which receives samples for mastitis diagnosis from the entire state of Minas Gerais and neighbouring states. All *E. coli* strains available in the collection were used in this study.

Environmental strains were isolated from dairy cow faeces collected in ten dairy farms in the South of Minas Gerais State. The samples were collected directly from the fresh faeces of lactating cows using sterile swabs. Up to three samples were collected in each farm, and one *E. coli* strain was isolated from each faecal sample. Faecal samples were plated onto MacConkey agar and incubated for 24 h at 37°C (Sigma-Aldrich Corporation, Saint Louis, MO, USA). Milk samples were plated in Tryptic Soy agar (Sigma-Aldrich Corporation, Saint Louis, MO, USA) enriched with 5% equine blood and incubated for 24 h at 37°C. Suggestive Gram-negative colonies were tested using KOH (potassium hydroxide) and oxidase tests. The isolates presumptively identified as *E. coli* were submitted for identification by phenotypic tests according to Markey *et al.* (Markey *et al.* 2013). The strains were stored at – 80°C in a solution containing Brain Heart Infusion broth (Sigma-Aldrich Corporation, Saint Louis, MO, USA) and 20% glycerol (Synth, Diadema, SP, Brazil).

DNA extraction

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

Species-specific PCR

All presumptive *E. coli* strains were submitted to PCR assays to confirm species identification. The PCR assays were performed using primers flanking the gene encoding the universal stress protein (*usp*A), according to Chen and Griffiths (1998). Some adaptations in the cycling conditions were made: 5 min initial denaturation at 95°C, 35 cycles of 1 min at 95°C, 1 min at 66.4° C, and 1 min at 72°C, followed by 7 min final extension at 72° C. *E. coli* strain ATCC 25922 was used as a positive control, and PCR mix without a DNA template was used as the negative control in all assays (Supplementary Materials Table S1). Amplicons were separated by electrophoresis in 1.2% agarose gels (w/v) and visualized using 0.5 × Gelred® (Biotium, Inc., Fremont, CA, USA).

Detection of virulence genes

PCR assays targeting three *E. coli* virulence factors were performed: *lpf*A (long polar fimbriae) (Blum and Leitner, 2013); *fli*C (flagella) (Dego *et al.*, 2012); *esc*N (type III secretion system) (Kyaw *et al.*, 2003). Positive controls, primers, and PCR conditions used in all PCR assays for the detection of virulence genes are summarized in the online Supplementary Materials (Table S1). PCR mix without a DNA template was also used as the negative control in all assays. Amplicons were separated by electrophoresis, as stated before.

REP-PCR

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

Fingerprints were analysed using the software BioNumerics® 7.6 (Applied Maths, Sint-Martens-Latem, East Flanders, Belgium), and similarity analyses were performed using the Dice coefficient (Dice, 1945), and the unweighted pair group method with arithmetic mean (UPGMA) (Sokal and Michener, 1958). The minimum spanning tree (MST), generated using the same software, was used to identify any clustering patterns among the strains based on the presence of virulence genes, source (milk or dairy farm environment), and mastitis clinical presentation (clinical or subclinical). MST was performed using

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

Virulence gene	Source of the isolates	Positive	Negative	OR	CI (95%)	*P*-value
lpfA	SM	7/33 (21.21%)	26/33 (78.79%)	Base category		
	CM	3/24 (12.50%)	21/24 (87.50%)	0.53	0.12–2.31	Non-significant
	DFE	18/31 (58.06%)	13/31 (41.94%)	5.14	1.71–15.42	$P < 0.05$
fliC	SM	26/33 (78.78%)	7/33 (21.21%)	6.75	2.22–20.54	$P < 0.05$
	CM	14/24 (58.33%)	10/24 (41.66%)	2.54	0.85–7.61	Non-significant
	DFE	11/31 (35.48%)	20/31 (64.51%)	Base category		
escN	SM	14/33 (42.42%)	19/33 (57.57%)	Base category		
	CM	16/24 (66.67%)	8/24 (33.33%)	3.71	0.91–8.11	Non-significant
	DFE	31/31 (100%)	0/31 (0%)			

SM, subclinical mastitis; CM, clinical mastitis; DFE, dairy farm environment; OR, Odds Ratio; CI, confidence interval.
*χ^2 test.

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the UPGMA to calculate the distance matrix Prim's algorithm associated with the priority rule and the permutation resampling. The tree with the highest reliability score was selected.

Statistical analyses

Descriptive analyses to compare the presence of virulence genes and the source of the isolate (subclinical mastitis, clinical mastitis and dairy farm environment) were performed using Microsoft Excel® (Microsoft Corporation, Redmond, Washington, EUA). The χ^2 test and the *odds ratio* were calculated using the EpiInfo™ software 7.2.2.6 (Centers for Disease Control and Prevention-CDC, Atlanta, GA, USA) to analyse possible associations between these variables, with the level of significance of $P < 0.05$.

Results

All environmental strains and 66.67% (16/24) of clinical isolates were positive for *esc*N (Table 1). However, subclinical isolates were 6.75 times more likely to harbour the *fli*C gene than the environmental isolates. Regarding the *lpf*A gene, environmental isolates showed 5.14 times more chance of exhibiting this gene than subclinical strains.

Eight virulence profiles were constructed from the results of three analysed genes (Fig. 1). Subclinical isolates exhibited mainly *fli*C gene [11/33 (33.33%)] and *fli*C + *esc*N genes [10/33 (30.30%)]. Clinical isolates exhibited mainly *fli*C + *esc*N genes [12/24 (50%)] and environment isolates *lpf*A + *esc*N genes [18/31 (58.04%)]. The occurrence of the three tested virulence genes according to the source of isolation and the association between these variables are shown in Table 1.

Thirty-four REP-PCR genotypic profiles were observed in the dendrogram among the studied isolates (Fig. 2a). The dendrogram and the MST (Fig. 2b) showed genetic proximity between clinical and environmental isolates and the segregation of some subclinical strains.

Discussion

The dynamic interaction among parasite, host and environment in bovine mastitis can be very complex and, as a result, studies about persistent infections caused by *E. coli* have drawn conflicting conclusions (Shpigel *et al.*, 2008; Blum *et al.*, 2015; Leimbach *et al.*, 2017). To contribute to this discussion, we conducted a comparative analysis of *E. coli* strains isolated from cows with clinical and subclinical mastitis and from the environment of dairy farms. Our goal was to determine the degree of genetic relatedness among these strains and identify potential genetic determinants that might be associated with the pathogen's ability to persist within the mammary gland. To do this, we consider that subclinical isolates would be more likely to be classified as MPEC due to the characteristics of contagious transmission and persistence in the mammary gland proposed for this pathotype. Our results showed some peculiarities in subclinical isolates, such as lower genetic diversity and significantly higher prevalence of *fli*C gene compared with the other groups of strains evaluated.

The genetic diversity was analysed using the REP-PCR technique. The results demonstrated the clustering of subclinical isolates (Fig. 2), suggesting that these strains are less genetically diverse than clinical and environmental isolates. In accordance, Blum and Leitner (2013) also observed lower genetic diversity in MPEC compared with environmental *E. coli* strains, using

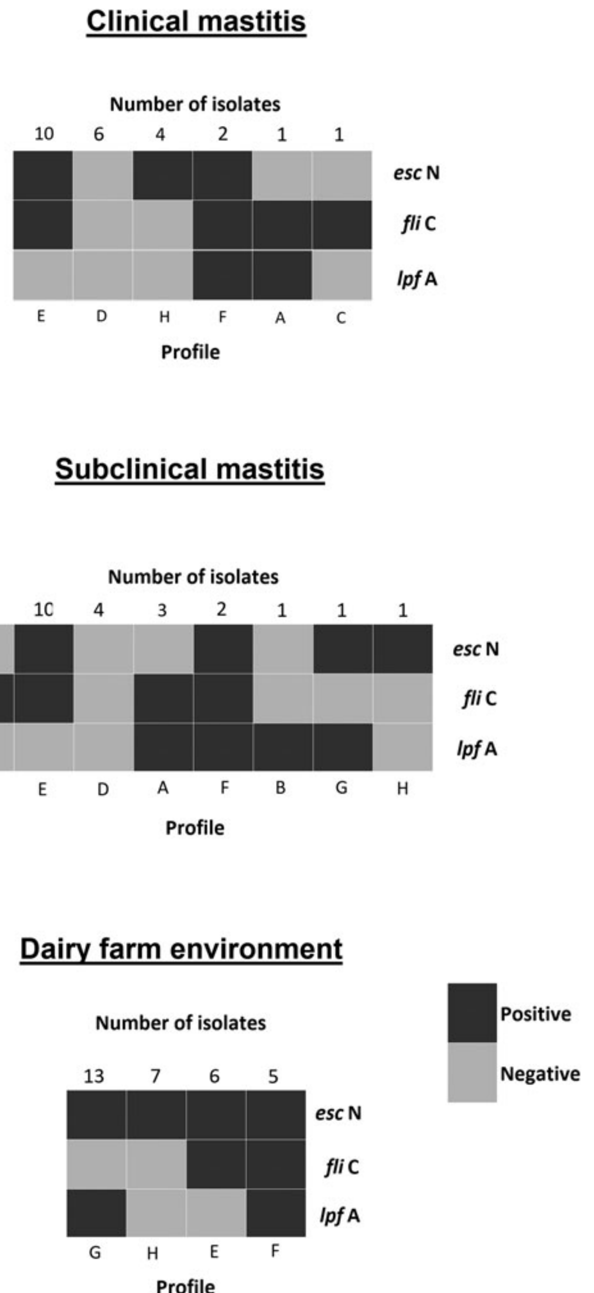

Fig. 1. Eight virulence profiles (A, B, C, D, E, F, G, H) observed in *Escherichia coli* isolated from subclinical mastitis, clinical mastitis, and dairy farm environment, performed using three virulence genes: *lpfA* (long polar fimbriae), *fliC* (flagella), escN (type III secretion system). Minas Gerais, Brazil, 2004–2017.

multi-locus sequence typing (MLST) and pulsed-field gel electrophoresis (PFGE) techniques. In addition, we observed that subclinical isolates had 6.75-fold more likelihood of being positive for the *fli*C gene – which encodes bacterial flagellin protein – compared with environmental isolates, which suggests that flagella are an important factor in subclinical and chronic infections caused by *E. coli* strains and, probably, by MPEC.

In several human and animal diseases, the pathogenicity of *E. coli* is highly associated with its capacity to adhere to and invade epithelial cells from different tissues (Kalita *et al.*, 2014). The flagellum is one of the mechanisms associated with this capacity. It has already been identified as a determinant virulence factor to

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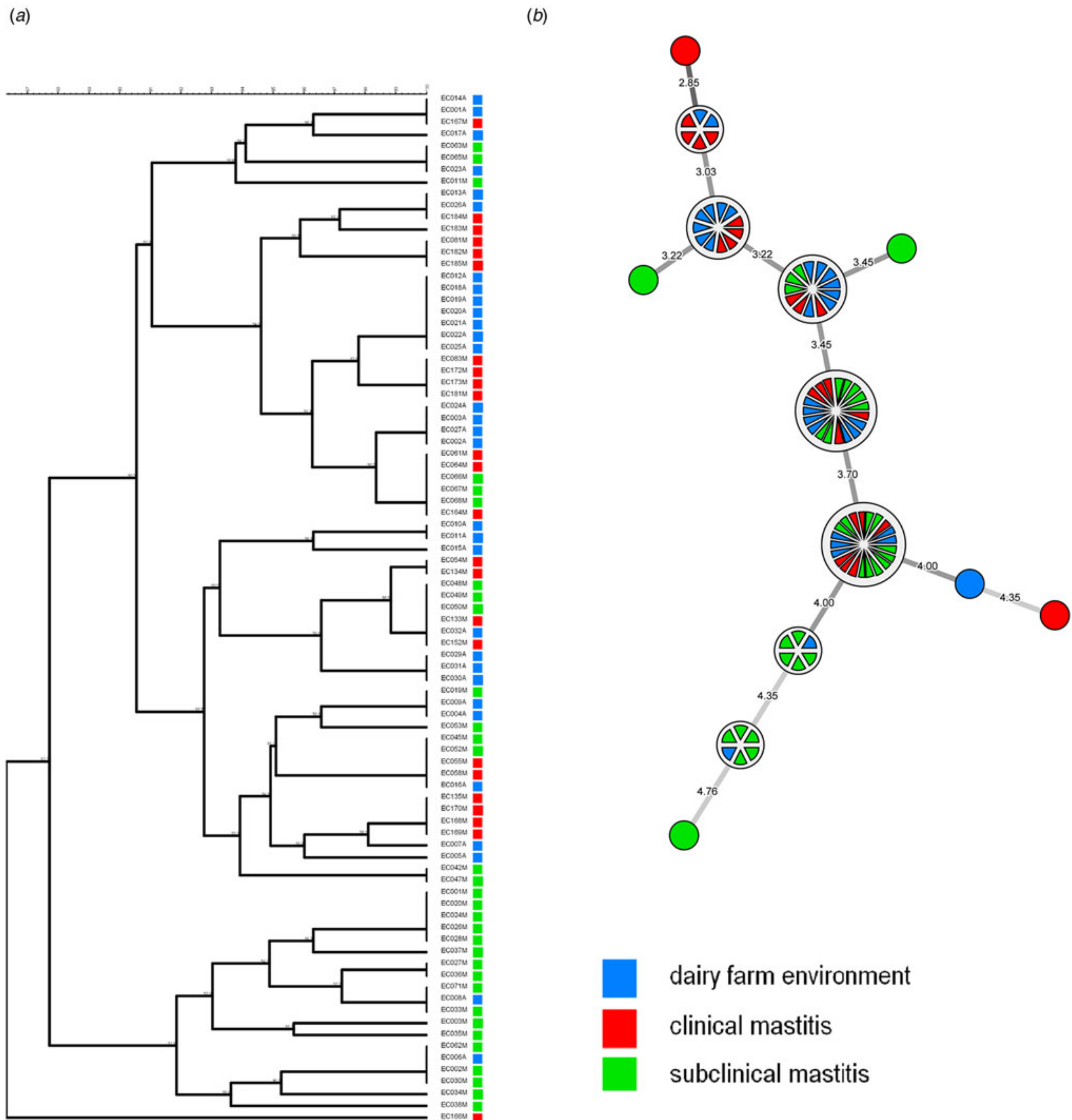

Fig. 2. Dendrogram (a) and minimum-spanning tree (MST) (b) performed using REP-PCR fingerprints of *Escherichia coli* isolated from cows showing subclinical and clinical mastitis and from dairy farm environment, Minas Gerais, Brazil, 2004–2017.

persistent gastrointestinal infections in chickens (Best *et al.*, 2005) and colonization of bovine intestinal epithelium by *E. coli* O157: H7 (Mahajan *et al.*, 2009), in addition to other types of infections in humans (Kalita *et al.*, 2014). Although not yet related to flagella, adherence capacity has already been pointed out as fundamental to *E. coli* chronic persistence in the mammary gland (Döpfer *et al.*, 2000; Dogan *et al.*, 2006). This virulence mechanism was well evidenced in an experiment conducted by Almeida *et al.* (2011) that compared chronic and acute *E. coli* mastitis strains and concluded that chronic strains were faster and more capable of adherence, invasion and multiplication in epithelial mammary gland cells. Since flagella allow great mobility in liquid environments (like milk) and are also related to adhesion and invasion of other epithelial cell types, this virulence factor may be the key to chronic and subclinical persistence of *E. coli* in the bovine udder as well. Additionally, flagella are also related to biofilm formation, another relevant virulence mechanism that could help in mammary gland persistence. It was observed that flagella are important to the early attachment of the microorganisms on the biofilm site, as well as to the development and maturation of the biofilm structure (Duan *et al.*, 2012; Haiko and Westerlund-Wikström, 2013), which can contribute to

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chronic and recurrent mammary infections (Pedersen *et al.*, 2021).

A strong immune response in mammary infections by *E. coli* with flagella was expected, as flagellin is a very immunogenic structure (Hayashi *et al.*, 2001; Hajam *et al.*, 2017). However, Porcherie *et al.* (2012) observed that bovine mammary glands have a low expression of type 5 toll-like receptors (TLRs-5), the innate immune system receptors responsible for recognizing bacterial flagellin (Hayashi *et al.*, 2001). Moreover, the same study demonstrated that bovine mammary epithelial cells do not recognize flagellin, which could allow the subclinical and chronic permanence of the pathogen inside the udder without being detected. Therefore, it is possible to speculate that MPEC may be determined by a set of virulence factors, including flagella that allows the persistence of the pathogen in the mammary gland (Blum *et al.*, 2008). Determining these factors is fundamental to understanding the pathogen and the disease, as well as proposing control measures, such as using bacterial flagellin as a target of intervention strategies for mastitis control.

Beyond flagella, long polar fimbriae (*lpf*A) are an *E. coli* virulence factor related to adhesion and invasion of host cells and have been mentioned as a key virulence factor in infections by MPEC (Dogan *et al.*, 2012; Blum and Leitner, 2013; Zhou *et al.*, 2019). Curiously, this gene was poorly found in our subclinical isolates, which probably indicates that MPEC needs a virulence factor that allows adhesion and invasion. Still, this factor does not necessarily have to be the long polar fimbriae, mainly when flagella is already present. Likewise, secretion systems are virulence factors previously related to pathogenic *E. coli* (Kyaw *et al.*, 2003; Buttner, 2012), including MPEC (Richards *et al.*, 2015). Although the type III secretion system (*esc*N gene) was found in about half of the subclinical isolates, it was found more frequently in clinical and environmental strains. These results suggest that the type III secretion system is a common virulence factor in pathogenic *E. coli* but not exclusively associated with infections caused by MPEC, as previously observed in the Type IV secretion system (Richards *et al.*, 2015).

Persistent *E. coli* can be characterized by periods of subclinical disease with intermittent clinical episodes (Döpfer *et al.*, 1999). The strains isolated from clinical mastitis showed more genetic proximity to environmental strains when compared with subclinical isolates (Fig. 1) in genotyping results. This might be evidence of the environmental route of transmission of these pathogens. However, we cannot state that all clinical strains analysed are opportunistic pathogens and strictly related to acute and transient clinical cases. Indeed, regarding virulence profile, clinical strains were more similar to subclinical than to environmental isolates, and 58.33% of the isolates showed the flagella gene. Therefore, assuming that MPEC are defined by a set of virulence factors and the strains present in the environment could have these factors (Blum *et al.*, 2008), environmental strains may also adapt to the mammary gland and cause persistent and contagious infections (Ruegg, 2012). This highlights the possible role of the environment as a source of MPEC to the mammary gland, although less important than cow-to-cow transmission.

The environment of the dairy farm as a source of clinical and subclinical mastitis isolates may explain the high genetic proximity among most of the isolates (>87% of similarity: Fig. 2) in REP-PCR. On the other hand, it is important to consider that even strains very close genetically can cause disease with variable degrees of severity and clinical signs, according to the capacity of the immune response and other factors attributed to infected cows (Burvenich *et al.*, 2003). Another issue that could explain the high similarity among all isolates is the lower discrimination power of REP-PCR compared with other molecular techniques, such as PFGE (Bae *et al.*, 2014), which is a limitation of this study.

In conclusion, our results suggest that flagella may be a determinant virulence factor in subclinical and persistent infections caused by *E. coli* in the bovine mammary gland and could be a target for intervention strategies for *E. coli* mastitis control. Moreover, REP-PCR genotyping results indicate that subclinical mastitis *E. coli* strains isolated from dairy farms in Minas Gerais state, Brazil, were less genetically diverse than clinical mastitis and dairy farm environmental isolates. However, it was not possible to determine a genotype associated with subclinical and persistent *E. coli* strains (MPEC).

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0022029923000353

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