molecular detection of diarrheagenic *escherichia coli* pathotypes isolated from children with diarrhea. A cross-sectional study in Mbarara city, South Western Uganda.

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Abstract
Background
Diarrheal diseases remain a global public health concern affecting children, with a high prevalence in resource-limited settings. In many poor nations, diarrheal diseases have been listed as 1 of the top 10 causes of mortality.
Objective
This study aimed to determine the prevalence and antibiotic susceptibility profiles of diarrheagenic *Escherichia coli* (*E. coli*) pathotypes with diarrhea using Polymerase Chain Reaction in Mbarara City.
Methodology
It was a cross-section hospital-based study where 391 stool samples were collected from children aged six months and 12 years presenting with diarrhea and not taking any antibiotic treatment for diarrhea at the time of the investigation.
Results
Out of 391 stool samples collected, 78 were positive for *E. coli* giving an overall prevalence of **19.95 %.** Of the 78 (19.95 %) positive samples, males were 18 (54.55%) and females were 25 (55.56%). Among the 78 *E. coli* isolates still, 43 (55.13 %) were pathogenic and belonged to the three common pathotypes including Enteropathogenic E. *coli* (EPEC), which was the most prevalent pathotype (**86.05** %), followed by Enterohaemorrhagic *E.coli* (EHEP) (9.30 %), while enter-invasive was the least (4.65**%).** The *E. coli* isolates were most sensitive to chloramphenicol, followed by imipenem (70%), tetracycline (30%), ceftriaxone (28%) and amoxicillin (26%) was the most resistant
Conclusion
Diarrheagenic *E. coli* (DEC) is prevalent in Mbarara City and is an important agent that should be considered in routine studies and surveillance for childhood acute diarrheal disease.
Recommendations
Strengthening molecular diagnostic capacity in health laboratories should be supported to adopt molecular diagnostic techniques (e.g., PCR) for routine detection of diarrheagenic *E. coli*, since conventional culture methods may not differentiate pathogenic E.coli pathotypes.

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Introduction

Diarrheal diseases remain a global public health concern affecting children, with a high prevalence in resource-limited settings (Omona *et al.*, 2020). Diarrhea is characterized by stools of decreased consistency and increased volume due to imbalance of secretion and absorption of water and salts in the intestine (Saka *et al.,* 2019). It is a major source of malnutrition in Low-Middle Income Countries (LMICs) (Saka *et al.,* 2019). Diarrhea have been listed as 1 of the top 10 causes of mortality and disability-adjusted life-years for persons in all age groups, and 1 of the 5 leading causes of mortality and disability-adjusted life-years among children aged <5 years (Troeger *et al.*, 2018; Fenta, Alemu and Angaw, 2020).

Globally, an estimated 1.6 million child mortality is recorded yearly due to chronic diarrhea, accounting for 1:5 child deaths (Manetu *et al*., 2021). Although global diarrhea mortality has decreased significantly over the last 25 years, diarrhea morbidity has not because of risk factors, including poor water supply, sanitation, and hygiene (WASH) in Sub-Saharan Africa (Thiam *et al.*, 2017). Diarrhea represents a major cause of childhood mortality across Africa, With Substantial geographic variation and particularly high burden in East and Uganda. Geographic analysis reveals Eastern Africa has the highest disease incidence (114,389 cases per 100,000 children <5), while Western Africa shows highest mortality rates (Thystrup *et al.,* 2024). In Uganda specifically, diarrhea accounts for 22% of deaths in children under 5 (Omona *et al.,* 2019) with overall child mortality reaching 2 in 10 children (Tumusiime *et al.,* 2024).

Rotavirus, *Cryptosporidium*, *Escherichia coli (E. coli),* enterotoxigenic *E. coli* (*ETEC*), and *Shigella* are utmost the main etiological agents of diarrhea (Mokomane *et al.*, 2018). Other causes include various genera of bacterial pathogens such as *Salmonella*, *Shigella*, *Yersinia*, *Vibrio*, *Bacillus*, *Enterobacter*, *Plesiomonas*, *Klebsiella*, *Proteus*, *Serratia*, *Aeromonas* (Onohuean and Igere, 2022). Diarrheagenic *E. coli* is classified into six major pathotypes (pathogenic variants), including enteroaggregative *E. coli* (EAEC), enteropathogenic *Escherichia coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enter-invasive *Escherichia coli* (EIEC), enterohaemorrhagic *E. coli* (EHEC also known as Shiga-toxin producing *E. coli*), and diffusely adherent *Escherichia coli* (DAEC) (Peirano *et al.*, 2018). EAEC and EPEC are the foremost causes of acute diarrhea outbreaks or leading potentially fatal infant and children in underdeveloped nations, while ETEC is the prominent cause of travelers' diarrhea.

A large number of verotoxigenic *E. coli* serotypes are associated with human intestinal infections, and some of these serotypes are recognized as important foodborne pathogens that may cause mild to severe bloody diarrhea and hemolytic uremic syndrome (citation). Cattle and their environment are among the most important sources of pathogenic *E. coli*, and they may be the origin of contamination of meat and meat products (Elder *et al*., 2000; Midgley and Desmarchelier, 2001). In Uganda, *E. coli* is frequently linked to acute infantile diarrhea.

In western Uganda, *E. coli* is commonly isolated from children who have diarrhea; however, the genetic background is not regularly assessed, thus it is unclear what percentage of diarrhea is caused by DEC. As a result, there is a dearth of research on the significance of DEC and particular DEC pathotypes as diarrheal disease causes in Mbarara Municipality and southwest Uganda. In order to provide baseline information on the circulating DEC pathotypes in the study locality and their clinical significance, this study examined the prevalence and frequency of DEC as a cause of infectious diarrhea in children under the age of 12 in Mbarara City, as well as their pattern of antibiotic susceptibility.

Methodology

Study Design

This study was a cross-sectional hospital-based study. Sample collection was carried out at health centers of Mbarara City including Mbarara Municipal Council Health Centre IV and Holy Innocents Children's hospital. The study involved children of 6 months to 12 years old with diarrhea during the study time.

Sample Collection

Out of the 391 children aged 6 months to 12 years old with diarrhea at the study centers. Diarrhea was well-defined, according to World Health Organization guidelines as the occurrence of 3 or more, loose, liquid or watery stools within 24 hours, as this was done on triage of the children at different Health Centre's (O'Reilly, *et al.,* 2018). The

study included only those children who had no antibiotics exposure for the last two weeks. There is variability in the validity of antibiotics to remain in the body, some antibiotics have shorter half-life when compared to others (Kong *et al.,* 2019). The study excluded those who never had diarrhea but visited the health units at the time of the study was done by (Zelelie *et al.,* 2023).

Sample handling and transportation.

Sterile stool sample containers were given to Children's parents / guardians and were instructed on stool sample collection into a dry, clean stool container and for children below 3 years a sterile rectal swab was used to collect the specimen to avoid contamination of the sample with urine. To ensure optimum recovery of *E. coli* all specimens obtained were transported immediately to the Mbarara University Microbiology Laboratory. The samples were handled by the researcher and a research assistant following standard Operating Procedures on sample handling. The bacterial isolation and identification, DNA extraction and antimicrobial susceptibility test was carried out.

Bacterial Isolation and Identification stool samples

The stool samples collected were inoculated on MacConkey medium and Levin eosin methylene blue media. All stool samples were processed in less than 6 hours from the time of specimen collection. The Plates were incubated for 18 – 24 hours at 37 ° C in an aerobic environment. The isolates of *E. coli* were identified by Gram-stain for morphology; the biochemical tests (indole, Simmons citrate, urea, triple sugar iron agar) was carried out to identify *E. coli*.

DNA extraction and pathotype identification.

DNA was extracted at DC Molecular Laboratories in Mbarara city from the isolates using the boiling method by boiling at 100 °C for 10 minutes in a preheated heating block as described by (Onohuean and Igere, 2022); and following the established standard operating procedure for DNA extraction. The different DEC pathotypes were speciated by Polymerase Chain reaction (PCR) techniques using the primers as shown in Table 1. The Fresh overnight cultures were placed in a sterile 1.5 ml Eppendorf tube and centrifuged for 10 minutes at 13,000 rpm. The cell pellets were then rinsed twice with phosphate-buffered saline pH

7.4 and suspended in 500 ml sterile and distilled water before lysed for DNA release. After centrifuging the suspension for 5 minutes at 15,000 rpm, the supernatant was cautiously pipetted into sterile cryogenic tubes and kept at 20 °C for use as probable genomic DNA *E. coli spp*. for PCR tests.

Antimicrobial Susceptibility test

For all DEC pathotypes were assessed using the disc diffusion method (kirby-bauer method) according to clinical and laboratory standard institute guidelines (CLSI). Antibiotics for the study were selected based CLSI guidelines for enterobacterales. The bacterias tested were chloramphenicol (30NgC30), Ceftriaxone(30NgCR30), Tetracycline (30NgTE30), Imipenum (10mcgIPM10), Ciprofloxacin (30NgCIP30), Amoxicillin (20NgAMC30). The results were interpreted using CLSI guidelines. *E. coli* ATCC (American type culture collection) 25922 was used as quality control strained for Antimicrobial Susceptibility strain testing.

Pathotype identification.

About 2.5 ml of crude template DNA in a 25-L reaction volume, 10 mM Tris-HCl, 2 mM $MgCl_2$, 1.5 U Taq polymerase (HybriPol Bioline, UK), 0.2 mM dNTPs, 0.2 mM primers (SBS Genetech Co, Ltd), and a Gene Amp 2700 thermocycler were used for the amplifications (Periano et al 2018). 30 cycles of 1 minute at 94 °C, 1 minute at different annealing temperatures, and 1 minute at 72 °C were conducted under similar conditions for all reactions, which included a 5-minute denaturation at 94 °C. A 10 minutes at 72 °C was the final extension period. To see the PCR bands, the resulting PCR products were subsequently electrophoresed with 0.5X T ethidium bromide stain on 2 percent agarose gels (Periano et al., 2018). The first PCR screens employed stx1/stx2 and eae primers to see if STEC or EPEC DEC were present. To distinguish between tEPEC and aEPEC, DNA with positive eae and negative stx1/stx2 PCR was examined using bfp primers. Negative eae and stx1/stx2 extracts were tested using pCVD432 primers for plasmidic EAEC sequences, ipaH primers for detecting genes coding the invasion plasmid antigen of EIEC (and Shigella), and PCR assays for ETEC labile and stable enterotoxins (Periano et al 2018). Table 1 shows the summary of the primers and the Sequence employed.

Table 1. Genes for PCR amplification, Primers and Their characteristics.

GENE	PRIMER	Sequence 5^1-3^1	Amplicon size (bp).	Annealing temp (°C).
eae	EAE 1 EAE 2	GAGAATGAAATAGAAGTCGT GCGGTATCTTTCGCGTAATCGCC	775	55
bfp	EP1 EP2	AATGGTGCTTGCGCTTGCTGC GCCGCTTTATCCAACCTGGTA	324	55
Stx1	VT1-A VT2-B	GAAGAGTCCGTGGATTACG AGCGATGCAGCTATTAATAA	131	55
Stx2	VT2-a VT2-b	TTAACCACACCCCACCGGGCAGT GCTCTGGATGCATCTCTGGT	348	55
pCDV	EAEC1 EAEC2	CTGGCGAAAGACTGTATCAT CAATGTATAGAAATCCGCTGTT	630	60
ipaH	EI1 EI2	GTTCCTTGACCGCCTTTCCGATACCGTC GCCGGTCAGCCCTCTGAGAGTAC	620	55
eltA	LT-A-1 LT-A-2	GGCGACAGATTATACCGTGC CCGAATTCTGTTATATATGTC	332	55
estA	STA-1 STA-2	ATTTTTATTTCTGTATTGTCTTT GGATTACAACACAGTTCACAGCAG	147	55

Data Analysis

The complete data was then verified for completeness, uniformity and accuracy to remove outliers. The data was coded and exported to MS Excel spreadsheets and STATA version 2.0). The analysis was done based on the study objectives and results were presented in tables and charts using frequency, proportion, percentages and bar charts. CLSI guidelines was used to classify the isolates based on the susceptible patterns (S, I, R) to determine variations in susceptibility of the isolates of E.coli from different patients.

Ethical Approval

An institutional review Board (IRB) approval (MUST-2023-799) was obtained from the Research Ethics Committee (REC) of Mbarara University Science and Technology. In line with Helsinki declaration all human participants were treated with utmost confidentiality. Informed consent was obtained from the parent or guardian of each participant prior to participation in the study and sample collection

Results

A total of 391 stool samples were collected from children aged 6 months to 12 years of presenting with diarrhea. About 78 tested positive for *E. coli* with an overall prevalence of 19.95 % (CI=95%, 16.27-24.22). Of the 78 (19.95 %) positives, the highest prevalence of *E. coli* by sex was found in females representing 55.56%. The overall proportion of Pathogenic E. *coli* isolated based on pathotypes was 55.13% (43.84-65.91, 95% CI) as shown in Table 1.

Table 2: Prevalence of *E. coli* based on sex and status.

E. *coli* Status		Frequency (n)	Percentage
Negative		313	80.05 (75.78-83.73)*
Positive		78	19.95 (16.20 -24.22)*
Sex	Pathogenic	Non-pathogenic	Total
Male	18 (54.55%)	15 (45.45%)	33 (100%)
Female	25 (55.56%)	20 (44.44%)	45 (100%)
Total	**43 (55.13%)**	**35 (44.87%)**	**78 (100%)**

* (95% Confidence Interval)

The Proportion of different pathogenic E. *coli* Pathotypes was identified. About 43 (55.13%) pathogenic E. *coli* belonged to the three common pathotypes as shown Table figure 2. The different pathotypes isolated were Enteropathogenic E. *coli* (EPEC) as the most prevalent pathotype representing 86.05 %, followed by Entero-haemorrhagic E. *coli* (EHEP) with 9.30 %.
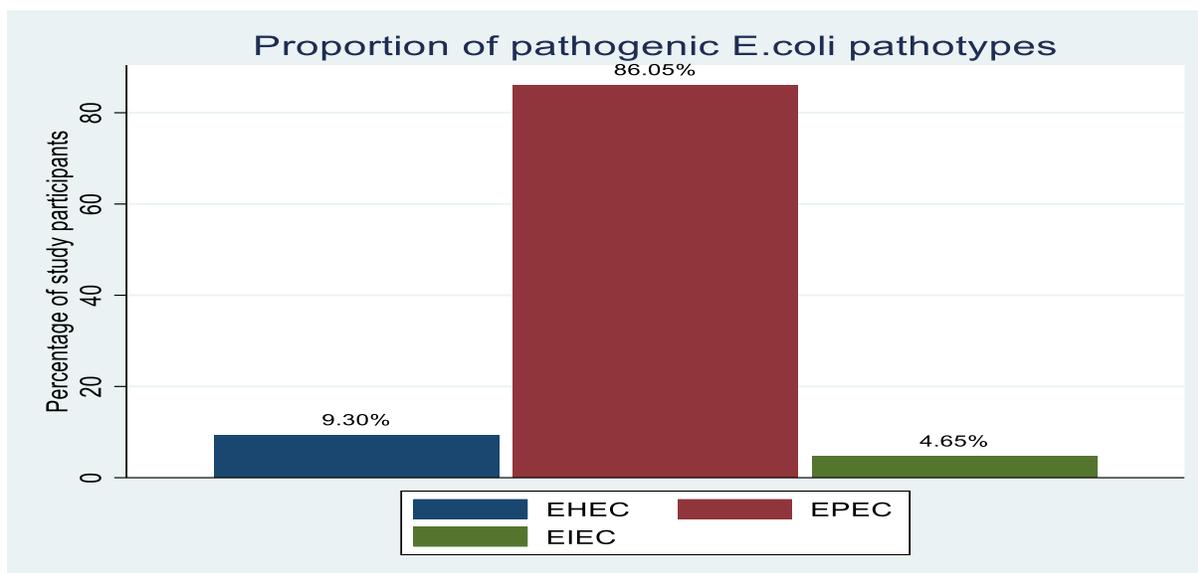

Figure 1: Proportion of different pathogenic E. *coli* Pathotypes identified

The Antimicrobial Profile on DEC Pathotypes as shown in Table 3. Drug susceptibility patterns were done on the pathogenic *E. coli* using the Kirby-Bauer disc diffusion method as described by the Clinical Laboratory Standards Institute (CLSI, 2022) to determine sensitivity, intermediate and resistance to different drugs that are commonly used to treat E.*coli* infections. The drug sensitivity indicate that the most sensitive drug was Imipenum with 79% (EPEC; 29/43,), followed by Chloramphenicol representing (EPEC; 27/43) The most resistant drug was amoxicillin with 70% (EPEC;25/43), followed by ciprofloxacin with 60% (EPEC; 25/43) as shown in Table 3.

Table 3: Antimicrobial Profile on DEC Pathotypes.

Drug	Category	Total n=43	EHEC n=4	EPEC n=37	EIEC n=2
Chlorophenical30NgC-30	Sensitive	33 (72%)	2 (50%)	27 (73%)	2 (100%)
	Resistant	12 (28%)	2 (50%)	10 (27 %)	0 (0%)
Ceftriaxone30Ng	Sensitive	23 (53%)	1 (25%)	19 (51%)	2 (100%)
CR-30	Resistant	2 0 (47%)	2 (50%)	18 (49%)	0 (0 %)
Tetracycline30Ng	Sensitive	1 8 (42%)	1(25%)	15 (41%)	2 (100%)
TE-30	Resistant	25 (58%)	3(75%)	22 (59%)	0 (0%)
Imepenum10mcg	Sensitive	34 (79%)	3 (75%)	29 (78%)	2 (100%)
IPM-10	Resistant	9 (21%)	1(25%)	8 (22%)	0 (0%)
Ciprofloxacin30Ng	Sensitive	17 (40%)	3(75%)	12 (32%)	2 (100%)
CIP-30	Resistant	26 (60%)	1(25%)	25 (68%)	0 (0%)
Amoxacillin20Ng	Sensitive	13 (30%)	1(25%)	12 (32%)	0 (0%)
AMC-30	Resistant	30 (70%)	3 (75%)	25 (68%)	2 (100%)

1 is the ladder

2,3,4 are the samples

5 is the negative control

2,3 are the samples

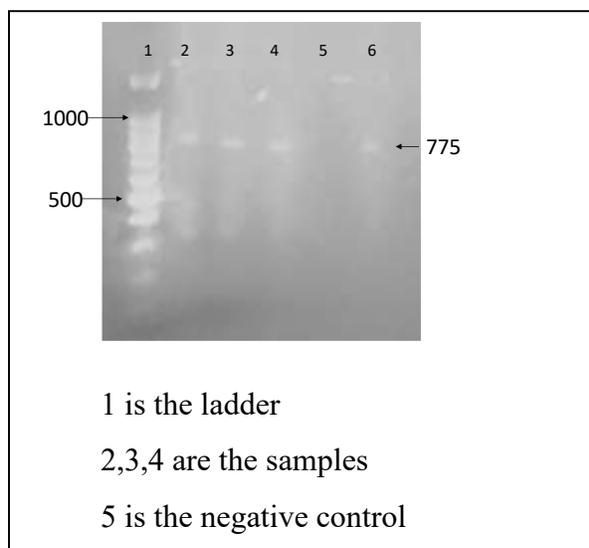4 is the negative control

5 is the positive control

Figure 2: Agarose gel electrophoresis of PCR products showing amplification of a 775 bp of EAE. Lane 1 represents the DNA ladder (1000bp). Lanes 2, 3, and 4 correspond to the tested samples, all showing bands at approximately 775 bp, indicating successful amplification of EAE. Lane 5 is the negative control, showing no detectable band. Lane 6 is the positive control, validating the PCR reaction.

Figure 3: PCR amplification showing a 630 bp of EAEC product in samples (lanes 2–3) and positive control (lane 5); no band observed in the negative control (lane 4).
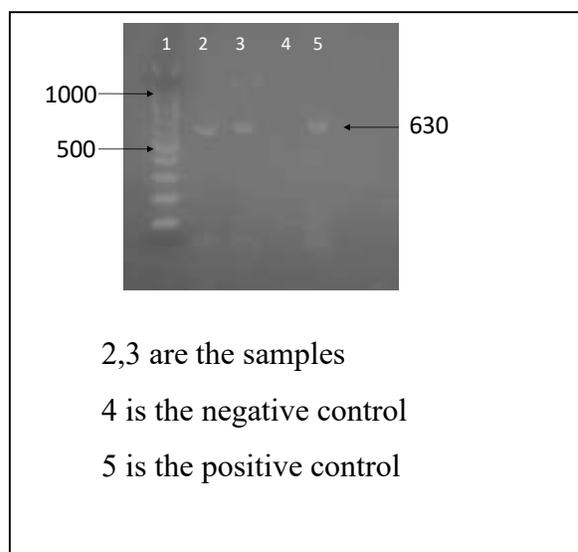
Discussion

Diarrheagenic E. coli infections are indistinguishable from gastroenteritis due to other bacterial or viral infection, and therefore isolation and identification of the specific DEC associated with a clinical case could allow caregivers to provide appropriate treatment (Saka *et al*.,2019). This study examined the prevalence and frequency of DEC as a cause of infectious diarrhea in children under the age of 12 in Mbarara City, as well as their pattern of antibiotic susceptibility.

Of the 391 stool samples collected from children with diarrhea aged 6 months to 12 years at two health facilities in Mbarara, 78 tested positive for E. *coli* given an overall prevalence of **19.95 %** with a 95% confidence interval of 16.27% to 24.22%. This is a low prevalence compared to the studies done Central Ethiopia (Zelelie *et al*.,2023) and Northwest Ethiopia, where 194 samples consisting of 144 stool samples from children and 50 fecal samples from calves were studied, presumptive E. *coli* isolates were identified from 74 (38.3%) of the samples (Belete, Demlie *et al.* 2022) and in Mozambique (35.7%) by (Manhique-Coutinho, Chiani *et al.* 2022) and in Branzil (Robins-browne *et al*., 2016), The variability in the prevalence could

be due to climate variability, study population, water supply that will influence DEC incidence (Zelelie *et al*., 2023).

Of the 391 samples, **187** (47.83%) were males and **204** (52.17%) were females. Of the 78 (19.95 %) E. *coli* positives, prevalence of E. *coli* by gender, males were 18 (54.55%) and females were 25 (55.56%). The *P*- value for chi - square test was above 0.05 therefore, there was no significant difference in the prevalence of E.*coli* by gender i.e. the prevalence of E. *coli* in males did not significantly differ from the prevalence of E.*coli* in females, this is in line with the study done in North East Ethiopia (Belete, Demlie *et al*. 2022).

Though *E. coli* is a microbe that can be found in both humans, animals and animal gut, normally, it is not harmful. However, some *E. coli* variants can acquire virulence genes and cause gastroenteritis, which can be deadly if left untreated. These strains are classified as diarrheagenic *E. coli* (DEC), and of six subtypes (Rodrigues *et a*l., 2023, Joffré, & Iñiguez, 2020).In this study, the 78 (19.95 %) E.*coli* positives isolates were subjected to multiplex PCR to identify the DEC.

Of the 78 *E.coli* isolates, 43 were pathogenic giving prevalence percentage of pathogenic

E.coli as 55.13% with a 95 % CI of 43.84% to 65.91%. This was higher compared to the studies done in Rakai hospital, Southern Uganda (38.2%) by (Masiga, Kigozi et al. 2022) higher than that done in informal settlements in Nairobi Kenya where the prevalence was 27%, and in Mozambique (48.6%) by (Manhique-Coutinho, Chiani *et al.* 2022) but lower compared to studies done in Amatole District Municipality of Eastern Cape, South Africa (Omolajaiye, Afolabi *et al.* 2020), this could be due to distribution of DEC pathotypes which varies geographically due to seasonal variations, animal interactions and poor sanitation and hygiene.

The 43 (55.13%) pathogenic E.*coli* belonged to the three common pathotypes i.e.

Enteropathogenic E. *coli* (EPEC) was the most prevalent pathotype with 86.05 %, followed by Enterohaemorrhagic *E.coli* (EHEP) with 9.30 % and enter-invasive E.*coli* (EIEC) was the least with a prevalence percentage of 4.65%. The other pathotypes like Enterotoxigenic

E. coli (ETEC), Enteroaggregative E. *coli* (EAEC), Adherent-Invasive *E. coli* (AIEC) and diffusely adhesion E. *coli* (DAEC) were not found. EPEC being the most prevalent is in agreement with other studies done in developing countries where it's classified as the leading cause of diarrhea and death in children, particularly in developing nations (Robins-browne *et al*., 2016). Also in agreement with results reported in West Africa where higher recovery rates for EPEC and EHEP have been found significantly associated with childhood diarrhea (Okeke , *et al, 2000,* 2009,). Though majority of the previous studies carried out also implicate enteroaggregative *E. coli* (EAEC) as the most prevalent like in Nigeria (Saka, Dabo *et al*. 2019) , and in Eastern Cape town in South Africa (Omolajaiye, Afolabi *et al*. 2020).

Antibiotic resistance is quickly becoming one of the most concerning public health issues today (Mobarki, Almerabi and Hattan, 2019), The rise of multi-drug resistance is due to the over-prescription, antimicrobial misuse in medicine, and their rampant use for agricultural purposes (Ayukekbong, Ntemgwa and Atabe, 2017). In this study, the highly sensitive drug Imepenum with 79%, followed by Chlorophenical with 72%, This is not in agreement with a study done in Bahir Dar city, Northwest Ethiopia (where antibiotics results indicated that norfloxacin was found the most effective drug with 85.1% sensitivity (norfloxacin is quinolone like ciprofloxacin which was the least sensitive) followed by chloramphenicol 83.8% which is in line with this study where chloramphenicol was the most sensitive (100%) (Belete, Demlie *et al.* 2022).

This study has several limitations; first was inability to determine prior use of antibiotics using laboratory techniques and only relied on information given by the parents or guardians. Secondly, the study did not consider the socio-economic variables of the participants. DEC is prevalent in children with diarrhea in Mbarara City western Uganda and its identification in children should be considered among strategies for combatting childhood diarrhea in Uganda.

Conclusion

The findings indicate that the prevalence of pathogenic *E.coli* is 55.13% with diarrheagenic *E. coli* (DEC), particularly *EPEC*, could contribute to childhood acute diarrheal disease.

Further Research

These observations highlight the need for further research to clarify the epidemiologic significance of DEC pathotypes in Uganda

Generalizability of the Study Findings:

The findings are most generalizable to children with diarrhea attending health facilities in Mbarara City or similar urban settings in southwestern Uganda. This is because the study population likely shares similar environmental conditions, sanitation practices, healthcare access, and socioeconomic characteristics

Recommendations

Strengthening molecular diagnostic capacity in health laboratories should be supported to adopt molecular diagnostic techniques (e.g., PCR) for routine detection of diarrheagenic *E. coli*, since conventional culture methods may not differentiate pathogenic pathotypes

Limitations of the study

This study did not focus on environmental or risk factors like sanitation practices, drinking water sources, hygiene practices and contact with animals which limits understanding transmission pathways.

Secondly since the study was conducted in Mbrarara City, therfore the results may not represent the distribution of diarrhaegenic E.coli pathotypes in other regions such as rural settings

Recommendations

Strengthening molecular diagnostic capacity in health laboratories should be supported to adopt molecular diagnostic techniques (e.g., PCR) for routine detection of diarrheagenic *E. coli*, since conventional culture methods may not differentiate pathogenic E.coli pathotypes

Competing interests

The author declared that there was not conflicting interest.

Acknowledgement

Consent for Publication

Not Applicable

Author Contributions Statement

Study Conceptualization: Phionah KA, Jemimah N., Halid K., Joel B., Charles NB.

Data Analysis and presentation: Phionah KA, Jemimah N., Halid K., Joel B., Charles NB.

Results and discussion: Phionah KA, Jemimah N., Halid K., Joel B., Charles NB and UMS

Manuscript draft and final revision: Phionah KA, Jemimah N., Halid K., Joel B., Charles NB and UMS

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Limitations of the study

The study was conducted only in Mbarara City, which may limit the generalizability of the findings to other regions of Uganda or other countries where environmental, sanitation, and socioeconomic conditions may differ.

Since samples were collected from children attending health facilities, the study may exclude children with diarrhea who did not seek medical care, leading to selection bias and reducing representativeness of the general population

Data Availability

Data used in the study were captured in the main text and also supplementary materials was provided.

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PUBLISHER DETAILS