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Multidrug-Resistance and Virulence-Related Properties of Diarrheagenic *Escherichia coli* in Urban River: A Possible Source and Dissemination of Human Infections

Nogueira, B. A.*[a]; Olivella, J. G. B.[b]; Sued-Karam, B. R. [b]; Ribeiro, P. M. A. P. [b]; Oliveira, V. W. [b]; Rocha-de-Souza, C. M. [c]; Fracalanzza, S. E. L.[d]; Mattos-Guaraldi, A. L. [b]; Ignácio, A. C. P. R. [b]

[a] *Laboratório de Tecnologia Bacteriana – LATEB, Fundação Oswaldo Cruz – FIOCRUZ, Rio de Janeiro, RJ, Brasil.* [b] *Departamento de Microbiologia e Imunologia, Faculdade de Ciências Médicas, Universidade do Estado do Rio de Janeiro – UERJ, Rio de Janeiro, RJ, Brasil.* [c] *Laboratório de Pesquisa em Infecção Hospitalar, Instituto Oswaldo Cruz, Fundação Oswaldo Cruz – FIOCRUZ, Rio de Janeiro, RJ, Brasil.* [d] *Instituto de Microbiologia Paulo Góes, Universidade Federal do Rio de Janeiro – UFRJ, Rio de Janeiro, RJ, Brasil.*

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ABSTRACT

The presence of multi-drug resistant (MDR)*E. coli* harboring virulence pathotypes in aquatic systems is a public health concern due to an increased number of cases of infections and outbreaks in industrialized and developing countries. The present study aimed to evaluate the microbiological quality of the Joana river, located in Rio de Janeiro, by analyzing *E. coli* bacteria contamination and investigating virulence properties and MDR profiles by phenotypic and genotypic methods, including bacterial interaction with Caco-2 cells. A total of 34 *E. Coli* were identified by MALDI-TOF and 20 *E. coli* were characterized as MDR when submitted to antimicrobial susceptibility test. Evaluation by multiplex-PCR of MDR *E. coli* demonstrated the presence of virulence pathotypes: EHEC (*stx1*, *stx2*, *eae* genes), STEC (*stx2* gene), and EIEC/STEC (*stx2*, *iaL* genes). Virulence potential was demonstrated by the ability to adhere and survive within Caco-2 cells of MDR *E. coli* pathotypes (n=4). In conclusion, this study demonstrates the presence of diarrheagenic MDR *E. coli* in river water in Rio de Janeiro. The possibility of aquatic environment dissemination of antimicrobial resistance and human contamination leading to community and nosocomial infections due to virulent MDR *E. coli* water-borne pathogens is a matter of concern.

INTRODUCTION

Urban rivers may act as a reservoir of several human pathogens, including *Escherichia coli*, a commensal inhabitant of the human gastrointestinal tract, considered the main indicator of water potability (Lanna *et al*., 2020; Guzmán *et al*., 2015). Although most *E. coli* strains live harmlessly in the colon, several pathogenic strains can cause intestinal and extraintestinal diseases, through different virulence factors, in healthy and immunocompromised individuals (Leimbach *et al*., 2013).

Diarrheal illnesses are a severe public health problem and a major cause of morbidity and mortality, especially in Africa, Asia, and Latin America due to poor living conditions, including poor environmental hygiene and sanitation. Diarrheagenic *E. coli*(DEC) is one of the most important of the various etiological agents of diarrhea, where strains have evolved to the acquisition, through horizontal gene transfer, of a particular set of characteristics that have persisted in the host. The DEC pathotypes are classified as Enteropathogenic *E. coli* (EPEC), Enterohaemorrhagic (Shiga toxin-producing) *E. coli* (EHEC/STEC), Enteroaggregative *E. coli* (EAEC), Enterotoxigenic *E. coli* (ETEC) and enteroinvasive. *coli* (EIEC - based on the presence of seven virulence genes: *eaeA*, *stx1*, *stx2*, *iaL*, *Lt*, *St,* and *egg* respectively. Each of these pathotypes represents a group of clones that share specific virulence factors. (Gomi*et al*., 2015; Gomes at al. 2016).

Several reports indicate ETEC as a major cause of diarrheal illness in poor areas of the world where they contribute to unacceptable mortality, particularly among young children (Fleckenstein; Kuhlmann, 2019); EHEC strains secret Shiga toxin (*Stx*) which can lead to hemolytic uremic syndrome; EPEC is an important cause of infant diarrhea and mortality worldwide; EIEC strains are involved in invasive intestinal infections and dysentery; EAEC strains cause persistent diarrhea due to a heat-stable enterotoxin activity(Abe et al., 2008; Serapio-Palacios, Finlay, 2020); STEC may cause outbreaks, sporadic cases of hemorrhagic colitis and are associated with the hemolytic uremic syndrome and thrombotic thrombocytopenic purpura (Beutin*et al*., 2004). Pathogenic *E. Coli* strains may colonize human tissues by attaching and/or invading host cells. Clinical manifestations induced by each of these strains are associated with a watery form of diarrhea or inflammatory presentation of the disease (Kalita, Hu, Torres, 2014; Wang *et al*., 2017; Lanna*et al*., 2020).

Population heterogeneity of *E. coli* has been linked to environmental changes and genome plasticity evolution of some lineages associated with human diseases due to new combinations of virulence genes and phenotypic diversity, contributing to survival, higher virulence profiles, and multi-resistance dissemination (Ashbolt, 2004; Brito *et al*., 2008; Hartland and Leong, 2013).

Although bacterial interaction within the gastrointestinal human tract is essential to maintain members of the normal microflora, it is also considered a critical phase in all diarrheal infections caused by pathogenic *E. coli* strains (Kalita, Hu, Torres, 2014). Epithelial cell invasion is a virulence mechanism expressed by EIEC strains leading to dysentery-like illness. Invasive properties to cultured epithelial cells have been also reported for EPEC strains (Luck *et al*., 2005). Therefore, understanding the occurrence of pathogenic *E. Coli* and their ability to adhere, invade and persist in the host cell will improve the knowledge of environmental transmission media to humans and its important role in emergence outbreaks creating potential threats and becoming a public health risk (Xiong*et al*., 2015). However, further studies remain necessary.

This present study aimed to evaluate the microbiological quality of the Joana river, located in the Rio de Janeiro metropolitan area, by analyzing *E. coli* bacteria contamination and investigating virulence-related properties and multidrug-resistance profiles by phenotypic and genotypic methods, including host-cell interaction.

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MATERIALS AND METHODS

Study area and sample collection.

Water samples were collected from the Joana River located in front of a University Hospital in the Rio de Janeiro metropolitan area, Southeast Brazil, which receives water from different sources including residences, State universities, rain, and hospital sewage.

Approximately 100 mL of water samples were collected, stored in sterilized bags, and transported to the laboratory for analysis. Samples were inoculated in 100mL of Brain Hearth Infusion broth – BHI (2x), incubated for 24h at 37ºC, and subsequently cultivated onto MacConckey ágar (24h at 37ºC), as previously described (Nogueira *et al*., 2015). *E. coli* strains were identified using Matrix Assisted Laser Desorption Ionization Time-Of-Flight (MALDI-TOF) mass spectrometry. This method analyzes the profiles of bacterial macromolecules that are

obtained from whole bacteria. The procedure provides a unique mass spectral fingerprint of the microorganisms, biopolymer molecules normally present in the condensed phase be converted into intact, isolated ionized molecules in the gas phase. Then, ions are separated according to their molecular weight after migration in an electric field. Each molecule detected is characterized by molecular mass, the charge, the ratio mass/charge, and the relative intensity of the signal (Carbonelle *et al*., 2011).

Antimicrobial susceptibility assays.

Antimicrobial susceptibility testing was done by the disc diffusion method (Bauer *et al*., 1966), and the results were interpreted according to Clinical Laboratory Standards Institute (CLSI) guidelines (CLSI, 2019). The following antimicrobial drugs were tested: cephalothin, cefazolin, cefoxitin, cefuroxime, cefotaxime, ceftriaxone, ceftazidime, cefepime, gentamicin, amikacin, kanamycin, tobramycin, ampicillin, piperacillin/taxobactam, amoxicillin/clavulanic acid, ampicillin/sulbactam, ciprofloxacin, norfloxacin, imipenem, ertapenem, meropenem, aztreonam, chloramphenicol, tetracycline, cotrimoxazole, and colistin. Multidrug resistance was considered when strains were resistant to three or more antimicrobial agents of interest class (beta-lactams, fluoroquinolones, aminoglycoside, and carbapenems) (Magiorakos *et al*., 2012).

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Biofilm formation on hydrophobic polystyrene surface.

Biofilm assays on polystyrene surfaces were performed for all *E. coli* strains. The optical density (OD) of the stained attached bacteria and control wells were read at λ = 570 nm. The cut-off OD (ODc) was defined as the mean OD of the negative control (TSB only). Based on the ODs of the bacterial biofilms, all strains were classified into the following categories: non-adherent (-: OD ≤ ODc), weakly adherent (+: ODc> OD ≤ 2x ODc), moderately adherent (++: 2x ODc> OD ≤ 4x ODc), or strongly adherent (+++:OD>4x ODc). Each assay was performed in triplicate and repeated three times. *S. epidermidis* strain ATCC 35984 was used as a positive controll (Van Belkum *et al*., 2007).

Biofilm formation on a hydrophilic glass surface.

Microorganisms were inoculated in glass tubes (15x100 mm) containing 5 mL of TSB medium and incubated at 37ºC for 48 h. The supernatants containing non-adherent bacterial cells were

discarded. Fresh sterile TSB (5 mL) was added to the test tubes and re-incubated for 48 h. This procedure was repeated twice. Glass-adherent bacteria created a confluent coat of cells on the sides of the tube. Microorganisms were classified as non-adherent (-: absence of adherence), weakly adherent (+: adherent bacteria appeared as a ring at the interface between the medium and the air), moderately adherent (++: bacteria attached on the side of the glass tubes), or strongly adherent (+++: bacteria attached on the side of the glass tubes and at the interface between the medium and the air). *S. epidermidis* strain ATCC 35984 was used as a positive control (Mattos-Guaraldi and Formiga, 1991).

Multiplex-polymerase chain reaction (PCR)assays

Twenty *E. Coli* strains were submitted to a simple boiling method for DNA extraction, suspended in water injection, and maintained at -20ºC (Nogueira *et al*., 2015) to search for EHEC, EPEC, STEC, EIEC, EAEC and ETEC, PCR targeting *eaeA* (917 bp), *stx1* (614 bp), *stx2* (779 bp), *ial* (630 bp), *Lt* (450 bp) and *St* (160 bp) genes was employed. The amplification was performed using a reaction mixture that contained 20 µL containing 1X PCR buffer (10 mM Tris HCl, pH 8,4; 25 mM KCl), 1.5 mM $MgCl_2$, 200 µM dNTPs (Promega, Madison, WI, USA), 0.5 µM of each primer (Integrated DNA Technologies, Coralville, IA, USA), 1 U Taq DNA polymerase (Phoneutria, Belo Horizonte, MG, Brazil), and 2 µL of DNA. In each batch of positive reactions, controls were employed. The amplification conditions used were as follows: an initial denaturation step at 95 ◦C for 5 min was performed, followed by 35 cycles of 94 ◦C for 45 s, annealing at 55 ◦C for 1 min, and elongation at 62 ◦C for 2 min. A final elongation step was executed at 72 ◦C for 5 min. Amplicons were resolved by electrophoresis on SYBR Safe DNA Gel Stain (ThermoFisher Scientific, Vilnius, Lithuania). Gels were evaluated using E-gel Imager, and amplicon sizes were compared with a 100 bp DNA ladder (ThermoFisher Scientific, Vilnius, Lithuania) (Chandra *et al*., 2013).

Bacterial interaction with human colon adenocarcinoma cell line Caco-2 –

Human colon adenocarcinoma cells Cao-2 were used in adherence, invasion, and persistence assays (Pereira *et al*., 2008). Epithelial cells were grown in 96-well cell culture clusters to confluent monolayers (10^7 cells per well) in Minimum Essential Medium Eagle (Sigma-Aldrich) supplemented with 10% bovine fetal serum. Mid-log-phase bacteria were cultured in Trip Soy

Broth – TSB for 24h at 37ºC and reach OD 580 nm of 0.2 were then added to each well with MOI of 10 and 100 bacteria per epithelial cell to test the influence of the amount of inoculated bacteria on the number of internalized bacteria. Internalization assays were allowed to occur for 2 h and 4 h at 37°C in an atmosphere of 94% air–6% CO_2.

To determine the level of bacterial adhesion, 96-well plates containing epithelial cells incubated with mid-log-phase bacteria had been prewashed three times with PBS and lysed with 100 ul PBS-triton (Sigma-Aldrich) to enumerate adherent bacteria added. All strains were shown to be susceptible to ≤ 150 mg/mL of gentamicin in the invasion experiments and were incubated for 1 h to determine the bacterial invasion. After the incubation period, monolayers were washed three times with saline and lysed with 0.1% Triton X-100 to determine the viable counts of released intracellular bacteria. Invasion ability was expressed as the percentage of inoculum that survived 150 mg of gentamicin per ml treatment. Following the invasion period as described above, an assay of resistance was demonstrated after 24 h. The infected cells were incubated at 37°C in 5% CO_2 using 150 mg of gentamicin per ml. The results were recorded as a percentage of the original inoculum. All assays were conducted in triplicate and were repeated independently at least three times (Hirata Jr. *et al*., 2004; Sahly*et al*., 2000).

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RESULTS

In this study, a total of 34 *E. coli* strains were identified by MALDI-TOF mass spectrometry with a≥ 2 score. Analysis of data showed that all strains presented resistance to at least one group of antimicrobial agents tested, and expressed a resistant (R) profile. Interestingly, 20 of 34 (58.8%) *E. coli* river isolates presented resistance to three or more antimicrobial groups of interest and were considered multi-drug resistance (MDR). Multi-drug resistance of *E. coli* river isolates included resistance to 3rd and 4th cephalosporin, aminoglycosides, fluoroquinolones, and carbapenems (**Table 1**).

Table No 1: Resistance profile of *E. coli* strains isolated at Joana river, located in Rio de Janeiro metropolitan area, Brazil

E. coli strains	*Antimicrobial resistance profiles*	
Ec10	MDR	CFZ/CFO/CRX/CTX/CRO/CAZ/CPM/GEN/AMI/KAN/TOB/PPT/AMC /ASB/CIP/NOR/IMI/ETP/ATM/SUT
Ec31	MDR	CFL/CTX/ CRO/CAZ/CPM GEN/KAN/TOB/AMP/AMC/ASB/NOR/SUT/TET
Ec21	MDR	CFL/CFZ/CRX/CTX/CRO/GEN/AMI/AMP/AMC/CIP/NOR/SUT/TET
Ec30	MDR	CFL/CFZ/CFO/CRX/CTX/CRO/CAZ/KAN/TOB/ETP/ATM/TET
Ec27	MDR	CFL/CFZ/CFO/CRX/CTX/CRO/CAZ/CPM/GEN/AMI/KAN/TOB/NOR/ ETP/ATM/SUT
Ec04	MDR	CFL/CFZ/CRX/CTX/CRO/CPM/AMI/KAN/AMP/AMC/ASB/CIP/NOR/ IMI/ETP/TET
Ec06	MDR	CFL/CFZ/CFO/CRX/CTX/CRO/CAZ/CPM/AMI/KAN/TOB/AMP/IMI/ ETP/ATM/SUT
Ec03	MDR	CFL/CFZ/CRX/CTX/CRO/CAZ/CPM/AMI/KAN/AMP/NOR/IMI/ETP/ ATM/CLO
Ec23	MDR	CFL/CFZ/CRX/CTX/CRO/GEN/AMP/PPT/AMC/ASB/CIP/NOR/SUT/T ET
Ec32	MDR	CFL/CFZ/CFO/CRX/CTX/CRO/CAZ/CPM/GEN/KAN/TOB/ETP/ATM
Ec33	MDR	CFL/CFZ/CTX/GEN/AMI/TOB/AMP/AMC/ASB/CIP/NOR/TET
Ec34	MDR	CFL/CFZ/CTX/GEN/AMI/TOB/AMP/AMC/ASB/CIP/NOR/TET
Ec01	MDR	CFL/CFZ/CRX/CTX/AMI/ CIP/NOR/IMI/ETP AMP/ATM/TET
Ec22	MDR	CFZ/CRX/CTX/GEN/KAN/TOB/AMP/CIP/NOR/SUT/TET
Ec16	MDR	CFL/CRX/CTX/GEN/AMI/TOB/AMP/AMC/SUT/TET
Ec15	MDR	CFL/CFZ/CRX/CRO/CPM/CIP/NOR/IMI/TET
Ec24	MDR	CRX/CTX/GEN/AMI/AMP/NOR/SUT/TET
Ec29	MDR	GEN/TOB//ETP/AMP/ASB/NOR/SUT/TET

Ec08	MDR	CFL/CFZ/CRX/CTX/GEN/AMP/NOR/TET
Ec18	MDR	CFL/CFZ/CFO/CRX/CAZ/KAN/TOB/ETP
Ec13	R	CFL/CFZ/CFO/CTX/CRO/CAZ/AMP/PPT/AMC/CIP/TET
Ec05	R	CFL/CFZ/CRX/CTX/CRO/CPM/AMP/IMI/ATM/SUT
Ec20	R	CFL/CFZ/CFO/CTX/GEN/AMI/TOB/AMP/SUT/TET
Ec19	R	CFZ/CTX/GEN/AMI/AMP/ASB/SUT/TET
Ec02	R	CFL/CFZ/CRX/CTX/CRO/AMP/IMI/SUT
Ec17	R	CFL/CFZ/CTX/GEN/AMP/SUT/TET
Ec07	R	CFL/CFZ/CRX/CTX/GEN/AMP
Ec26	R	GEN/AMP/AMC/SUT/TET
Ec11	R	CFL/CFZ/CRX/AMP/SUT
Ec12	R	CFL/CFZ/CRX/CRO/AMP
Ec25	R	CTX/GEN/AMP/SUT/TET
Ec28	R	CTX/GEN/AMP/SUT/TET
Ec09	R	CFL/CFZ/AMP/AMC
Ec14	R	CFL/CFZ/CRX/AMP

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Legend: R- resistant, MDR-multi-drug resistant; CFL-cephalothin, CFZ-cefazolin, CFO-cefoxitin, CRX-cefuroxime, CTX-cefotaxime, CRO-ceftriaxone, CAZ-ceftazidime, CPM-cefepime, GEN-gentamicin, AMI-amikacin, KAN-kanamycin, TOB-tobramycin, AMP-ampicillin, PPT-piperacillin/taxobactam, AMC-amoxicillin/clavulanic acid, ASB-ampicillin/sulbactam, CIP-ciprofloxacin, NOR-norfloxacin, IMI-imipenem, ETP-ertapenem, MER-meropenem, ATM-aztreonam, CLO-chloramphenicol, TET-tetracycline, SUT-cotrimoxazole, COL-colistin

Analysis of the biofilm formation ability of resistant and MDR *E. coli* strains demonstrated that all strains were able to promote biofilm formation on polystyrene surfaces at different levels. Of the 20 MDR *E. coli* strains, 40% (n=8) were considered as strongly adherent on both polyestinere and glass surfaces, followed by 20% (n=7) moderated adherent, 20% (n=4) weakly adherent, and 5% (n=1) non-adherent. Resistant *E. coli* also demonstrated heterogeneity among both polyestinere and glass biofilm formation: 42.8% (n=6) were able to promote biofilm

formation and were considered as weakly adherent, followed by, 35.7% (n=5) as moderated adherent and 7.2% (n=1) as strongly adherent, while 14.3% (n=2) of resistant *E. coli* strains were considered as non-adherent (**Table 2**).

Table No 2: Biofilm formation ability of *E. coli* strains isolated at Joana river, located in Rio de Janeiro metropolitan area, Brazil

***E. COLI* STRAIN**	**BIOFILM FORMATION**	
	Polystyrene Surface	**Glass surface**
EC10	SA	+++
EC31	SA	+++
EC21	SA	+++
EC30	SA	+++
EC27	MA	++
EC04	WA	+
EC06	MA	++
EC03	WA	+
EC23	MA	++
EC32	MA	++
EC33	MA	++
EC34	MA	++
EC01	MA	++
EC22	SA	+++
EC16	WA	+
EC15	SA	+++
EC24	NA	-
EC29	SA	+++
EC08	SA	+++
EC18	WA	+
EC13	MA	++
EC05	WA	+

EC20	MA	++
EC19	SA	+++
EC02	MA	++
EC17	WA	+
EC07	WA	+
EC26	MA	++
EC11	WA	+
EC12	WA	+
EC25	WA	+
EC28	NA	-
EC09	MA	++
EC14	NA	-

Legend: SA/ +++: strongly adherent, MA/++: moderated adherent; +: adherent bacteria appeared as a ring at the interface between the medium and the air.

Further analysis of virulence pathotypes of MDR *E. Coli* strains was displayed in Table 3 and Figure 1. Data showed that 20% (n=4) of MDR *E. coli* river isolates expressed virulence pathotypes: EHEC (Ec31); STEC and EIEC – hybrid (Ec21) and STEC (Ec30 and Ec10) while 80% (n=16) did not present any virulence pathotypes analyzed. Evaluation of the presence of virulence genes demonstrated heterogeneity among the isolates. All four strains presented *stx2* gene followed by *stx1* (n=1), *iaL* (n=1) and *eaeA* (n=1). Multiplex-PCR for *Lt*, *St,* and *aegg* genes presented negative results in all opportunities (**Figure 1**).

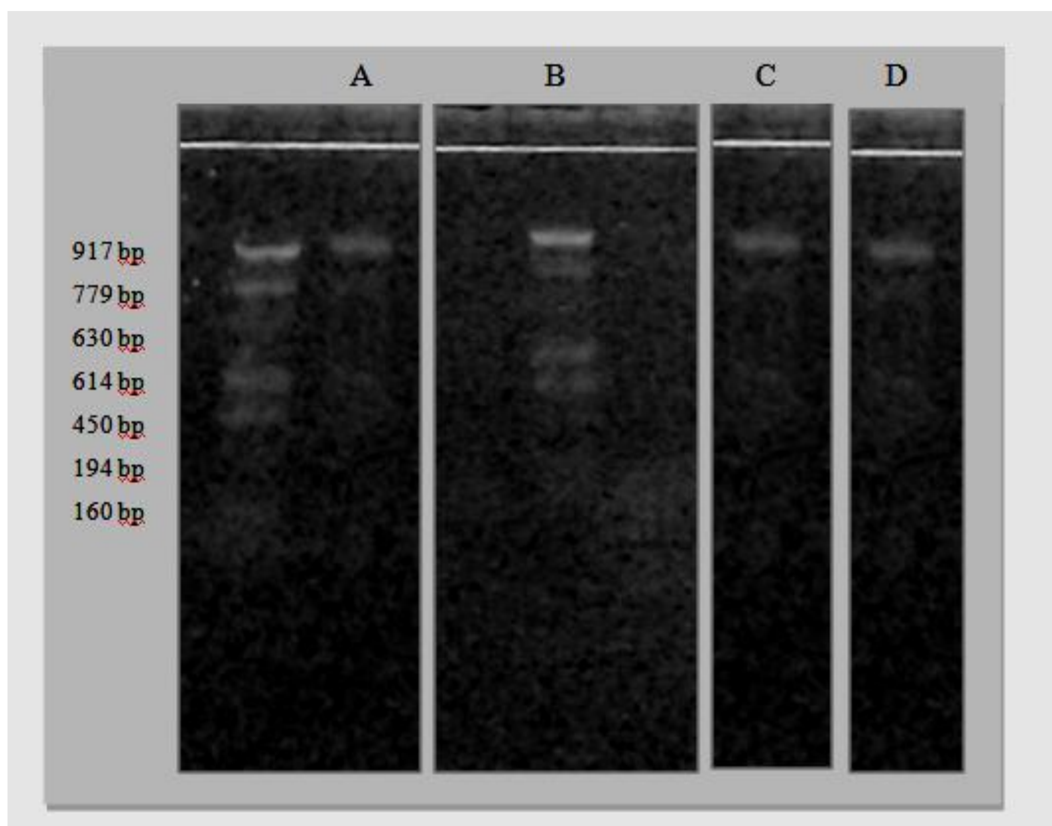

Figure 1: Amplification profile scheme generated by mPCR for multidrug-resistant *Escherichia coli* and determination of virulence genes: *eaeA* (917 bp), *stx1* (614 bp), *stx2* (779 bp), *iaL* (630 bp), *Lt* (450 bp) and *St* (160 bp) corresponding to A- STEC and EIEC (Ec21); B-EHEC (Ec31) and C and D-STEC (Ec30 and Ec10) strains.

The results of the quantitative cell-associated MDR *E. coli* river isolates (n=4) harboring virulence patothypes were shown in Table 2. The highest level of adherence to Caco-2 cells was observed with STEC (Ec10 and Ec30) strains to present the *stx2* gene: 63.1% and 45.9% respectively. EIEC/STEC (Ec21) and EHEC (Ec31) strains expressed lower ability of adherence to human epithelial cell line: 35.4% and 27.8% respectively. Viable internalized bacteria were detected at 1 h post-infection of the monolayers, regardless of the *E. coli* pathotypes but at different levels. The highest percentages of viable intracellular bacteria deduced from Caco-2 cell-associated bacteria were observed for both STEC (Ec10 and Ec30) strains: 3.5% and 0.1% respectively. EIEC/STEC (Ec21) and EHEC (Ec31) strains presented lower percentages of viable intracellular bacteria: 0.007% and 0.01% respectively. Bacterial persistence following a long period of incubation (24h) was displayed in Table 2. All four MDR *E. coli* analyzed strains were

able to survive in the presence of Caco-2 cells at different levels: EIEC/STEC (Ec21) 1.8%; EHEC (Ec31) 3.14%, STEC (Ec30) 18.3% and STEC (Ec10) 145.8%. Interestingly, the STEC (Ec10) isolate not only persisted viable but also was capable to multiply within the Caco-2 cell (**Table 3**).

Table 3: Virulence genes, pathotypes, and cell interaction results of MDR Escherichia coli isolated from river environment located at Rio de Janeiro metropolitan area, Brazil.

Strains	*Virulence genes*	*Pathotypes*	*Caco-2 epithelial cells*			
			Control	Adherence	Internalized bacteria (1h)	Persistence (24h)
EC31	*Stx1, stx2, eaeA*	EHEC	$6.1x10^8$	$1.7x10^8$ (27.8%)	$2.1x10^4$ (0.01%)	$6.6x10^2$ (3.14%)
EC21	*Stx2, iaL*	EIEC; STEC	$4.8x10^8$	$1.7x10^8$ (35.4%)	$1.2x10^4$ (0.007%)	$2.2x 10^2$ (1.8%)
EC30	*Stx2*	STEC	$3.7x10^8$	$1.7x10^8$ (45.9%)	$6x10^4$ (3.5%)	$1.1.x10^3$ (18.3%)
EC10	*Stx2*	STEC	$3.8x10^8$	$2.4x10^8$ (63.1%)	$2.4x10^5$ (0.1%)	$3.5x10^5$ (145.8%)

DISCUSSION

E. Coli strains are highly affected by the propagation of resistance and virulence genes in urban rivers when compared to other *Enterobacteriaceae* (Tortora, Funke, Case, 2008, Kittinger *et al*., 2016). However, there is still a poor understanding of the environmental factors that may alleviate the spread of antibiotic resistance. At present, it is not clear to what extent environmental antibiotic-resistant bacteria (ARB) and antibiotic-resistant genes (ARGs) promote the acquisition and spread of antibiotic resistance among clinically relevant bacteria, or whether ARGs that are acquired by both clinically relevant bacteria and strictly environmental bacteria originate from the same reservoirs (Berendonk, *et al*., 2015).

In this present study, we documented the presence of resistance and MDR*E. Coli* strains are isolated from the Joana river, located in Rio de Janeiro metropolitan area, Brazil. Heterogeneity among virulence pathotypes as well as host-pathogen interaction with Caco-2 cells was verified among MDR river isolates. The presence of antimicrobial-resistant *E. coli* in aquatic systems released from anthropogenic sources such as communities, industries, veterinary, and hospitals, is a public health concern in industrialized and developing countries due to its relevance to the environmental dissemination of antimicrobial resistance (Djordjevic, Stokes, Chowdhury, 2013; Berendonk, *et al*., 2015).

Although generally harmless, *E. coli* strains may express virulence potential properties that account for human localized and invasive infections in both communities and hospital environments (Hall-Stoodley, Costerson, Stoodley, 2004). The plasticity of the *E. coli* genome has hindered the identification of certain *E. coli* isolates as a pathotype, because some isolates combine the main virulence characteristics of different pathotypes and are thus considered potentially more virulent hybrid pathogenic strains. In this study, MDR *E. Coli River* isolates presented the following distinct pathotypes: STEC, EHEC, and EIEC/STEC.

MDR *E. coli* Ec31isolated strain was classified as EHEC due to the ability to produce *stx1* and *stx2*Shiga toxin (Stx) cytotoxin associated with *eaeA* gene. MDR *E. coli* strains Ec21, Ec30 and Ec10 presented *stx2* Shiga toxin (Stx) cytotoxins. The association of Stxcytotoxins, especially *stx2*, with severe diseases has been extensively studied by using endothelial cell lines and their ability to adhere is related to EHEC/STEC pathogenesis (Rivas *et al*., 2016). In addition, biofilm may act as bacterial protection against adverse environmental conditions, especially in aquatic environments. A study conducted by Biscola and co-workers (2011) evaluated the capacity of biofilm formation in EHEC/STEC strains isolated from different reservoirs and demonstrated a strong ability to adhere on both glass and polystyrene surfaces. Cell invasion and survival of EHEC/STEC strains in cultured human intestinal epithelial cells have been previously described (Cordeiro *et al*., 2013) and may be related to biofilm strongly adherence. It should be mentioned that this invasive characteristic has been identified in EHEC/STEC serotypes, responsible for human infections (Mateus-Guimarães *et al*., 2014; Cordeiro *et al*., 2013) and isolated from water representing an important vehicle of transmission (Lascowski *et al*., 2013).

Our studies also demonstrated the presence of MDR enteroinvasive. *coli* Ec21 strain. EIEC is a causative agent of dysentery in humans, especially in developing countries, due to its ability to invade and penetrate cells by endocytosis, as shown in Table 2. Despite the similarities in invasion mechanism and symptoms of the disease, the infectious dose of EIEC appears to be a milder and self-limiting form when compared to *Shigella, which* leads to an exacerbation of proinflammatory response. EIEC was responsible for several outbreaks, but there are few reports on routes of transmission and distribution of this bacterium in nature, including water and cheese (Marier*et al*., 1973; Valentini *et al*., 1992) as well as the direct transmission through person-to-person contact. The isolation of EIEC in Brazil has ranged from 0.5 to 15%, depending on the population investigated (Moreno *et al*., 2010; Lozer *et al*., 2013). Toledo and Trabulsi (1990) investigated the presence of this microorganism in different areas of the city of São Paulo. This bacterium has been found related to children with diarrhea (15.9%). Studies performed outside the city of São Paulo showed a low prevalence of these bacteria, 0.5–2.5% (Oliveira *et al*., 1989).

Few studies have been conducted to investigate pathogenic *E. Coli* strains in urban rivers, although pollution of surface waters with these pathogens has been implicated in an increased number of disease outbreaks and consequent deaths (Masters *et al*., 2011).

In an attempt to investigate the virulence potential of *E. coli* environmental isolates expressing MDR profiles and virulence genes, were investigated for the ability to interact with Caco-2 human intestinal epithelial cells. All MDR *E. Coli* strains of STEC, EHEC, and EIEC/STEC pathotypes were able to adhere to epithelial cell surfaces. MDR *E. coli* (Ec 10 and Ec30) isolates, classified as STEC pathotypes and presenting *stx2* gene, expressed a higher ability of adherence, internalization, and persistence within Caco-2 epithelial cells. Previous reports documented that STEC annually was responsible for 2,801,000 cases of acute illness, 3890 HUS cases, 270 permanent end-stage renal disease, and 270 deaths worldwide and cases of infections have been traced to person-to-person transmission (Duffy, Burgess, Bolton, 2014; Majowicz *et al*., 2014).

Presently, the MDR *E. coli* strain (Ec21)of EIEC pathotypes and harboring gene, showed the ability of adherence, internalization, and persistence within Caco-2 epithelial cells. EIEC infection occurs via fecal-oral route by the ingestion of contaminated food or water and invasion of colonic epithelium, causing abdominal cramps, and bloody and mucous diarrhea. During the

last decades, there are an increasing number of EIEC cases in varied countries, including two large outbreaks in Europe (Thong *et al*., 2005; Bueris *et al*., 2007; Michelacci *et al*., 2020).

Moreover, one MDR *E. coli* (Ec31) river isolate was characterized as EHEC, presenting lower levels of adherence, internalization, and persistence within Caco-2 epithelial cells. The virulence potential of these pathotypes is partially demonstrated by the ability to attach intimately and effacing microvilli of epithelial intestinal cells that can directly induce renal and endothelial lesions due to the expression of *eaeA, Stx1* and*Stx2* genes (Donnenberg, 1993; Maule, 2000; Gomes *et al*., 2016). Survival and persistence of EHEC in contact with surfaces and exposure to water environments among other conditions should be recognized as important risk factors in the spread of this pathogen, including rivers located in metropolitan areas. Data that deserves attention.

In this study, one MDR *E. coli* (Ec31) river isolate was characterized as EHEC, presenting lower levels of adherence, internalization, and persistence within Caco-2 epithelial cells, possibly related to previously described cytotoxicity abilities - whether apoptosis and/or necrosis (Donnenberg, 1993; Maule, 2000; Gomes *et al*., 2016; Abul-Milh *et al*., 2001). Data reinforce the fact that survival and persistence of EHEC in contact with surfaces and exposure to water environments among other conditions should be recognized as important risk factors in the spread of this pathogen, including rivers located in metropolitan areas. Information that deserves attention concerning the virulence potential and risk of contamination by EHEC pathotypes is the ability to cause acute infections with only ten bacterial cells indicating a high virulence level (Maule, 2000).

CONCLUSION

In conclusion, this study demonstrates the presence of diarrheagenic *E. coli* strains in the river water source in Rio de Janeiro metropolitan area, Brazil. However, a subset of these strains demonstrated a high pathogenic potential as they exhibited a multi-drug resistant phenotype and virulence genes. The possibility of contamination leading to human infection and causing gastrointestinal disease due to MDR *E. coli* presenting virulence pathotypes of water-borne pathogens is a matter of concern. The presence of diarrheagenic *E. coli* in river waters warrants the implementation of environmental safety strategies to avoid the dissemination of clones to

people leaving in the area but particularly those more vulnerable communities who utilize these waters for domestic purposes, including Rio de Janeiro.

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