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E-mail: ambi.agua@gmail.com

Study of *Escherichia coli*, EPEC, ETEC AND STEC, in water offered in schools in a municipality with low human development index in Maranhão State, Brazil

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Iran Alves da Silva[1]; Greiciene dos Santos de Jesus[1]; Larissa Sarmento dos Santos Ribeiro[2]; Hamilton Pereira Santos[2]; Danilo Cutrim Bezerra[3]; Viviane Correa Silva Coimbra[1]; Amanda Mara Teles[1]; Nancyleni Pinto Chaves Bezerra[1*]

[1]Programa de Pós-graduação Profissional em Defesa Sanitária Animal. Universidade Estadual do Maranhão (UEMA), Cidade Universitária Paulo VI, Avenida Lourenço Vieira da Silva, n° 1000, CEP: 65055-310, São Luís, MA, Brazil. E-mail: iranalves46@gmail.com, greicy2403@hotmail.com, vivianecorrea@yahoo.com, amandatelespesq@gmail.com
[2]Departamento de Engenharia de Pesca. Universidade Estadual do Maranhão (UEMA), Cidade Universitária Paulo VI, Avenida Lourenço Vieira da Silva, n° 1000, CEP: 65055-310, São Luís, MA, Brazil. E-mail: larissa.sarmento@uema.br, hpsluiza@yahoo.com.br
[3]Departamento de Zootecnia. Universidade Estadual do Maranhão (UEMA), Cidade Universitária Paulo VI, Avenida Lourenço Vieira da Silva, n° 1000, CEP: 65055-310, São Luís, MA, Brazil. E-mail: danilocbezerra15@gmail.com
[*]Corresponding author. E-mail: nancylenichaves@hotmail.com

ABSTRACT

This study examined the EPEC, ETEC and STEC pathotypes in *E. coli* isolates from water supplied to schools in a municipality in Maranhão, Brazil with a low human development index. For this, 57 bacterial strains isolated from 19 water samples were used. All strains were confirmed as belonging to the *E. coli* species, by Gram staining and phenotypic tests. From pure cultures of E. coli, DNA was extracted followed by characterization of the isolates by polymerase chain reaction (PCR). This is the first study carried out in the state of Maranhão on the research of the diarrheagenic strains EPEC, ETEC and STEC in water for human consumption offered in schools, with the detection of virulent genes characteristic of enterotoxigenic E. coli (26.31%; n = 15/57) and E. coli producing Shiga toxin (8.78%; n= 5/57). Of the strains identified, 8.78% (n= 5/57) corresponded to combinations of est + stx1 genes. It is concluded that the detection of the diarrheagenic strains ETEC and STEC in *E. coli* isolates from water samples for human consumption indicates that, in addition to the water being contaminated, it harbors strains with pathogenic potential to cause diarrheal infection in its users.

Keywords: ETEC, school environment, STEC.

Estudo de *Escherichia coli*, EPEC, ETEC E STEC, em água ofertada a escolas de um município com baixo índice de desenvolvimento humano, Estado do Maranhão, Brasil

RESUMO

Esse estudo examinou os patótipos EPEC, ETEC e STEC em isolados de *E. coli* oriundos de água ofertada em escolas de município maranhense de baixo índice de desenvolvimento humano, Brasil. Para isso, foram utilizadas 57 cepas bacterianas isoladas de 19 amostras de água. A totalidade das cepas foram confirmadas como pertencentes a espécie *E. coli*, por coloração de Gram e testes fenotípicas. Das culturas puras de *E. coli* realizou-se a extração do DNA seguido da caracterização dos isolados por reação em cadeia da polimerase (PCR). Esse é o primeiro estudo realizado no estado do Maranhão sobre a pesquisa das estirpes diarreiogênicas EPEC, ETEC e STEC em água para consumo humano ofertadas em escolas, com a detecção de genes de virulência característicos de *E. coli* enterotoxigênica (26,31%; n= 15/57) e *E. coli* produtora da toxina de Shiga (8,78%; n= 5/57). Das estirpes identificadas, 8,78% (n= 5/57) corresponderam a combinações de genes *est* + *stx1*. Conclui-se que a detecção das estirpes diarreiogênicas ETEC e STEC em isolados de *E. coli* oriundos de amostras de água para consumo humano indica que, além da água estar contaminada, abriga estirpes com potencial patogênico para causar infecção diarreica em seus usuários.

Palavras-chave: ambiente escolar, ETEC, STEC.

1. INTRODUCTION

Food and waterborne diseases (FWBDs) - seen as relevant emerging public health issues - are mainly caused by intake of water and food contaminated with enteric-origin pathogens. Adults and children living in developing countries, such as Brazil, are susceptible to infectious diarrheal diseases whose main toxic infection source is in water (Grube *et al.*, 2014). According to the World Health Organization (WHO and UNICEF, 2017), 361 thousand children under the age of 5 years die yearly worldwide due to diarrhea caused by lack of basic sanitation and contaminated water consumption.

Maranhão State's rural areas present a low human development index, which is evidenced by their precarious sanitary and infrastructure conditions (Monteiro *et al.*, 2021). Most of the population living in these areas suffers from lack of water supply, and it forces citizens to find alternative solutions, such as consuming water from collective sources like artesian and cacimba-type wells, which are mostly susceptible to contamination (Ferreira *et al.*, 2016; Monteiro *et al.*, 2021; Silva *et al.*, 2023).

Bacteria belonging to the coliform group are biological bioindicators addressed in Brazilian laws about the potability standard of water used for human consumption (Brasil, 2021) and aimed at monitoring water microbiological quality. *Escherichia coli*, which is a fecal-origin thermotolerant bacteria, has significant clinical-epidemiological relevance within this group (Grube *et al.*, 2014).

E. coli is a bacterial species belonging to the family Enterobacteriaceae. It is widely distributed in nature and uses both human and animal intestinal tracts as its main habitat (Grube *et al.*, 2014; Ferreira *et al.*, 2016; Gomes *et al.*, 2016). Commensal *E. coli*, which is part of the intestinal microbiota, is non-pathogenic and plays a key physiological role in body functioning. However, there are eight pathogenic *E. coli* categories that cause intestinal infection in both humans and animals. They are called diarrheagenic *E. coli* (APHA *et al.*, 2017) and are differentiated by the presence of virulence factors, such as fimbrial and afimbrial adhesins, toxins and invasions. These categories comprise Enteropathogenic (EPEC),

Enterotoxigenic (ETEC), Enteroinvasive (EIEC), Shiga toxin-producing (STEC), Enteroaggregative (EAEC), diffusely-adherent (DAEC), adherent-invasive (AIEC) and Shiga toxin-producing enteroaggregative (STEAEC) *E. coli* (Clements *et al.*, 2012; Schuroff *et al.*, 2014; Vendruscolo *et al.*, 2017).

Diarrheagenic *E. coli* differentiation and classification processes are based on the presence of different chromosomal and/or plasmid virulence genes lacking in commensal *E. coli*. Molecular techniques enabled faster identifying different pathotypes, since conventional phenotypic methods, such as toxin detection, adherence and invasion tests, require longer time and the conduction of many tests (Gomes *et al.*, 2016).). In light of the foregoing, the current study researched the EPEC, ETEC and STEC pathotypes in *E. coli* isolates from water supplied to schools in a municipality in Maranhão, Brazil with a low human development index.

2. MATERIAL AND METHODS

2.1. *Escherichia coli* isolation and characterization

Fifty-seven (57) bacterial strains isolated from 19 samples of water used for human consumption in 11 elementary schools were used in the current study (the selection criteria were based on the logistics of access to communities and the interest of managers in participating in the project). All strains were confirmed as species belonging to genus *Escherichia*, based on dye (Gram staining) and phenotypic tests (gas production from glucose, lactose fermentation, lysine decarboxylation, indole production from tryptophan, urea non-degradation, and lack of hydrogen sulfide production from sulfur amino acids in Rugai medium added with lysine), according to Monteiro *et al.* (2021). Biochemical tests, such as Methyl Red and Voges Proskauer, were also performed.

2.2. Molecular Tests

DNA extraction from pure *E. coli* cultures was performed based on using Instalx Matrix® (bio)resin, according to the manufacturer's instructions. Initially, 200 µL of culture was centrifuged at 12,000 revolutions per minute (RPM), for 1 minute. The resulting pellet was resuspended in 1 mL of ultrapure sterile water and centrifuged (12 rpm/1 min). The supernatant was discarded and 200 µL of resin was added to the samples, which were then incubated at 56°C for 30 minutes. Subsequently, samples were homogenized in a vortex for 10 seconds and incubated for the last time at 100°C, for 8 minutes. Then, they were homogenized again in vortex for 10 seconds and centrifuged at 12,000 rpm for additional 3 minutes - 50 µL of the supernatant were removed and the precipitate was discarded.

The extracted DNA was quantified in spectrophotometer based on absorbance reading carried out at 260 nm; 260/280 nm ratio was used to determine samples' purity (Samincok and Russel, 2001). The samples' concentration was adjusted to approximately 200 ng/µL based on using the TE buffer at pH 8.0. DNA was stored at -20°C until polymerase chain reaction technique (PCR) application to detect diarrheagenic *E. coli* pathotypes.

In the amplification, 25 µL of solution containing 3.0 µL of DNA sample, 12.5 µL of GoTaq® colorless master mix (Promega®), 7.5 µL of ultrapure water, and 10 pmol of each oligonucleotide were used. Target primers and amplification conditions are summarized in Table 1. Positive controls for *eae*, *Stx1* and *Stx2* (*E. coli* CDC EDL-933, INCQS 00171), *bfpA* (*E. coli* CDC O126, INCQS 000184), *elt* (*E. coli* 0761-2) and *est* (*E. coli* 0122-4), as well as internal negative control (ultrapure sterile water), were included in each batch of reactions.

The PCR products were visualized by applying 5 µL of the amplified product on 2% agarose gel, staining with SYBR Safe® and horizontal electrophoresis for 30 min at 90V in 1X TBE buffer. The bands were visualized under ultraviolet light and digital images were recorded with the L-PIX Image EX image capturing system (Locus Biotechnology, Brazil).

Table 1. Sequence of oligonucleotides used as primer in polymerase chain reaction (PCR) and amplific+5ation conditions set for *Escherichia coli* diarrheagenic pathotypes.

Primer's name	Primers (5'-3')	Program	Amplicon size (bp)	Reference
eae1	CTGAACGGCGATTACGCGAA CCAGACGATACGATCCAG	94°C/5 min 94°C/1 min 53°C/2 min 72°C/3min 30x 72°C/7 min	917	Reid *et al.* (1999)
bfpA	AATGGTGCTTGCGCTTGCTGC GCCGCTTTATCCAACCTGGTA	94°C/5 min 94°C/30 s 56°C/1 min 72°C/2 min 29x 72°C/7 min	326	Gunzburg *et al.* (1995)
Stx1	CAGTTAATGTGGTGGGGAAGG CACCAGACAATGTAACCGCTG	95°C/5 min 95°C/20 s 61°C/40 s 72°C/90 s 30x 72°C/7 min	348	Vidal *et al.* (2004)
Stx2	ATCCTATTCCCGGGAGTTTACG GCGTCATCGTATACACAGGAGC	95°C/5 min 95°C/20 s 61°C/40 s 72°C/90 s 30x 72°C/7 min	584	Vidal *et al.* (2004)
Elt	GGCGACAGATTATACCGTGC CGGTCTCTATATTCCCCTGTT	95°C/5 min 95°C/45 s 50°C/1 min 72°C/1 min 40x 72°C/7 min	450	Aranda *et al.* (2004)
Est	ATTTTTMTTTCTGTATTRTCTT CACCCGGTACARGCAGGATT	95°C/5 min 95°C/45 s 50°C/1 min 72°C/1 min 40x 72°C/7 min	190	Aranda *et al.*, (2004)

Wherein: bp= basis pairs; s= seconds; min= minutes.
Source: Elaborated by the authors.

3. RESULTS AND DISCUSSION

All 57 bacterial isolates used in the current study were tested for the presence of six virulence genes accounting for featuring different *E. coli* diarrheagenic pathotypes.

Diarrheagenic strains were identified in 15 (26.31%) of these isolates, which derived from five (n=5/19; 26.31%) water samples collected in five different schools.

Virulence genes characteristic of enterotoxigenic *E. coli* (ETEC) and Shiga toxin-producing *E. coli* (STEC) were detected (Table 2). This is the first study carried out in the state of Maranhão on the detection of diarrheagenic *E. coli* in water for human consumption offered in municipal schools. ETEC is known as the main etiological agent of "traveler's diarrhea" (Gomes *et al.*, 2016), whereas STEC is associated with non-bloody diarrhea, abdominal pain and emesis episodes. However, some people affected by STEC may present bloody diarrhea and intense abdominal pain (Vidal *et al.*, 2005). Diarrheal disease caused by both aforementioned pathotypes may result from contaminated water and food intake (Dumke *et al.*, 2006; Grube *et al.*, 2014; Gomes *et al.*, 2016).

Table 2. *Escherichia coli* diarrheagenic pathotypes detected in samples of water used for human consumption in municipal schools of a municipality presenting low human development index.

Water samples (n= 19)	Bacterial isolates (n= 57)	Identified genes	*Escherichia coli* diarrheagenic pathotypes
05	15	*est*	ETEC
01	03	*stx1*	STEC

ETEC exclusively associated with the *est* gene (heat-stable enterotoxin producer) was detected in 15 bacterial isolates, although the *elt* gene (heat-labile enterotoxin producer) was not detected in them. Similar results were observed by Macedo *et al.* (2020) who assessed 58 samples of fresh groundwater used for consumption purposes in a rural settlement and identified ETEC in three samples (8.33%) - two of them only presented the *est* gene.

Cestari *et al.* (2016) have found 36 (12.2%) ETEC isolates in 295 *E. coli* strains isolated from fresh water used for human consumption in Londrina City, Paraná State, Brazil. A study conducted in France detected high rates (74.19%; n = 46/62) of ETEC isolates in river surface-water samples (Kambire *et al.*, 2017). A study conducted with 0-5-year-old Nigerian children with acute diarrhea has shown that 51 of 400 collected stool samples presented diarrheagenic *E. coli* and that 16 of them presented ETEC features (Ifeanyi *et al.*, 2015).

ETEC has been often identified in developing countries, as reported in different studies. Ali *et al.* (2012) have found 3.2% of ETEC in water used for human consumption in Libya, whereas Widmer *et al.* (2013) have found 1.2% of ETEC in surface water in Bangladesh. Lack of basic sanitation was defined as the main factor contributing to the high incidence of this pathotype in the investigated countries (Akter *et al.*, 2013; Sidhu *et al.*, 2013).

According to Mainil (2013), humans, pigs, cattle and dogs are the main hosts of the ETEC pathotype, with emphasis on cattle. According to Silva *et al.* (2023), schools in the investigated municipality are supplied by wells, whose water supply source undergoes little or no maintenance at all, a fact that increases the risk of waterborne disease incidence. The aforementioned authors added likely contamination sources to water supplied to the investigated schools, such as livestock animals' breeding, with emphasis on cattle and pigs.

Five (5) isolates presented virulence genes typical of STEC (*stx1*). Despite the relevance of this finding, it should be analyzed with caution because seasonality is the main variable influencing water microbiological quality degradation. Consequently, intense runoff increases the transport of particles contaminated by fecal material and it can increase the contamination of water supply sources used by municipal schools in the investigated region over the year. According to Brennan *et al.* (2013), E. coli's survival ability, which is associated with both simple nutritional needs and its underground adaptability, turns the environment into a microbiological contamination spreading system towards water bodies and it increases the likelihood of diarrheagenic pathotypes' transmission.

The herein amplified genes were *est* (26.31%; n = 15/57) and *stx1* (8.78%; n = 5/57) - 8.78% (n = 5/57) of the identified pathotypes corresponded to *est* + *stx1* gene combinations. According to Amaral *et al.* (2003) and Ferreira *et al.* (2016), the main water supply sources in rural environments are underground - mostly untreated - sources susceptible to contamination due to their low depth and closeness to the top soil, a fact that exposes them to rainwater flows carrying impurities. This set of information, in addition to that reported by Silva *et al.* (2023), who mentioned lack of sanitation infrastructure and livestock animals' breeding, mainly cattle and pigs, in the assessed region, can feature contamination sources for the analyzed water samples.

Lack of basic sanitation, environmental conditions, as well as human and agricultural activities, are critical variables capable of increasing the diversity of *E. coli* diarrheagenic pathotypes in the water supplied to schools in the assessed municipality. Consequently, they lead to FWBDs, since ETEC and STEC can be ingested by both adults and children through water supplied to schools and cause mild to severe diarrhea cases.

4. CONCLUSION

The identification of ETEC and STEC diarrheagenic pathotypes in *E. coli* isolates deriving from samples of water used for human consumption in municipal schools has indicated that, besides being contaminated, the analyzed water harbors strains with pathogenic potential to cause diarrheal infection in the school community. Thus, it is of paramount importance to monitor the microbiological quality of water in school environments, mainly due to the amount of time community members spend at school and to the likelihood of having water working as vehicle for straight pathogenic bacterial strains' transmission to humans, or even as source of contamination of food produced and provided to its users.

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