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Characterization of mammary pathogenic *Escherichia coli* reveals the diversity of *Escherichia coli* isolates associated with bovine clinical mastitis in Brazil

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ABSTRACT

Mammary pathogenic *Escherichia coli* (MPEC) is one of the most common pathogens associated with clinical mastitis. We analyzed isolates obtained from milk samples of cows with clinical mastitis, collected from 10 farms in Brazil, to verify molecular and phenotypic characteristics. A total of 192 (4.5%) mammary pathogenic *E. coli* isolates were obtained from 4,275 milk samples analyzed, but we tested 161. We assigned most of these isolates to *E. coli* phylogroups B1 (52.8%) and A (36.6%), although phylogroups B2, C, D, E, and unknown also occurred. All isolates were assessed for the presence of several genes encoding virulence factors, such as adhesins (*sfaDE*, *papC*, *afaBC III*, *ecpA*, *fimH*, *papA*, and *iha*), toxins (*hlyA*, *cnf1*, *sat*, *vat*, and *cdt*), siderophores (*iroN*, *irp2*, *iucD*, *ireA*, and *sitA*), an invasion protein (*ibeA*), and serum resistance proteins (*traT*, *KpsMTII*, and *ompT*), and isolates from phylogroups B1, B2, and E showed up to 8 genes. Two isolates harbored the locus of enterocyte effacement ($escN^+$) and lack the bundle-forming pilus ($bfpB^-$) operon, which corresponds to a molecular profile of a subgroup of diarrheagenic *E. coli* (aEPEC), thus being classified as hybrid MPEC/aEPEC isolates. These isolates displayed a localized adherence-like pattern of adherence in HeLa cells and were able to promote F-actin polymerization underneath adherent bacteria. Based on the pulsed-field gel electrophoresis analyses, considerable genetic variability was observed. A low index of antimicrobial resistance was observed and 2 extended-spectrum β-lactamase–producing *E. coli* were identified, both harboring $bla_{CTX\text{-}M15}$ gene, and were classified as ST10 and ST993 using multilocus sequence typing. A total of 148 (91.2%) isolates were weak biofilm producers or formed no biofilm. Because raw milk is still frequently consumed in Brazil, the occurrence of virulence factor–encoding genes from extraintestinal or diarrheagenic *E. coli* added to the presence of extended-spectrum β-lactamase–producing isolates can turn this veterinary medicine problem into a public health concern.

Key words: phylogroup, MLST, bovine intramammary infection

INTRODUCTION

Bovine mastitis is the disease with the greatest impact on dairy farming, affecting health, welfare, and productivity and causing huge losses worldwide due to lower milk value, in addition to treatment costs, increased risk of culling, and high risk of disease transmission within herd (Down et al., 2017).

Mastitis can be classified as clinical (score 1, 2, or 3) or subclinical. Clinical mastitis includes various signs such as pain; fever; swelling; changes in rumination rate, hydration, and behavior; and animal death. The milk is also altered, and the presence of flakes and clots may occur, with changes in color and consistency (Adkins and Middleton, 2018). As named, in subclinical mastitis, the animals do not present systemic signs nor do visible changes occur in the milk, but there is an increase in SCC (Ruegg, 2017).

Mastitis pathogens are conventionally classified as contagious or environmental agents. Contagious pathogens are mainly transmitted directly between animals (e.g., by handlers or by milking equipment), whereas environmental pathogens are ubiquitous in the farm

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environment and can be found in bedding material, water, flies, and even the bovine gastrointestinal tract, such as *Escherichia coli* (Klaas and Zadoks, 2018).

Escherichia coli is one of the main environmental agents causing clinical mastitis (Vangroenweghe et al., 2020; Yu et al., 2020), and it is named mammary pathogenic *E. coli* (**MPEC**) (Shpigel et al., 2008). This species is characterized by great intraspecific diversity and is classified into 8 phylogenetic groups—A, B1, B2, C, D, E, F, and G (Denamur et al., 2021)—with the majority of MPEC isolates assigned into phylogroup A or B1 (Zhang et al., 2018).

Mammary pathogenic *E. coli* isolates encompass a wide variety of genes responsible for encoding virulence factors and include toxins, adhesins, siderophores, membrane proteins that help evade the host's immune system (protactins), and lipopolysaccharide (Steimle et al., 2016; Chen et al., 2017), although the nonspecific profile of the virulence factors has yet to be directly related to the occurrence of mastitis (Leimbach et al., 2017). Biofilms are also considered a virulence factor, presenting complex polysaccharide structures that easily allow bacteria to adhere to environmental surfaces or animal tissues. Bacteria in biofilms are more resistant to phagocytosis, antimicrobial agents, and disinfectants due to low diffusion through the matrix and altered cellular metabolism (Pedersen et al., 2021).

The most characteristic virulence factors found in pathogenic *E. coli* are in mobile genetic elements (Croxen et al., 2013), which can lead to an intense exchange of genes, thus resulting in the emergence of hybrid isolates. These hybrid isolates can occur by gene exchange between distinct diarrheagenic *E. coli* (**DEC**) pathotypes, such as the enteroaggregative *E. coli* (**EAEC**)–Shiga toxin-producing *E. coli* hybrid of serotype O104:H4 (Mora et al., 2011), or between DEC and extraintestinal pathogenic *E. coli* (**ExPEC**) isolates (Lindstedt et al., 2018).

Regarding antimicrobial resistance, food-producing animals could be one of the main causes of the increase in multidrug resistance, including resistance to colistin (Cyoia et al., 2019; García et al., 2018). In addition, the rapid spread of extended-spectrum β-lactamases (**ESBL**) and multidrug resistance is mostly associated with ExPEC isolates (Manges et al., 2019).

The present study assessed the presence of a set of genes encoding virulence factors, as well as assigning the MPEC isolates obtained from the milk of cows with clinical mastitis, in the distinct *E. coli* phylogroups described thus far. Other important features of pathogenic *E. coli*, such as patterns of adherence in epithelial cells, biofilm, and ESBL production, were also investigated.

MATERIALS AND METHODS

Study Design and Origin of E. coli Isolates

This study was approved by the Ethics Committee on Animal Use in the School of Veterinary Medicine and Animal Science, São Paulo State University, Botucatu, São Paulo State, Brazil (No. 2015/19688-8).

Ten farms located in São Paulo and Minas Gerais states, Brazil, with mastitis control programs with available electronic health and production records were used. The eligible criteria were herds composed of Holstein or Holstein crossbred cows, herd size of greater than 200 lactating cows producing greater than 20 kg of milk per day, bulk tank somatic cell count (less than 400,000 cells per mL), and use of milking equipment.

Diagnosis of clinical mastitis was performed at every milking. The first milk streams were visually inspected in a strip cup with black background to detect any abnormality, such as the presence of flakes, blood, pus, or color changes. The clinical severity scores of mammary infections were defined as mild, or score 1, when the milk presented macroscopic changes in appearance (e.g., presence of flakes, pus, and blood). Mastitis was considered moderate, or score 2, when both abnormal appearance of milk and udder inflammation (swelling, pain, or redness in the affected mammary gland) occurred, whereas cases with additional signs of inappetence, fever, tachycardia, tachypnea, decubitus, or ruminal hypomotility were classified as severe (score 3) (Pinzón-Sánchez and Ruegg, 2011).

Milk samples were collected between 2017 and 2019. Beforehand, milkers were trained by veterinarians to recognize the different severity scores of clinical mastitis. Milk samples were collected upon diagnosis of clinical mastitis, before treatment. After the milking technicians performed the premilking hygiene procedures (stripping, predipping, and drying of teats with paper towels), the teat end was disinfected with a cotton pad soaked with 70% alcohol and 15 mL of milk was collected into a sterile plastic vial. Samples were kept refrigerated (4°C to 8°C) until microbiological culturing. A volume of 10 μL of each milk sample was streaked onto defibrinated sheep blood agar (5.0%) and MacConkey agar (Oxoid). The plates were incubated at 37°C/72 h, under aerobic conditions. Microorganisms were classified according to the National Mastitis Council (NMC, 1999; Procop et al., 2016). Colonies compatible with *E. coli* were also identified based on conventional phenotypic tests, such as gas/glucose and sulfuric acid production, urea hydrolysis, L-tryptophan deamination, motility, indole production, lysine decarboxylation, and Simmons citrate. Intramammary

infection was defined as the presence of at least 3 cfu of *E. coli*. Milk samples that yielded more than 3 different colony types were considered contaminated and discarded (NMC, 1999).

The isolates were kept frozen at −70°C in brain heart infusion (**BHI**) (Oxoid) broth added by 15% glycerol until the time of testing. The isolates were frozen for almost a year and a half (due to quarantine by the COVID-19 pandemic). After this period, for molecular and phenotypic tests, all isolates frozen at −70°C were subcultured in BHI, incubated at 35°C/24 h. The turbid tubes were plated onto MacConkey agar to verify the purity and observation of the morphology of colonies compatible with *E. coli*.

Detection of Virulence Genes

For DNA extraction, 1 colony of each isolate was suspended in 200 μL of sterile water, boiled for 10 min, and maintained in an ice bath for an additional 10 min. The suspensions were centrifuged at 10,000 × *g* for 1 min at 4°C (Centrifuge 5424 R, Eppendorf), and the supernatants were frozen (Dias et al., 2016).

Extraintestinal pathogenic *E. coli* virulence factor–associated genes encoding for adhesins (*sfaDE*, *papC*, *afaBC III*, *ecpA*, *fimH*, *papA*, and *iha*), toxins (*hlyA*, *cnf1*, *sat*, *vat*, and *cdt*), siderophores (*iroN*, *irp2*, *iucD*, *ireA*, and *sitA*), an invasion protein (*ibeA*), and serum resistance proteins (*traT*, *KpsMTII*, and *ompT*) were investigated by PCR using primers and PCR conditions as listed in Supplemental Table S1 (https://doi.org/10.6084/m9.figshare.21082000; Rall, 2022).

Furthermore, to identify DEC isolates, we searched for the main virulence factor–encoding genes associated (*eae*, *bfpA*, *aatA*, *aggR*, *stx1*, *stx2*, *eltA*, *est*, *ipaH*, *daaE*, and *ehxA*), and the reactions were performed according to the references cited in Supplemental Table S2 (https://doi.org/10.6084/m9.figshare.21082000; Rall, 2022).

The PCR products were subjected to electrophoresis (Electrophoresis Power Supply Model EPD 600; Amersham Pharmacia Biotech Inc.) in a 1.5% agarose gel (Sigma-Aldrich) in Tris-borate-EDTA buffer, and the bands were stained with SYBR Safe (Invitrogen). The images were captured using SmartView Pro Imager System 1200 (Major Science).

Molecular Characterization

Phylogenetic Grouping. *Escherichia coli* isolates were classified using a quadruplex PCR among the different phylogenetic groups already recognized (A, B1, B2, C, D, E, F, G, and *Escherichia* clades), according to Clermont et al. (2013, 2019).

Pulsed-Field Gel Electrophoresis. Pulsed-field gel electrophoresis (**PFGE**) assay was performed according to PulseNet (CDC, 2017), using Xba (Thermo Scientific) (50 U for 2 h at 37°C) as the restriction endonuclease to digest *E. coli* DNA. We used clamped homogeneous electric field (Bio-Rad), with an initial switch time of 6.76 s, final switch time of 35.38 s, voltage of 6 V, and an included angle of 120°, through a run time of 18 h. In addition, the electrophoresis buffer for the PFGE run was supplemented with 50 μ*M* thiourea, when necessary (Goering, 2010). We used Bionumerics software v.7.6 to analyze the images and a band position tolerance of 1.5% to analyze the fingerprints. Clustering was carried out by the unweighted pair-group method with arithmetic mean, using the Dice coefficient, and a similarity index of 80% according to Kitchel et al. (2009). The DNA fragments of ATCC BAA-664 (*Salmonella* ser. Braenderup H9812) were used as molecular weight markers for the XbaI digestion standard.

Multilocus Sequence Typing. The PCR products of housekeeping genes were purified using AMPure XP (Beckman Coulter) according to manufacturer recommendations and then sequenced using Sanger (https://pubmlst.org). In the multilocus sequence typing (**MLST**) assay, we used the highest biofilm producer, considering the clusters obtained in PFGE, with 3 or more isolates (5 cluster) and both ESBL$^+$ isolates.

HeLa Cell Assays

In vitro Adhesion Test. HeLa cells were maintained at 37°C and 5% CO_2 in Dulbecco's Modified Eagle Medium (Sigma), supplemented by 10% bovine fetal serum (Sigma) and 1% PenStrep antibiotic (10,000 U of penicillin + 10 mg of streptomycin per milliliter) (Sigma). The assays were performed according to Cravioto et al. (1979).

Fluorescent-Actin Staining Test. Two isolates that harbored the *escN* gene (located within the locus of enterocyte effacement region) and a molecular marker of enteropathogenic *E. coli* isolates were submitted to a fluorescent-actin staining (**FAS**) test, according to procedures previously described by Knutton et al. (1989), to observe whether the isolates can induce F-actin polymerization underneath adherent bacteria, a main feature of the attaching and effacing lesion. Typical enteropathogenic *E. coli* prototype E2348/69 (FAS-positive) and diffusely adherent *E. coli* C1845 (FAS-negative) strains were used as positive and negative controls, respectively.

ESBL Production and Resistance Genes. All isolates were tested for ESBL production, according to CLSI (2020), using ceftriaxone (30 μg), cefotaxime

Table 1. Distribution of genes encoding virulence factors in *Escherichia coli* isolates obtained from milk from cows with clinical mastitis, considering the phylogenetic groups

	Phylogenetic group							
Gene	A (n = 59)	B1 (n = 85)	B2 (n = 2)	C (n = 4)	D (n = 1)	E (n = 8)	Unknown (n = 2)	Total (%) (n = 161)
ExPEC[1]								
fimH	58	85	2	4	1	8	2	160 (99.4)
ecpA	38	59	—	3	1	6	1	108 (67.1)
hlyA	1	9	1	—	—	—	1	12 (7.5)
vat	1	—	2	—	—	—	—	3 (1.9)
cdt	1	—	1	—	—	2	—	3 (1.9)
iroN	—	4	—	—	—	1	—	5 (3.1)
irp2	1	11	1	—	—	—	1	14 (8.7)
iucD	—	—	—	—	—	1	—	1 (0.6)
sitA	3	7	2	—	1	1	—	14 (8.7)
ibeA	—	—	2	—	—	—	—	2 (1.2)
ompT	7	42	2	—	1	6	2	60 (37.3)
traT	48	65	1	2	1	7	2	126 (78.3)
kpsMTII	4	—	1	—	1	1	—	7 (4.4)
DEC								
escN	—	2	—	—	—	—	—	2 (1.2)
astA	—	7	—	—	—	2	—	9 (5.6)
aaiA	1	1	—	—	—	—	1	3 (1.9)
aaiC	1	1	—	—	—	—	1	3 (1.9)

[1]ExPEC = extraintestinal pathogenic *E. coli*; DEC = diarrheagenic *E. coli*. Dashes indicate negative results.

(30 μg), ceftazidime (30 μg), and aztreonam (30 μg) (Cefar Disks). The isolates considered resistant to all those drugs were submitted to a confirmatory test, using amoxicillin-clavulanic acid (30 μg) disc at 20 mm β-lactamase inhibitor. Distorted halos or "ghost zones" characterized ESBL-producing *E. coli*.

All isolates were also tested to investigate β lactam resistance–encoding genes, such as bla_{TEM}, bla_{SHV}, bla_{CTX-} (bla_{CTX-M2}, bla_{CTX-M8}, and $bla_{CTX-M15}$), bla_{CMY2}, and carbapenemases (bla_{KPC} and bla_{NDM}), as well as colistin resistance (*mcr*-1 and *mcr*-2). We also used a general bla_{CTX-M} primer, so positive isolates were sequenced by Sanger for classification. Before, the PCR products were purified using AMPure XP (Beckman Coulter), according to manufacturer recommendations. The references and reactions are listed in Supplemental Table S3 (https://doi.org/10.6084/m9.figshare.21082000; Rall, 2022).

Biofilm Formation. The biofilm formation test was performed according to Stepanović et al. (2007). Briefly, *E. coli* isolates were incubated in BHI at 35°C/24 h. After this period, each culture was diluted to approximately 1.5×10^8 cfu/mL (0.5 MacFarland scale), using Vitek Densicheck (Biomerieux) with BHI plus 0.5% glucose, and an aliquot of 200 μL was seeded in quadruplicate into a 96-well microplate, incubated at 35°C/24 h. The wells were washed 3 times with PBS (pH 7.4) and 250 μL of methanol was added in each well (15 min) to fix the biofilm. Methanol was removed and the microplate stained with crystal violet (1%) for 5 min. The plates were washed 3 times or more with water to remove excess dye and the biofilm was resuspended in 200 μL of 33% (vol/vol) glacial acetic acid (10 min). Optical density was then measured on an ELISA plate reader (Babsystems, MultiSkan EX) at 570 nm. The tests were performed in triplicate.

We used sterilized BHI + 0.5% glucose as a negative control and *E. coli* 042 as a positive control. From the average of the replicates and according to the relationship between optical density and the negative control, the samples were classified according to Yang et al. (2018).

RESULTS

MPEC Isolates from Cows with Clinical Mastitis

From the 10 farms involved in the present study, 4,275 milk samples from cows with clinical mastitis were collected. Mammary pathogenic *E. coli* isolates were obtained from 192 (4.5%) of the total of milk samples analyzed. Unfortunately, some isolates died during the storage period, leaving 161 isolates.

MPEC Isolates Assigned in Phylogroups A and B1 and Harboring Several Virulence Factor–Encoding Genes Associated with ExPEC Pathogenicity

Most of the virulence factor–encoding genes investigated were observed at low frequencies, as shown in

Table 1. Genes *sfaDE*, *iha*, *papA*, *papC*, *afaBCIII*, *cnf1*, *sat*, and *ireA* were absent in all MPEC isolates studied.

Regarding classification of the MPEC isolates in the distinct *E. coli* phylogroups, we observed that 85 (52.8%) were assigned in the phylogroup B1, 59 (36.6%) in group A, 8 (4.97%) in group E, 4 (2.48%) in group C, 2 (1.24%) in group B2, and 1 (0.62%) in group D. Two (1.24%) isolates were assigned to the unknown group. We observed a great diversity of virulence profiles among the MPEC isolates studied, even among isolates assigned in the same phylogroup. Table 2 shows the presence of a range of 1 to 8 genes in phylogroups B1, B2, and E.

Occurrence of DEC Genetic Diagnosis Markers in the MPEC Isolates Studied

Regarding the occurrence of genes frequently used for DEC pathotype classification, we observed that 2 (1.2%) harbored the *escN* gene but lacked *bfpB* and could thus represent MPEC–atypical enteropathogenic *E. coli* (**aEPEC**) hybrid isolates. These 2 hybrid MPEC-aEPEC isolates displayed the localized adherence-like pattern on HeLa cells (Figure 1A) and were able to recruit F-actin polymerization underneath adherent bacteria (FAS-positive) (Figure 2).

Together, the 2 phenotypic features (localized adherence-like and FAS-positive) indicate that the 2 hybrid MPEC/aEPEC isolates presented characteristics commonly found in aEPEC isolates obtained from patients with diarrhea and correlated with the presence of the locus of enterocyte effacement region ($escN^+$).

Three isolates (2.5%) harbored genes previously identified in the EAEC in a chromosomal pathogenicity island from the prototype EAEC O42 ($aaiA^+$ and $aaiC^+$) but were nonadherent in HeLa cells (Figure 1B). Moreover, 9 isolates (5.6%) harbored the *astA* gene, responsible for encoding the enteroaggregative heat-stable toxin 1, which was primarily found in EAEC and further in several other DEC pathotypes, as well as in nonpathogenic *E. coli*, and thus is not currently used for a diagnosis proposer (Savarino et al., 1996; Dudley et al., 2006).

Detection and Molecular Characterization of MPEC Isolates with ESBL Phenotype

Just 2 isolates were ESBL producers, showing the ghost zone, after the addition of the amoxicillin-clavulanic acid disc as a β-lactamase inhibitor, and we found the $bla_{\text{CTX-M-15}}$ gene in both isolates. Both isolates also belonged to phylogroup 1, but different sequence types (**ST**) (ST10 and ST993). The other resistance genes (bla_{TEM}, bla_{SHV}, bla_{KPC}, bla_{NDM}, *mcr*-1, and *mcr*-2) were not observed.

Biofilm Production May Vary Among MPEC Isolates

Out of 161 isolates, 68 (42.2%) were classified as nonproducing, 80 (49.7%) as weak, 11 (6.8%) as moderate, and only 2 (1.2%) as strong producers. The distribution of production capacity found within the phylogenetic groups is represented in Figure 3. The strong producers were classified in phylogroups A and B1.

Pulsotypes Found Among the MPEC Isolates Studied

Using a similarity coefficient of 80% in the PFGE analysis, the MPEC isolates were classified in 21 clusters, but only 5 of them grouping 3 or more isolates, according to the dendrogram (Supplemental Figure S1; https://doi.org/10.6084/m9.figshare.21082000; Rall, 2022). Of particular importance, in the pulse type 0004 we observed the persistence of an isolate in the same animal during 3 collections, which occurred every 5 d.

MLST Analyses of Selected MPEC Isolates Reveal ST10 as the Most Frequent

The positive ESBL isolates belonged to ST10 and ST993. Regarding the 5 other isolates, 2 were classified as ST10, followed by ST101, ST392, and ST2614.

DISCUSSION

There is no doubt that the host immune system plays a role in the development of mastitis (White et al., 2010), but *E. coli* also must be considered to possibly carry a specific set of virulence genes related to its clinical signs. A huge variety of these genes have been described (Blum and Leitner, 2013; Zhang et al., 2018), but until now there has been no gene or a group of genes to characterize these MPEC isolates.

In Brazil, despite prohibition on the sale of raw milk throughout the country (MAGR, 1969), raw milk consumption still exists. According to the Brazilian Agricultural Research Corporation (BARC, 2020), in 2018, the consumption of raw milk and dairy products represented 27% of total production, in a volume of approximately 9.2 billion liters. In any case, the presence of diarrheagenic genes in ExPEC isolates is concerning, as it demonstrates that important virulence factors may be acquired by these isolates, with the formation of hybrids (Lindstedt et al., 2018; Tanabe et al., 2022), turning a veterinary medicine problem into a public health concern.

Table 2. Distribution profile of virulence genes among *Escherichia coli* phylogroup isolates

Phylogroup	Virulence genes	n
A	*fimH*, *traT*	10
	fimH, *ecpA*	6
	fimH, *kpsMTII*	1
	ecpA, *traT*	1
	fimH, *ecpA*, *traT*	23
	fimH, *ompT*, *traT*	3
	fimH, *traT*, *kpsMTII*	2
	fimH, *cdt*, *traT*	1
	fimH, *sitA*, *traT*	1
	fimH, *vat*, *traT*	1
	fimH, *ecpA*, *irp2*	1
	fimH, *ecpA*, *ompT*	1
	fimH, *ecpA*, *kpsMTII*	1
	fimH, *ecpA*, *sitA*	3
	fimH, *ecpA*, *ompT*, *traT*	3
	fimH, *aaiA*, *aaiC*, *traT*	2
	fimH, *ecpA*, *hlyA*, *sitA*, *traT*	1
B1	*fimH*	7
	fimH, *ompT*	1
	fimH, *traT*	5
	fimH, *ecpA*	4
	fimH, *sitA*	1
	fimH, *ecpA*, *traT*	14
	fimH, *ompT*, *traT*	6
	fimH, *ecpA*, *ompT*	6
	fimH, *irp2*, *traT*	1
	fimH, *sitA*, *traT*	1
	fimH, *ecpA*, *irp2*, *traT*	2
	fimH, *hlyA*, *traT*, *astA*	1
	fimH, *hlyA*, *ompT*, *traT*	1
	fimH, *ecpA*, *traT*, *astA*	1
	fimH, *ompT*, *traT*, *astA*	1
	fimH, *ecpA*, *sitA*, *traT*	1
	fimH, *ecpA*, *ompT*, *traT*	18
	fimH, *ecpA*, *irp2*, *sitA*, *traT*	1
	fimH, *ecpA*, *ompT*, *traT*, *astA*	2
	fimH, *ecpA*, *irp2*, *ompT*, *traT*	2
	fimH, *ecpA*, *sitA*, *ompT*, *traT*	1
	fimH, *aaiA*, *aaiC*, *hlyA*, *traT*	1
	fimH, *escN*, *ecpA*, *hlyA*, *traT*	1
	fimH, *ecpA*, *hlyA*, *traT*, *astA*	1
	fimH, *escN*, *ecpA*, *hlyA*, *irp2*	1
	fimH, *ecpA*, *iroN*, *irp2*, *ompT*, *traT*	1
	fimH, *ecpA*, *hlyA*, *iroN*, *irp2*, *ompT*, *traT*	1
	fimH, *ecpA*, *hlyA*, *iroN*, *irp2*, *sitA*, *ompT*, *traT*	2
B2	*fimH*, *vat*, *irp2*, *sitA*, *ibeA*, *ompT*	1
	fimH, *hlyA*, *vat*, *sitA*, *ibeA*, *ompT*, *traT*, *kpsMTII*	1
C	*fimH*	1
	fimH, *ecpA*	1
	fimH, *ecpA*, *traT*	2
D	*fimH*, *ecpA*, *sitA*, *ompT*, *traT*, *kpsMTII*	1
E	*fimH*, *traT*	2
	fimH, *ecpA*, *ompT*, *traT*	2
	fimH, *ecpA*, *cdt*, *ompT*	1
	fimH, *ecpA*, *ompT*, *traT*, *astA*	1
	fimH, *ecpA*, *cdt*, *ompT*, *traT*, *astA*	1
	fimH, *ecpA*, *iroN*, *iucD*, *sitA*, *ompT*, *traT*, *kpsMTII*	1
Unknown	*fimH*, *ecpA*, *hlyA*, *ompT*, *traT*	1
	fimH, *aaiA*, *aaiC*, *irp2*, *ompT*, *traT*	1

As previously reported (Zhang et al., 2018; Bag et al., 2021), most of the isolates were classified into phylogroups A and B1, confirming the environmental source of this opportunistic pathogen, as these phylogroups are very common in bovine feces (Higgins et al., 2007).

We observed a great diversity of virulence factors among and within phylogroups, showing the genetic

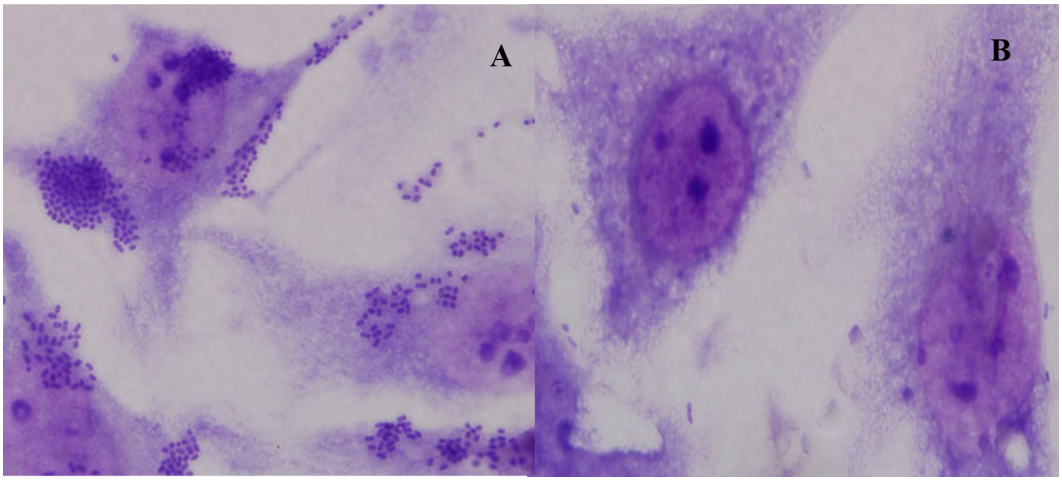

Figure 1. (A) *Escherichia coli escN*$^{+}$ isolates presenting localized-like pattern; (B) *aaiA*$^{+}$/*aaiC*$^{+}$ isolates showing no adhesion patterns.

diversity and the possibility of these environmental lineages becoming increasingly pathogenic over time. Although isolates from phylogroups B1, B2, and E had up to 8 genes, only isolates assigned in the B2 phylogroup had at least 1 gene from each class studied (adhesins, toxins, siderophores, invasins, and serum resistance), and the B2 group was the only one to present the invasin *ibeA*, responsible for tissue invasion, which can be considered the isolate with the greatest pathogenic potential. It is important to mention that the fact that we obtained only 2 isolates of these phylogroups means that this particular scenario cannot be extrapolated to other isolates of this phylogroup, thus becoming a limitation of our study.

Despite the great variability reported, we observed that MPEC isolates mostly lack known genes of virulence, confirming previous observations (Suojala et al., 2011; Blum and Leitner, 2013). The exception would obviously be LPS, the main cell-wall component of gram-negative bacteria and considered one of the main virulence factors of *E. coli* (Steele et al., 2019), inducing apoptosis in mastitic mammary tissue (Long et al., 2001), as well as cytokine and chemokine production in the mammary gland (Bannerman et al., 2003). Also, different kinds of fimbrial and afimbrial adhesins are widely distributed in these isolates (Guerra et al., 2020; Ismail and Abutarbush, 2020; Lan et al., 2020). There is wide variability among the other genes assessed in this study (Blum et al., 2008; Blum and Leitner, 2013; Guerra et al., 2020).

In MLST we sequenced housekeeping genes, but *E. coli* isolates already showed the huge potential for acquiring genes related to virulence factors, so MLST classification is often not in agreement with pathotype classification (Wirth et al., 2006) or virulence gene profile, explaining the great variety in mastitis isolates, with ST10 seeming to be one of the most frequent (Wu et al., 2012; Blum and Leitner, 2013; Keane, 2016). We can consider the low number of isolates classified by MLST as a limitation of this study, due to the high cost of this technique in Brazil. Among the 7 isolates, 3 (42.9%) were classified as ST10, and all of them belonged to phylogroup A, as observed by Blum and Leitner (2013). As far as we know, ST993 was not reported to cause mastitis, but it was found in red meat and carried the *stx* gene (Bai et al., 2015; Wieczorek and Osek, 2020). As observed by Shen et al. (2022), ST993 was also classified in phylogroup A. Besides, our isolate was EBSL producing, harboring the $bla_{\text{CTX-M-15}}$ gene.

In observing the PFGE dendrogram, we noted a large number of pulsotypes. The same cluster was found in different farms, showing a process of dispersion of the isolates. There were simultaneous infections of different animals caused by the same clone and chronic infection in a single animal (cluster 0004). Cases of chronic clinical mastitis caused by *E. coli* are uncommon, although they have already been reported (Bradley and Green, 2001; Dogan et al., 2006). This type of infection is related to immune system evasion mechanisms (Dogan et al., 2006), such as biofilm production, and among the 3 isolates involved, 2 were strong producers and the other was moderate. The 3 isolates involved in the case of persistent mastitis did not have this gene, and 2 of them were strong biofilm producers. Furthermore, Lippolis et al. (2014) performed shotgun proteomic analysis and observed the expression of 28 different proteins in *E. coli* strains associated with persistent versus transient mastitis. Many of them were related to flagellar and chemotaxis proteins, important in bacterial motility phenotypes (swimming and swarming). The 3 isolates

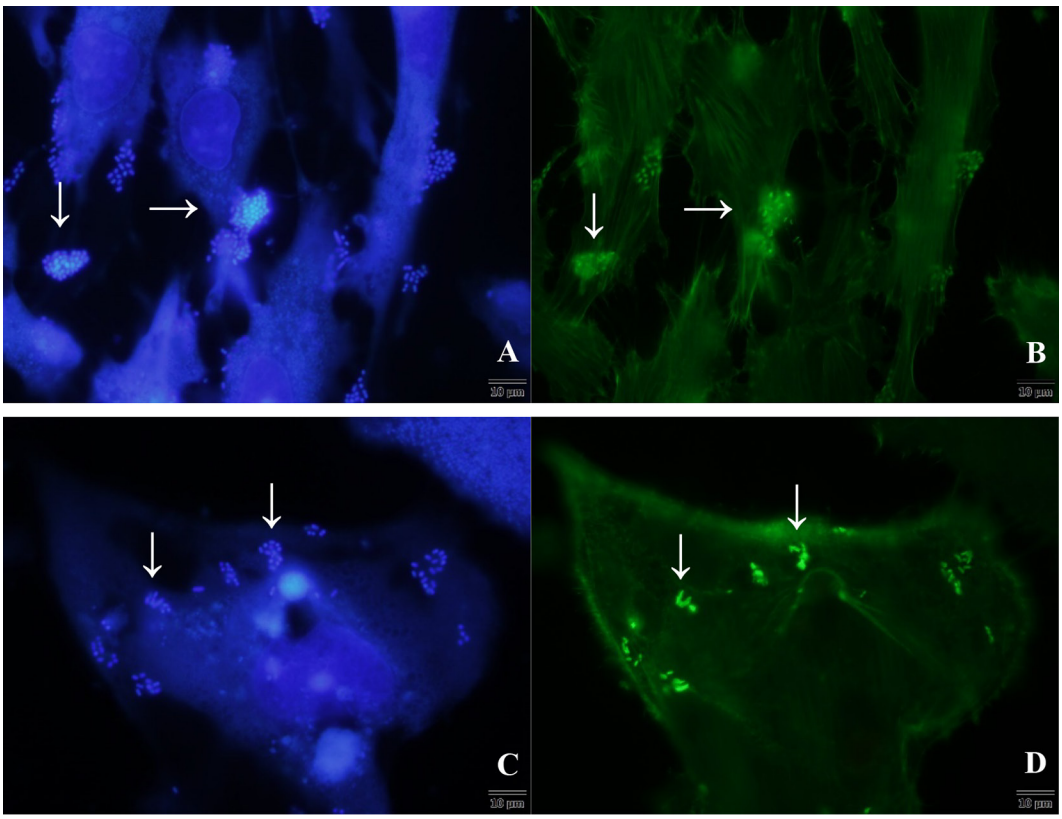

Figure 2. Both *Escherichia coli escN*$^{+}$ isolates present actin accumulation at the adhesion site in fluorescent-actin staining test. (A and C) Arrows indicate region of bacterial accumulation due to 4′,6-diamidino-2-phenylindole (DAPI) action. (B and D) Visible actin accumulation, evidenced by the action of phalloidin. (Bar = 10 μm).

considered persistent, observed in phylogroup 0004 in PFGE, were positive just for swimming test (data not shown), but the transient isolates were not tested, making it difficult to speculate about the relation of this phenotype with persistent isolates.

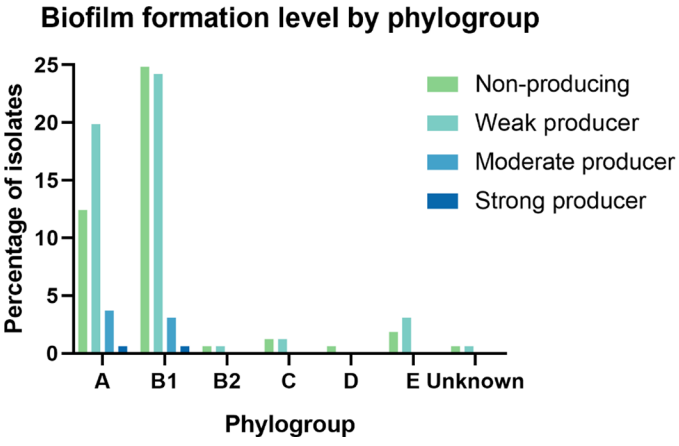

Figure 3. Biofilm production by *Escherichia coli* isolates that cause clinical bovine mastitis, considering the phylogenetic groups.

Escherichia coli causing clinical mastitis can form biofilm (Silva et al., 2009), and 57% of the isolates in the current study were producers. As previously mentioned, isolates involved in the case of persistent mastitis were strong producers, but specific clusters (0005 and 0006) were detected in different animals for more than a year, showing the permanence of these isolates in the environment. Unlike persistent infection, these isolates were nonproducing or weak biofilm producers. These isolates may have been housed in biofilms produced by other bacteria, even from different species, as biofilms are usually polybacterial (Kostakioti et al., 2013), or at the time of the tests there may have been insufficient expression of the genes responsible to produce biofilm due to inappropriate environmental factors (Parsek and Singh, 2003).

Although multiresistance of MPEC isolates is a concern all around the world (Yang et al., 2018; Zhang et al., 2018), in the present study, the only 2 isolates were ESBL+, both $bla_{CTX\text{-}M\text{-}15}$. This subtype also seems to be the most common in China (Ali et al., 2016; Yang et al., 2018), Japan (Ohnishi et al., 2013), and Korea (Tark et al., 2017), showing its spread in East Asian countries.

Like Yang et al. (2018) and Bag et al. (2021), we did not detect bla_{SHV}, demonstrating that it is not yet a common gene. On the other hand, bla_{TEM} was found in 68.5% (Yang et al., 2018), 83.1% (Yu et al., 2020), and 38.9% (Bag et al., 2021) of the isolates, showing that the MPEC isolates in this study are more susceptible. In the same way, we did not detect *mcr*-1 or *mcr*-2, unlike Filioussis et al. (2020).

Most clinical bovine mastitis infections were caused by *E. coli* classified mainly in phylogroups A and B1, confirming their environmental origin. Despite the set of genes assessed (22), none of them can be used as a marker for MPEC, due to the high genetic variability found. Even occurring in a few strains, the presence of ESBL plasmids in *E. coli* is concerning because they could be transmitted to humans, as raw milk is still consumed in Brazil.

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