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Molecular characterization of diarrheagenic *Escherichia coli* isolates from children with diarrhea: A cross-sectional study in four provinces of Mozambique
Diarrheagenic *Escherichia coli* in Mozambique

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ABSTRACT

Objectives: Analyze the frequency of diarrheagenic *Escherichia coli* (DEC) pathotypes and their antimicrobial resistance profiles among children aged <15 years with diarrhea in four Mozambican provinces.
Methods: A cross-sectional hospital-based surveillance program of diarrhea was implemented in Maputo, Sofala, Zambézia, and Nampula. A single stool sample was collected from each child from May 2014 to May 2017. Culture methods and biochemical characterization were performed to detect *E. coli* strains. DEC pathotypes were determined by conventional polymerase chain reaction targeting specific virulence genes. Antimicrobial susceptibility was assessed by the Kirby–Bauer method.
Results: From 723 specimens analyzed by culture, 262 were positive for *E. coli*. A total of 208 samples were tested by polymerase chain reaction for DEC identification, of which 101 (48.6%) were positive for a DEC pathotype. The predominant pathotypes were enteroaggregative (66.3%, 67/101), enteropathogenic (15.8%, 16/101), enterotoxigenic (13.9%, 14/101), and enteroinvasive *E. coli* (4.0%, 4/101). No Shiga toxin–producing *E. coli* was identified. Regardless of the province, the most frequent pathotype was enteroaggregative *E. coli*. Isolated DEC presented high frequency of resistance to ampicillin (97.8%), tetracycline (68.3%), chloramphenicol (28.4%), nalidixic acid (19.5%), and gentamicin (14.4%).
Conclusion: Children with diarrhea in Mozambique had DEC and higher resistance to ampicillin and tetracycline.

Introduction

Diarrheal disease is one of the major causes of childhood mortality in resource-limited countries (Vos et al., 2017). In 2019, diarrhea caused 499,950 deaths globally among children under 5 years old (Paulson et al., 2021); the highest number of deaths was in Africa (Abba et al., 2009; Vos et al., 2017).

Escherichia coli is a normal inhabitant of the human gut and part of the intestinal flora. However, certain *E. coli* types can be pathogenic to humans and cause diarrhea (Azevedo Feitosa Ferro et al., 2012; Kotloff et al., 2012), particularly in children (Okeke and Lamikanra, 2000).

Diarrheagenic *E. coli* (DEC) are classified on the basis of their pathogenicity mechanisms, involving different adhesion and

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virulence mechanisms in the interaction with the host cells (Govindarajan et al., 2020; Kaper et al., 2004). Notably, DEC strains cannot be easily distinguished from the normal fecal flora using conventional phenotypic methods such as culture or basic biochemical tests (Rappelli et al., 2005). To date, six main pathotypes of DEC have been described: enteropathogenic (EPEC), enterotoxigenic (ETEC), Shiga toxin–producing *E. coli* (STEC), enteroinvasive (EIEC), enteroaggregative (EAEC), and diffusely adherent *E. coli* (DAEC) (Kaper et al., 2004). These pathotypes are characterized by the presence of different virulence genes: *aggR* and *aaiC* genes, needed for enteroaggregative adhesion to the intestinal mucosa, characterize EAEC strains (Boisen et al., 2012; Nataro et al., 1994); *ipaH* gene, involved in the invasion process of the host intestinal cells, characterizes EIEC strains (Vargas et al., 1998); the gene *elt*, encoding heat-labile toxin, and genes *sth* and *stp*, encoding human and porcine variants of the heat-stable toxins, respectively, characterize ETEC strains (China et al., 1998; Dubreuil, 2008; Vargas et al., 1998); *eae* gene, which encodes the intimin and is involved in attaching and effacing lesion formation, characterizes EPEC strains (Kaper et al., 2004); and *stx1* and *stx2* genes, encoding the two main types of Shiga toxins, characterize STEC strains (Kaper et al., 2004; Paton and Paton, 1998; Schmidt et al., 1999).

In the south of Mozambique at *Centro de Saúde de Xipamanine* (health center located in Maputo city), EAEC, DAEC, and diarrheagenic *E. coli* in general were significantly associated with diarrhea cases in children (Rappelli et al., 2005). However, in another study performed at *Hospital Central de Maputo* (also located in Maputo city), EIEC was reported to be the most prevalent DEC pathotype, whereas EAEC, ETEC, EPEC, and DAEC were all identified in children with acute diarrhea (Sumbana et al., 2015). Limited information is available about the frequency of DEC pathotypes in other provinces (Garrine et al., 2020; Mandomando et al., 2007; Rappelli et al., 2005; Sumbana et al., 2015). This can possibly be attributed to the lack of routine application of molecular techniques needed to distinguish DEC pathotypes in clinical laboratories in Mozambique due to high implementation costs, hindering a proper characterization of *E. coli* virulence profile and therefore identification of DEC pathotypes.

According to diarrhea management guidelines, antibiotics are not recommended for all diarrhea cases, unless in cases of bloody diarrhea, co-infection, severe undernutrition, or specific chronic conditions (Farthing et al., 2013). However, antimicrobial resistance is a growing concern due to overuse in the treatment of diarrhea in countries with limited resources (Lim et al., 2016). Thus, characterization and monitoring of the circulating strains would be important in those settings. Widespread resistance among enteric strains has been reported in rural areas in south Mozambique, where high levels of resistance to ampicillin and trimethoprim-sulfamethoxazole were previously reported (Mandomando et al., 2007). Regular surveillance activities are important for providing useful information for guiding antibiotic therapy. Thus, the aim of our study was to analyze the frequency of DEC pathotypes and their antimicrobial resistance profiles among children with diarrhea aged <15 years in four Mozambican provinces.

Material and methods

Study population

These analyses were based on the National Surveillance of Diarrhea (ViNaDia) data from May 2014 to May 2017 from four provinces of Mozambique. The following hospitals were included: in the south region, *Hospital Central de Maputo, Hospital Geral de Mavalane* and *Hospital Geral José Macamo*; in the central region, *Hospital Central da Beira* and *Hospital Geral de Quelimane*; and in the north region, *Hospital Central de Nampula*. The surveillance began in *Hospital Geral de Mavalane*, and the other facilities were gradually included. All sites are located in urban areas.

The study population were children from 0–14 years, who presented to the sentinel sites with diarrhea disease, defined as three or more loose liquid stools within the last 24 hours (World Health Organization, 2005). Patients with nosocomial diarrhea were not eligible.

Sample collection and transportation and culture procedures

A total of 732 stool samples were collected using fecal swabs and transported in cool boxes to the microbiology laboratory in Cary–Blair medium.

The laboratories performed the standard culture methods after sample reception, and all isolates were sent to the microbiology laboratory at *Instituto Nacional de Saúde* (INS) in Maputo, with the exception of samples collected in the south region, which were directly sent to INS to perform culture method. Isolate confirmation was performed by plating onto MacConkey agar, and isolates were incubated for 24–48 hours at 35 ± 2°C. Confirmation was obtained by a biochemical microbiology method based on positive lactose-glucose and gas production, non-hydrogen sulfide-producing bacteria, negative citrate and urease, and positive indole and motility. After identification, *E. coli* isolates were stored in skim milk, with glycerol at −80°C for the polymerase chain reaction (PCR) test.

Multiplex PCRs for the detection of DEC-specific virulence genes were performed at INS, and all suspected DEC isolates were transported in room-temperature boxes in trypticase soy agar medium to *Istituto Superiore di Sanità* in Italy, Department of Food Safety, for PCR confirmation. Assessment of the antimicrobial susceptibility test was performed at INS.

Detection of DEC virulence genes by PCR

DNA was extracted from all *E. coli* strains isolated by the boiling method and subjected to single and multiplex conventional PCR with specific primers for the identification of specific DEC pathotypes (Table 1). For the STEC pathotype, in addition to primers targeting *stx1* and *stx2*, specific primers targeting *stx2f* subtype were used to ensure the detection of this subtype of *stx2*, which presents sequences different from those of all the other *stx2* subtypes, and thus could not be detected using the *stx2* primer pair. For the EAEC and ETEC pathotypes, the strains showing the presence of at least one specific virulence gene were considered positive for the corresponding pathotype.

Antimicrobial susceptibility test

The antimicrobial susceptibility of the PCR-positive *E. coli* pathotypes was determined by standard Kirby–Bauer disk diffusion method (Weinstein, 2018). The antibiotic-impregnated discs used were ampicillin (AMP, 10 mg), gentamicin (GEN, 10 mg), tetracycline (TET, 30 mg), amikacin (AMI, 30 mg), chloramphenicol (C, 30 mg), ciprofloxacin (CIP, 30 mg), ceftriaxone (CTR, 30 mg), and nalidixic acid (NA, 30mg) according to laboratory's antibiotic disc availability at the moment. *E. coli* ATCC 25922 was used as the reference strain for quality control in the antimicrobial susceptibility test. Results were interpreted according to the Clinical and Laboratory Standards Institute guidelines (Weinstein, 2018).

Data management and statistical analysis

Samples and forms received in the laboratory were inserted in the laboratory logbook in an Excel spreadsheet, which was used to perform descriptive univariate and bivariate analysis.

Table 1
Primers used to test specific pathotypes associated with DEC.

DEC pathotype	Primers	Target genes	Product size (bp)	Reference
EAEC	F: GCAATCAGATTAARCAGCGATACA R: CATTCTTGATTGCATAAGGATCTGG	aggR	426	(Boisen, 2012)
	F: TGGTGACTACTTTGATGGACATTGT R: GACACTCTCTTCTGGGGTAAACGA	aaiC	313	(Boisen, 2012)
EPEC	F: GACCCGGCACAAGCATAAGC R: CCACCTGCAGCAACAAGAGG	eae	384	(Paton, 1998b)
ETEC	F: GCGTTACTATCCTCTCTATG R: ATTGGGGGTTTTATTATTCC	elt	320	(Keuth and Bisping, 1994)
	F: TTAATAGCACCCGGTACAAGCAGG R: CTTGACTCTTCAAAAGAGAAAATTAC	sth	147	(Vargas, 1998)
	F: TTTCTGTATCTTTCCCCTCTT R: AGCACAGGCAGGATTACAACAA	stp	179	(China, 1998)
STEC	F: ATAAATCGCCATTCGTTGACTAC R: AGAACGCCCACTGAGATCATC	stx1	180	(Paton, 1998b)
	F: GGCACTGTCTGAAACTGCTCC R: TCGCCAGTTATCTGACATTCTG	stx2	255	(Paton, 1998b)
	F: AGATTGGGCGTCATTCACTGGTTG R: TACTTTAATGGCCGCCCTGTCTCC	stx2f	500	(Schmidt, 2000)
EIEC	F: TGGAAAAACTCAGTGCCTCT R: CCAGTCCGTAAATTCATTCT	ipaH	423	(Vargas, 1998)

bp, base pair; DEC, diarrheagenic *Escherichia coli*; EAEC, enteroaggregative *E. coli*; EIEC, enteroinvasive *E. coli*; EPEC, enteropathogenic *E. coli*; ETEC, enterotoxigenic *E. coli*; STEC, Shiga toxin–producing *E. coli*.

Table 2
Frequency of DEC pathotypes in the isolates obtained from fecal samples from children with acute diarrhea in four provinces of Mozambique and details of identified virulence genes.

DEC pathotypes	Virulence genes EAEC			EIEC	ETEC				EPEC
Province	*aggR*	*aaiC*	Total (%)	*ipaH* (%)	*elt*	*sth*	*Stp*	Total (%)	*eae* (%)
Maputo(n=126)	34	24	40(31.7%)	3(2.4%)	7	0	0	7(5.6%)	10(7.9%)
Nampula(n= 50)	14	13	18(36.0%)	0	5	0	1	5(10.0%)	3(6.0%)
Sofala(n= 10)	1	2	3(30%)	1(10%)	1	0	0	1(10%)	0
Zambézia(n= 22)	4	3	6(27.3%)	0	1	0	0	1(4.5%)	3(13.6%)
Total(n=208)	53	42	67(32.2%)	4(1.9%)	14	0	1	14(6.7%)	16(7.7%)

The total numbers in the columns for EAEC and ETEC correspond to the total number of strains showing at least one of the virulence genes specific to each pathotype.
DEC, diarrheagenic *Escherichia coli*; EAEC, enteroaggregative *E. coli*; EIEC, enteroinvasive *E. coli*; EPEC, enteropathogenic *E. coli*; ETEC, enterotoxigenic *E. coli*.

Ethics statement

The protocol was approved by the National Bioethics Committee for Health of Mozambique (IRB00002657, reference number: 348/CNBS/13), and each caregiver gave written informed consent to authorize their child's participation.

Results

From 732 samples collected from the sentinel sites in the four provinces of Mozambique, 262 isolates were identified by culture as *E. coli*. Because of insufficient reagents, 208 were tested by convenience sampling using the PCR technique, from which 101 DEC (48.6%) were characterized. The predominant DEC pathotype was EAEC (66.3%, 67/101), followed by EPEC (15.8%, 16/101). No STEC was isolated (Table 2).

There were geographical differences in terms of DEC pathotype distribution, with four different isolated pathotypes, of which 40 EAEC (31.7%), 10 EPEC (7.9%), seven ETEC (5.6%), and three EIEC strains (2.4%) in Maputo city (n = 126). The same pathotypes were isolated in Nampula and Zambézia provinces, with the exception of EIEC. In Sofala Province, all pathotypes were isolated except EPEC (Table 2).

Virulence genes of multiple pathotypes were detected in 8.9% of the samples (9/101), mostly combinations between EAEC and EPEC (5/9), ETEC and EAEC (2/9), ETEC and EPEC (1/9), and EIEC and EAEC (1/9) features.

Isolated DEC showed higher resistance to ampicillin (97.8%). Less than 10% of the strains showed resistance to ciprofloxacin, ceftriaxone, and amikacin (Table 3).

Discussion

DEC were identified in pediatric patients admitted with diarrhea in four provinces of Mozambique, representing an important bacterial agent causing diarrhea in the communities, as already described globally (Croxen et al., 2013; Odetoyin et al., 2016).

The DEC strains have been frequently isolated in low-income countries such as Nigeria (Aworh et al., 2019) and Burkina Faso (Konaté et al., 2017), and their detection varies according to many factors, including size of population studied, microbiological tech-

Table 3
The antibiotic susceptibility profile of DEC strains isolated from children with acute diarrhea attended in four provinces of Mozambique.

	Maputo		Nampula		Sofala		Zambézia		Total	
	R	S	R	S	R	S	R	S	R	S
Ciprofloxacin	1 (1.9%)	53 (98.1%)	1 (4.2%)	23 (95.8%)	2 (40.0%)	3 (60.0%)	0 (0.0%)	8 (100%)	4 (4.4%)	87 (95.6%)
Ampicillin	52 (96.3%)	2 (3.7%)	24 (100.0%)	0 (0.0%)	4 (100.0%)	0 (0.0%)	8 (100%)	0 (0.0%)	88 (97.8%)	2 (2.2%)
Tetracycline	31 (62.0%)	19 (38.0%)	16 (80.0%)	4 (20.0%)	3 (75.0%)	1 (25.0%)	6 (75.0%)	2 (25.0%)	56 (68.3%)	26 (31.7%)
Gentamicin	7 (13.2%)	46 (86.8%)	4 (16.7%)	20 (83.3%)	1 (20.0%)	4 (80.0%)	1 (12.5%)	7 (87.5%)	13 (14.4%)	77 (85.6%)
Ceftriaxone	4 (7.4%)	50 (92.6%)	2 (8.7%)	21 (91.3%)	0 (0.0%)	5 (100%)	1 (12.5)	7 (87.5%)	7 (7.8%)	83 (92.2%)
Amikacin	0 (0.0%)	53 (100.0%)	2 (8.3%)	22 (91.7%)	2 (40.0%)	3 (60.0%)	0 (0.0%)	8 (100%)	4 (4.4%)	86 (95.6%)
Chloramphenicol	11 (21.2%)	41 (78.8%)	7 (30.4%)	16 (69.6%)	2 (40.0%)	3 (60.0%)	5 (62.5%)	3 (37.5%)	25 (28.4%)	63 (71.6%)
Nalidixic acid	7 (13.5%)	45 (86.5%)	5 (21.7%)	18 (78.3%)	2 (40.0%)	3 (60.0%)	3 (42.9%)	4 (57.1%)	17 (19.5%)	70 (80.5%)

DEC, diarrheagenic *Escherichia coli*; R, resistant; S, sensitive.

niques employed, and the geographical differences (Rappelli et al., 2005; Santona et al., 2013).

The most frequent pathotype regardless of the province was EAEC. Previous reports in Mozambique indicated EAEC as the second and third most common pathotype in *Centro de Saúde de Xipamanine* and *Hospital Central de Maputo*, respectively. Both health facilities are located in urban areas of Maputo city (Rappelli et al., 2005; Sumbana et al., 2015). However, as in our findings, EAEC was the most common pathotype in *Manhiça District Hospital*, located in a rural area approximately 80 km north of Maputo city (Mandomando et al., 2007). Sampling periods and geographical differences may explain the observed differences, which highlight the importance of surveillance systems for detecting pathotype variability. Our data show relatively low frequency of EIEC, ETEC, and EPEC, although these pathotypes are frequently reported as etiological agents of diarrhea among children (Santona et al., 2013). The STEC pathotype was not identified, supporting findings from previous studies in Mozambique (Rappelli et al., 2005; Sumbana et al., 2015). A low frequency of STEC and EIEC was previously reported in a study conducted in children in rural areas of Mozambique (Garrine et al., 2020). This is consistent with previous reports indicating that in Africa, the STEC pathotype is commonly found more often in adults than in children, being usually a coincidental finding (Kotloff et al., 2012; Smith et al., 2011).

The frequency of the different DEC pathotypes varied between the different provinces, in which the study was conducted, and only Maputo showed the presence of all the detected pathotypes, namely EAEC, EIEC, ETEC, and EPEC. This may be attributable to Maputo city samples having been collected in three different hospitals, resulting in a higher number of samples tested and of isolated *E. coli* strains compared with the other provinces, in which samples were collected only from one hospital per province.

Our results confirm the co-detection of multiple DEC pathotypes in stool samples, as mentioned in other studies among children (Mandomando et al., 2007; Sumbana et al., 2015). Our analyses showed frequent combination between EAEC and EPEC. Each of the pathotypes has been described as the leading cause of diarrhea in children from developing countries in Africa (Boisen et al., 2012; Ochoa and Contreras, 2011).

As for the antimicrobial susceptibility profile, our study showed high resistance to ampicillin and tetracycline, as reported in the Manhiça District (Mandomando et al., 2007). This could be due to the frequent use of ampicillin in combination with gentamicin as the first-choice antibiotic treatment prescribed at admission to the public hospitals in Mozambique to treat non-pneumococcal pneumonia in children (Mandomando et al., 2007).

This study was limited by the different numbers of subjects in each province, dependence on enrollment at the surveillance site, and limited available resources, causing lack of testing of some *E. coli* strains for DEC pathotype virulence genes and limited numbers of antimicrobials tested for susceptibility profiles. Nevertheless, a comprehensive analysis of circulating DEC pathotypes provides novel insights (on the high frequency of EAEC in particular) and hints on their antimicrobial resistance and represents an important step in increasing the knowledge on DEC circulation in different areas of Mozambique.

Conclusion

DEC were identified in children with diarrhea in Mozambique, with variable frequency of the pathotypes in the different provinces. The antibiotic resistance profile of DEC showed high frequency of resistance to ampicillin and tetracycline and was similar in all provinces. These data emphasize the importance of monitoring DEC frequency and testing the antimicrobial resistance profile, which could prompt a revision of the current practice of antimicrobial use in Mozambique. Knowledge of antimicrobial resistance of DEC is important for selecting the appropriate therapy in serious diarrheagenic infections and formulating local guidelines on the use of antimicrobials.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Lena Manhique-Coutinho: Conceptualization, Methodology, Formal analysis, Investigation, Resources, Writing – original draft, Writing – review & editing, Visualization. **Paola Chiani:** Conceptualization, Methodology, Investigation, Resources, Writing – original draft, Writing – review & editing, Visualization. **Valeria Michelacci:** Methodology, Resources, Writing – review & editing, Visualization. **Elisa Taviani:** Methodology, Resources, Writing – review & editing, Supervision. **Adilson Fernando Loforte Bauhofer:** Formal analysis, Resources, Writing – review & editing, Visualization. **Assucênio Chissaque:** Formal analysis, Resources, Writing – review & editing, Visualization. **Idalécia Cossa-Moiane:** Resources, Writing – review & editing, Visualization. **Júlia Sambo:** Resources, Writing – review & editing, Visualization. **Jorfélia Chilaúle:** Resources, Writing – review & editing, Visualization, Project administration. **Esperança Lourenço Guimarães:** Resources, Writing – review & editing, Visualization. **Judite Salência:** Resources, Writing – review & editing, Visualization. **Marta Cassocera:** Resources, Writing – review & editing, Visualization. **Diocreciano Matias Bero:** Resources, Writing – review & editing, Visualization. **José Paulo Langa:** Conceptualization, Resources, Writing – review & editing, Visualization, Supervision. **Nilsa de Deus:** Conceptualization, Resources, Writing – review & editing, Visualization, Supervision, Funding acquisition.

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Ethical approval

The protocol was approved by the National Bioethics Committee for Health of Mozambique (IRB00002657, reference number: 348/CNBS/13), and each caregiver gave written informed consent to authorize their child's participation.

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