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Original Article
Livestock Diseases

Identification of enteropathogenic *Escherichia coli* as the cause of mastitis in cows from Brazil[1]

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ABSTRACT.- Pereira E.S., Crippa B.L., Morasi R.M., Almeida J.M., Gebara C., Langoni H., Neto A.T., Gonçalves M.C & Silva N.C.C. 2024. **Identification of enteropathogenic *Escherichia coli* as the cause of mastitis in cows from Brazil.** *Pesquisa Veterinária Brasileira 44:e07430, 2024.* Departamento de Ciência de Alimentos e Nutrição, Faculdade de Engenharia de Alimentos, Universidade Estadual de Campinas, Campinas, SP 13083-862, Brazil. E-mail: ncirone@unicamp.br

Escherichia coli is recognized as one of the main microorganisms responsible for triggering clinical mastitis, a disease that causes considerable economic losses in the dairy industry. In this context, this study aimed to identify *E. coli* isolates present in individual milk samples collected from cows diagnosed with clinical mastitis from various regions of Brazil. Additionally, through polymerase chain reaction (PCR), the presence of virulence genes *eae, bfp*B, *esc*N, *aat*A, *agg*R, *ipa*H, *stx*1, *stx*2, *est,* and *elt*A was investigated; all associated with the pathotypes of diarrheagenic *Escherichia coli* (DEC). As an integral part of the study, a comprehensive assessment of the sensitivity profile of the isolates to 11 different antimicrobials widely used in mastitis treatment was also conducted. A total of 198 milk samples were collected from cows diagnosed with clinical mastitis. Among these samples, 12 isolates (6.07%) demonstrated bacterial growth greater than three Colony-Forming Units (CFU) when grown on MacConkey agar medium and morphological characteristics of *E. coli*. The disc-diffusion test was used to evaluate the susceptibility of these isolates to antimicrobials, and the most predominant resistance was observed concerning streptomycin and tetracycline, affecting 16.67% of the strains analyzed. Notably, all isolates investigated did not demonstrate the presence of the genes *eae, aat*A, *agg*R, *ipa*H, *stx*1, *stx*2, *est,* and *elt*A. These results indicate that these isolates do not fit the pathotypes known as diarrheagenic *Escherichia coli* (DEC). However, one of the isolates tested was positive for the *bfp*B and *esc*N genes. The detection of resistant *E. coli* associated with clinical mastitis points to possible gaps in the treatment of the disease. Additionally, the presence of resistance genes in *E. coli* strains indicates the potential to transmit these genes between animals and, perhaps, along the food chain.

INDEX TERMS: *Escherichia coli*, clinical mastitis, antimicrobial resistance, dairy farms, Brazil.

RESUMO.- [**Identificação de *Escherichia coli* enteropatogênica como causa de mastite em vacas no Brasil.**] *Escherichia coli* é reconhecida como um dos principais microrganismos responsáveis pelo desencadeamento da mastite clínica, doença que causa perdas econômicas consideráveis na indústria de laticínios. Neste contexto, este estudo teve como objetivo principal a identificação de isolados de *E. coli* presentes em amostras individuais de leite coletadas de vacas com diagnóstico de mastite clínica, de diversas regiões do Brasil. Adicionalmente, por reação em cadeia da polimerase (PCR),

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foi investigada a presença dos genes de virulência *eae, bfp*B, *esc*N, *aat*A, *agg*R, *ipa*H, *stx*1, *stx*2, *est* e *elt*A, todos associados aos patótipos de *E. coli* diarreiogênica (DEC). Como parte integrante do estudo, foi realizada uma avaliação abrangente do perfil de sensibilidade dos isolados a 11 antimicrobianos diferentes amplamente utilizados no tratamento da mastite. Foram coletadas 198 amostras de leite de vacas com diagnóstico de mastite clínica. Dentre essas amostras, 12 isolados (6,07%) demonstraram crescimento bacteriano superior a três Unidades Formadoras de Colônia (UFC) quando cultivadas em meio ágar MacConkey e características morfológicas de *E. coli*. Para avaliar a suscetibilidade desses isolados aos antimicrobianos foi utilizado o teste de disco-difusão, sendo observada a resistência mais predominante em relação à estreptomicina e à tetraciclina, afetando 16,67% das cepas analisadas. É relevante ressaltar que todos os isolados investigados não demonstraram a presença dos genes *eae, aat*A, *agg*R, *ipa*H, *stx*1, *stx*2, *est* e *elt*A. Estes resultados indicam que estes isolados não se enquadram nos patótipos conhecidos como *Escherichia coli* diarreiogénica (DEC). Porém, um dos isolados testados apresentou positividade para os genes bfpB e escN. A detecção de *E. coli* resistente associada à mastite clínica aponta para possíveis lacunas no tratamento da doença. Além disso, a presença de genes de resistência em estirpes de *E. coli* indica a capacidade potencial de transmitir estes genes entre animais e talvez ao longo da cadeia alimentar.

TERMOS DE INDEXAÇÃO: *Escherichia coli*, mastite clínica, resistência antimicrobiana, fazendas leiteiras, Brasil.

INTRODUCTION

Mastitis is characterized by the inflammatory process of the mammary glands of dairy cows and is the disease that negatively impacts dairy farming the most. Clinical cases of the disease involve direct costs, such as milk disposal, treatment of infected animals, and veterinary services (Down et al. 2017, 2013).

Regarding its manifestation, mastitis can be classified as either clinical or subclinical. In its clinical condition, inflammatory changes in the mammary glands of the affected animal can be observed. It can affect changes in milk consistency (presence of lumps, pus, and blood) and milk color using the black-bottomed mug test. Systemic and milk changes vary according to the severity of the case, which can be defined as mild, moderate, or severe. The subclinical form is mainly diagnosed by somatic cell count (SCC), as it is not possible to visualize inflammatory changes in the gland nor visible changes in milk (Adkins & Middleton 2018).

Mastitis could be classified as contagious and environmental. Despite involving etiological agents such as yeasts, algae, and fungi, bacteria are the primary etiological agents responsible for triggering intramammary infections (IMI) (Bag et al. 2021). Contagious bacterial agents are those transmitted between animals during the milking process and through infected ceilings, milkers' hands, or milking equipment. In contrast, the agents that cause the so-called environmental mastitis are present throughout the farm environment (milking parlor floor, bedding material of the animals, drinking fountains). Furthermore, they can also be found in the gastrointestinal tract of cattle, such as *E. coli* (Mahmmod et al. 2018, Addis et al. 2020).

E. coli, being ubiquitous in the environment, takes on significance as a relevant bacterial agent in cases of clinical cattle mastitis. This species has a wide variety of diarrheagenic *E. coli* pathotypes (DEC), such as Enteropathogenic (EPEC), Enteroinvasive (EIEC), Enterotoxigenic (ETEC), Enteroaggregative (EAEC), Enterohemorrhagic (EHEC) and Diffusely Adherent (DAEC) (Orsi et al. 2023).

E. coli isolates have a wide diversity of genes that encode virulence factors and encompass adhesins, siderophores, toxins, lipopolysaccharides, and protactins, which help circumvent the host's immune system (Steimle et al. 2016, Chen et al. 2017, Orsi et al. 2023). However, it cannot be stated that the nonspecific profile of virulence factors is directly associated with cases of mastitis (Leimbach et al. 2017).

Antimicrobials have been widely used in veterinary medicine to control mastitis for decades. However, the excessive use of these drugs encouraged the emergence of resistance in mastitis-causing *E. coli* isolates, reducing the effectiveness of the various groups of antibiotics (Zhang et al. 2018). This condition occurs due to a complex communication of different means that grant resistance to several classes of antimicrobials. A commonly identified resistance medium in *E. coli* is the expression of extended-spectrum beta-lactamases (ESBLs), which collaborates with the transmission of resistance between *E. coli* isolates (Bandyopadhyay et al. 2015, Nagy et al. 2015, Orsi et al. 2023).

The aim of this study is to estimate the frequency of ESBL-producing *E. coli* in milk samples from cows with clinical mastitis from dairy farms in different geographic regions of Brazil. Additionally, it aims to estimate the prevalence of resistance to commonly used antimicrobials in mastitis treatment and perform molecular identification of virulence genes associated with enteropathogenic *E. coli* (DEC) pathotypes.

MATERIALS AND METHODS

Animal Ethics. This study was approved by the Genetic Heritage Management Council of the National System of Management of Genetic Heritage and Associated Knowledge (registration number: A4784B5) and by the Ethics Chamber in the Use of Animals (CEUA) of the "Universidade Federal de Goiás" (MB number 057/21).

Study design and origin of *Escherichia coli* isolates. In total, 198 milk samples were collected from cows with clinical mastitis on 12 farms. Of these, 11 farms use mechanical milking, while one farm employs manual milking. These farms employ different management strategies and are located in the states of Goiás, Pará, Paraíba, São Paulo, and Santa Catarina, Brazil. The collection process was carried out between the first half of 2021 (March) and the first half of 2022 (February). All samples were collected aseptically in sterile tubes after performing pre-milking hygiene procedures, and the ceiling tips were disinfected with cotton containing 70% alcohol. The transport of samples containing 15mL of milk each was carried out under refrigeration (4°C to 8°C) until microbiological culture.

The methodology and interpretation criteria used to diagnose mastitis in individual samples were based on detecting clinical mastitis cases by local and/or systemic changes in the animal.

Isolation and identification. The isolation and identification of *E. coli* were performed by sowing 10μL of each sample onto plates of MacConkey agar. The plates were incubated under aerobic conditions at 37°C for 72 h. The *E. coli* as a causative of intramammary infection was defined by at least three CFU of *E. coli*. Identifying the pathogen in the samples was carried out according to the morpho-dyeing characteristics and biochemical and culture tests as described by Quinn et al. (2005).

The characteristic colonies were submitted to biochemical tests recommended for differentiation of enterobacteria, such as Simmons citrate, indole production, Voges-Proskauer test (VP), and methyl red (VM), following the methodology described by Koneman (1997) and Macfaddin (2000).

The strains that were positive in the indole and methyl red (MV) production tests and negative in the Simmons citrate and Voges-Proskauer (VP) tests were classified as *E. coli* (Silva et al. 2001, Trabulsi et al. 2004, Clermont et al. 2019). For confirmation, the isolates were also identified by mass spectrometry (MALDI-TOF).

Antimicrobial susceptibility test. The disc-diffusion technique was used to evaluate the antimicrobial resistance profile of *E. coli* isolates (Bauer et al. 1966), according to criteria recommended by the Clinical and Laboratory Standards Institute (CLSI 2022). The isolates were cultured on BHI agar at 35°C/6 h, diluted in saline solution (0.9% NaCl) to the 0.5 McFarland scale (1.5 x 108 CFU) and sown in Mueller Hinton agar (MH, Oxoid) with the aid of a sterile swab. The antibiotics, ampicillin (AMP, 10µg), cefepime (CPM, 30µg), cefotaxime (CTX, 30µg), ceftriaxone (CRO, 30µg), cefoxitin (CFO, 30µg), ceftazidime (CAZ, 30µg), ceftiofur (CTF, 30µg), aztreonam (ATM, 30µg), gentamicin (GEN, 10µg), streptomycin (EST, 10µg) and tetracycline (TET, 30µg) were added to the MH plates and incubated at 35±2°C/18 h, to determine the diameters of the inhibition halos. The strain of *Escherichia coli* ATCC 25922 was used as quality control for the tests.

Disc-diffusion test for investigation of extended-spectrum β-lactamase (ESBL)-producing *E. coli.* For the investigation of ESBL-producing *E. coli*, the isolates were submitted to a screening test by disk diffusion (Bauer et al. 1966) using the antibiotics CRO, CTX, CAZ, and ATM. Isolates with a halo less than or equal to that recommended by CLSI (2022) (CRO ≤ 25mm or CTX ≤ 27mm or CAZ ≤ 22mm or TMJ ≤ 27mm) were selected for the confirmatory test consisting of the same test with the addition of the amoxicillin-clavulanic acid disc (CAM, 30µg) at a distance of 20mm from the antibiotic discs, as an inhibitor of β-lactamases. The presence of distorted halos or phantom zones characterizes ESBL-producing *E. coli.*

DNA extraction and molecular detection of virulence markers associated with the main pathotypes of diarrheagenic *Escherichia coli* (DEC). Each *E. coli* isolate was seeded on a brain heart infusion broth (BHI) agar plate, transferred to a microcentrifuge tube with 200µL of sterile Milli-Q water, and boiled for 10 min. After cooling in ice, each isolate was centrifuged at 10,000 x g for 1 min, and the supernatant was transferred to a new microtube and frozen for future polymerase chain reactions (PCRs) (Dias et al. 2016).

To identify isolates of diarrheagenic *E. coli* (DEC), the main genes associated with virulence for these pathotypes were investigated, namely: *eae* (Reid et al. 1999), *bfp*B (Müller et al. 2007), *esc*N (Dias et al. 2016), *aat*A (Schmidt et al. 1995), *agg*R/*ipa*H (Toma et al. 2003), *stx*1/*stx*2 (Paton & Paton 1998), *est* (Aranda et al. 2007) and *elt*A (Schultsz et al. 1994). The reactions were performed according to the references cited in Table 1.

The PCR products were submitted to 1.5% agarose gel electrophoresis (Electrophoresis Power Supply Model EPD 600; Amersham Pharmacia Biotech Inc.), and the bands were stained with SYBR Safe (Invitrogen). The images were obtained using the SmartView Pro Imager System 1200 (Major Science).

RESULTS

Identification of isolates

A total of 12 (6.07%) from the 198 milk samples from cows with clinical mastitis had growth greater than three CFU on MacConkey agar, and *Escherichia coli* was identified in these samples.

Susceptibility to antibiotics

The frequency of susceptibility for each antimicrobial and classes used in the disc-diffusion test is available in Table 2. Despite the low number of isolates, the susceptibility test results to antimicrobial agents showed that 25% (3/12) of

Table 1. Virulence genes and primer sequences used in PCR reactions to investigate virulence factors in diarrheagenic *Escherichia coli*

Gene	Sequence (5' → 3')	Annealing temperature (°C)	Amplicon (pb)	Reference
eae	F: CTGAACGGCGATTACGCGAA	52	917	Reid et al. (1999)
	R: CGAGACGATACGATCCAG			
*bfp*B	F: GACACCTCATTGCTGAAGTCG	63	910	Müller et al. (2007)
	R: CCAGAACACCTCCGTTATGC			
aggR	F: GTATACACAAAAGAAGGAAGC	52	254	Toma et al. (2003)
	R: ACAGAATCGTCAGCATCAGC			
*stx*1	F: ATAAATCGCCATTCGTTGACTAC	52	180	Paton & Paton (1998)
	R: AGAACGCCCACTGAGATCATC			
*stx*2	F: GGCACTGTCTGAAACTGCTCC	52	255	Paton & Paton (1998)
	R: TCGCCAGTTATCTGACATTCTG			
*elt*A	F: GGCGACAGATTATACCGTGC	50	696	Schultsz et al. (1994)
	R: CCGAATTCTGTTATATATGTC			
est	F: ATTTTTMTTTCTGTATTRTCTT	52	190	Aranda et al. (2007)
	R: CACCCGGTACARGCAGGATT			
*ipa*H	F: GTTCCTTGACCGCCTTTCCGATACCGTC	52	619	Toma et al. (2003)
	R: GCCGGTCAGCCACCCTCTGAGAGTAC			
*aat*A	F: CTGGCGAAAGACTGTATCAT	55	630	Schmidt et al. (1995)
	R: CAATGTATAGAAATCCGCTGTT			
*esc*N	F: CGACGACTATTGCAGAGT	52	670	Hernandes et al. (2009)
	R: GCCTTATCTGCTTCAGGA			

the strains exhibited resistance to at least one of the eleven antimicrobials used. Ampicillin, cefepime, cefoxitin, ceftazidime, ceftiofur, aztreonam, and gentamicin were the antimicrobials to which all strains displayed susceptibility. Intermediate resistance to streptomycin was observed in 25% (3/12) of the strains. There was resistance to cefotaxime at 8.33% (1/12), streptomycin at 16.67% (2/12), and tetracycline at 16.67% (2/12).

Disc-diffusion test for investigation of extended-spectrum β-lactamase-producing *E. coli* (ESBL)

None of the 12 isolates submitted to the disc-diffusion test for investigating extended-spectrum β-lactamase-producing *E. coli* presented the phenotypic characteristic.

Molecular characterization of *E. coli* isolates

Only the *bfp*B and *esc*N genes, which encode virulence factors associated with the atypical EPEC pathotype of DEC, were observed in a single strain. In contrast, the *eae*, *agg*R, *stx*1, *stx*2, *elt*A, *est*, *ipa*H, *aat*A genes were absent in any of the 12 DEC isolates.

DISCUSSION

Bovine mastitis is one of the most impactful and costly diseases in dairy production, with bacterial infections being one of the most prevalent causes. The present study aimed to perform the phenotypic and genotypic characterization of *Escherichia coli* isolates from individual raw milk samples from cows with clinical mastitis in five regions of Brazil. In this study, we observed the prevalence of *E. coli* in cows with clinical mastitis at a rate of 6.07%, which is higher compared to the rate of 4.5% reported by Orsi et al. (2023) and approximately equal to the rate of 6.9% mentioned by Oliveira et al. (2022). Lactating cows are susceptible to mastitis caused by *E. coli,* and the numbers demonstrate the relevance of this microorganism in the cases that this form of mastitis presents.

In the early lactation phase, the mammary gland is colonized by *E. coli*, which is considered one of the main etiological agents of environmental clinical mastitis. Infections caused by *E. coli* may present mild, moderate, or severe symptoms. Several virulence factors influence this classification, including genes encoding toxins, adhesion proteins, invasives, biofilm formation, and the ability to eliminate iron and resist serum complement. However, because it causes endotoxic shock, induces apoptosis of the mammary gland, and causes a higher degree of pain, lipopolysaccharides (LPS) are considered the main virulence factor of *E. coli* (Chen et al. 2017, Steele et al. 2019).

Despite regulatory and structural changes in milk production, the prevalence of resistant Gram-negative microorganisms, such as *E.* coli, has steadily increased in cases of mastitis, causing serious public health problems (Bandyopadhyay et al. 2015). The indiscriminate use of antimicrobials in mastitis treatment has become a worldwide concern due to favoring the occurrence of the resistance process (Ahmed & Shimamoto 2011). In this study, the antimicrobial susceptibility test revealed that *E. coli* isolates in individual milk samples with clinical mastitis showed greater resistance to cefotaxime at about 8.33% and streptomycin and tetracycline at about 16.67%. Similarly, other studies have shown a higher resistance in *E. coli* isolates to streptomycin and tetracycline; however, it is worth noting that our study involves a smaller number of isolates (Bandyopadhyay et al. 2015, Tadesse et al. 2018, Messele et al. 2019).

Cephalosporins, aminoglycosides, and tetracyclines are widely used in veterinary medicine, including in treating bovine bacterial mastitis. ESBL-producing *E. coli* can attribute resistance to cephalosporins through the hydrolysis of these antimicrobials. In contrast, tetracycline resistance occurs through ribosomal protection and efflux pumping, both associated with the *tet* gene, which triggers antimicrobial resistance to tetracyclines (Pereira et al. 2011).

The treatment of clinical mastitis cases should be based on the type of etiological agent and antimicrobial sensitivity. Farm culture systems have been widely employed in identifying infectious agents of mastitis before determining a therapeutic approach (McDougall et al. 2018, Sipka et al. 2021). Implementing culture systems on the farm minimizes the indiscriminate use of antibiotics, establishing antibiotic therapy only in cases of positive breast quarters and with chances of cure (Cameron et al. 2014).

The highest susceptibility of isolates to antimicrobials (>100%) studied was observed for ampicillin, cefepime, cefoxitin, ceftazidime, ceftiofur, aztreonam, and gentamicin, indicating that mainly cephalosporins should be considered in the treatment of cases of intramammary infections (IMI) by *E. coli* (Tomazi et al. 2018).

Table 2. In vitro sensitivity profile (disc diffusion method) in *Escherichia coli* strains isolated from individual samples of cows with clinical mastitis

Antimicrobial	Class	Sensitive % (N)	Partially sensitive % (N)	Resistant % (N)
Ampicillin 10 mcg	Penicillin	100 (12/12)	0 (0/12)	0 (0/12)
Cefepime 30 mcg		100 (12/12)	0 (0/12)	0 (0/12)
Cefotaxime 30 mcg		83.34 (10/12)	8.33 (1/12)	8.33 (1/12)
Ceftriaxone 30 mcg	Cephalosporin	91.67 (11/12)	8.33 (1/12)	0 (0/12)
Ceftazidime 30 mcg		100 (12/12)	0 (0/12)	0 (0/12)
Ceftiofur 30 mcg		100 (12/12)	0 (0/12)	0 (0/12)
Cefoxitin 30 mcg	Cefamycin	100 (12/12)	0 (0/12)	0 (0/12)
Aztreonam 30 mcg	Monobactamic	100 (12/12)	0 (0/12)	0 (0/12)
Gentamicin 10 mcg	Aminoglycoside	100 (12/12)	0 (0/12)	0 (0/12)
Estreptomycin 10 mcg		58.33 (7/12)	25 (3/12)	16.67 (2/12)
Tetracycline 30 mcg	Tetracycline	75 (9/12)	8.33 (1/12)	16.67 (2/12)

None of the *E. coli* isolates evaluated in this study presented phenotypic characteristics for producing extended-spectrum β-lactamase (ESBL). However, considering the indiscriminate use of antimicrobials in the treatment of mastitis, a certain frequency of ESBL-producing *E. coli* in raw milk is expected (Saei et al. 2022). Other studies reported a higher prevalence in identifying isolates of ESBL-producing *E. coli* in milk samples, these being 3.42% and 21.56%, respectively (Parussolo et al. 2019, Saei et al. 2022).

This study investigated the main genes associated with DEC virulence factors in *E. coli* isolates. The occurrence of DEC in milk samples with clinical mastitis from different regions of Brazil is unknown. All isolates tested negative for the *eae*, *aat*A, *agg*R, *ipa*H, *stx*1, *stx*2, *est,* and *elt*A genes, with only one positive isolate for the *bfp*B and *esc*N genes. The non-identification of genes such as *stx* in isolates is in accordance with Bag et al. (2021) and Lan et al. (2020). However, the occurrence of these genes in *E. coli* has been reported in clinical mastitis cases by other authors (Lan et al. 2020, Bag et al. 2021). Although the association between virulence genes and the severity of clinical mastitis cases is not well elucidated, the presence of the *bfp*B and *esc*N genes may contribute to clinical mastitis caused by DEC. Therefore, many samples are needed to investigate the presence of virulence genes associated with DEC pathotypes in *E. coli* isolates from individual milk samples with clinical mastitis in various states of Brazil. However, these preliminary results suggest a low number of bovine clinical mastitis cases caused by DEC.

CONCLUSIONS

Based on the presented results, we identified *Escherichia coli* in 6.07% of milk samples from cows with clinical mastitis. The antibiotic susceptibility analysis revealed that 25% of the strains showed resistance to at least one of the eleven tested antimicrobials. However, it is crucial to highlight that none of the isolates subjected to the disk diffusion test for extended-spectrum β-lactamase-producing *E. coli* (ESBL) presented this phenotypic characteristic. Additionally, the *bfp*B and *esc*N genes, associated with the atypical enteropathogenic *E. coli* (EPEC) pathotype of diarrheagenic *E. coli* (DEC), were detected in only one strain. In contrast, other virulence genes, such as *eae*, *agg*R, *stx*1, *stx*2, *elt*A, *est, ipa*H, and aatA, were absent in all 12 DEC isolates.

These results suggest a low prevalence of bovine clinical mastitis cases caused by DEC despite the presence of resistance to some antimicrobials. The absence of ESBL isolates is optimistic regarding resistance to broad-spectrum β-lactams. However, there is a continued need for surveillance and comprehensive studies to understand the epidemiology and antimicrobial resistance in *E. coli* strains associated with bovine clinical mastitis.

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