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Molecular Characterization of Shiga Toxin Genes (stx1 and stx2) and Extended-Spectrum β-Lactamase Genes in Escherichia Species Isolated from Children with Diarrhea in Eastern Sudan

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Abstract: Shiga toxin-producing Escherichia coli (STEC) and extended-spectrum β-lactamase (ESBL)-producing strains are a major threat to paediatric health, especially in resource-limited settings. The present study was conducted to investigate the molecular characteristics of Shiga toxin genes (stx1 and stx2) and ESBL genes (blaCTX, blaTEM, blaSHV) in Escherichia species isolated from children with diarrhea, Kassala State, Sudan. 2. A descriptive cross sectional study was conducted from the year 2021-2024 among 404 children less than five years of age presenting with diarrhoea. Stool specimens were cultured on MacConkey agar and E. coli isolates were identified by conventional microbiological MicroScan system was used for determination of antimicrobial susceptibility. Multiplex PCR was used for the detection of Shiga toxin genes (stx1, stx2) and extended spectrum beta-lactamase (ESBL) genes (blaCTX, blaTEM, blaSHV). Data were analyzed using chi-square tests in SPSS version 23. E. coli isolates were recovered from 123 (30.4%) out of 404 stool samples. Molecular analysis revealed the presence of stx2 in 25 isolates (20.3%) and stx1 in 15 isolates (12.2%). The ESBL genes were highly prevalent as follows: blaCTX (n=89, 72.4%), blaTEM (n=85, 69.1%) and blaSHV (n=37, 30.1%). The rates of antimicrobial resistance were >85% for all the tested cephalosporins. There was no statistically significant correlation of the presence of the Shiga toxin genes or ESBL genes with the resistance to cephalosporins (p > 0.05). STEC and ESBL producing E. coli were common among children in Kassala State. The higher occurrence of stx2 than stx1 is worrying as stx2 was associated with poor clinical outcomes. The high prevalence of blaCTX genes and the extensive cephalosporin resistance highlight the need for further surveillance and antimicrobial stewardship in this region.

Keywords: *Escherichia Coli; Shiga Toxin; stx1; stx2; ESBL; Antimicrobial Resistance; Children; Sudan.*

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I. INTRODUCTION

The presence of diarrhea as a disease continues to be one of the leading causes of morbidity and mortality among children less than five years of age in low and middle-income countries. The presence of Escherichia coli as a pathogen is one of the most common isolates to-date; and out of this, Shiga toxin- producing E. coli (STEC) has the greatest concern due to its association with severe clinical outcomes, including both hemorrhagic colitis and hemolytic uremic syndrome (HUS) [1], [2]. Shiga toxins, which are encoded by the genes stx1 and stx2, inhibit the synthesis of protein in host cells, causing damage to the intestinal epithelial layer and systemic complications resulting from these cellular injuries [3]. Furthermore, the toxin stx2 has a much higher frequency

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of severe disease and increased virulence than does the toxin stx1 [4].

Antimicrobial resistance (AMR) among E. coli strains is making it more difficult to treat children that have diarrhoea disease. ESBL-producing E. coli strains that produce enzymes that degrade third generation cephalosporin have increased in hospitals and the community [5]. CTX-M type ESBLs, specifically CTX-M-15, are now the predominant ESBL family globally having displaced TEM and SHV ESBLs from the predominant position they previously held [6]. There is a growing body of literature describing the co-existence of virulence and resistance genes in E.coli isolates, raising concerns over the emergence of highly pathogenic and multidrug resistant E. coli [7].

In Sudan, there is little data to describe the molecular epidemiology of STEC and ESBL-producing E. coli in children. In an earlier study in Khartoum's hospital, 38% of E. coli isolates produced ESBL and 78% contained blaCTX-M markers [8] but little information comes from eastern Sudan, particularly in Kassala state. A thorough understanding of virulence and antibiotic resistance genes in this region will be necessary for appropriate clinical management and effective public health interventions.

The objectives of this study were to: (1) determine the frequency of Shiga toxin genes (stx1 and stx2) among Escherichia strains isolated from children with diarrhoea; (2) examine the occurrence of extended-spectrum beta-lactamase (ESBL) genes (blaCTX, blaTEM and blaSHV) among the same Escherichia isolates; and (3) assess the association between genetic and phenotypic resistance to antimicrobial compounds in Escherichia isolates.

II. MATERIALS AND METHODS

➢ *Study Design and Setting*

The study was conducted as a descriptive cross-sectional hospital-based research study conducted in Kassala State, eastern Sudan from 2021-2024. This work was conducted at both Kassala Teaching Hospital and Alqueity Pediatric Hospital. The Kassala Teaching Hospital is one of the most important referral centers in the region.

➢ *Study Population and Sample Size*

The study involved children lower than five years of age who presented with diarrhea. The sample size was considered as 404 participants utilizing the mathematical formula such as $N = (Z^2 \times P(1-P)) / e^2$, where Z = 1.96 (95% confidence level), P = 0.5 (estimated prevalence), and e = 0.05 (margin of error).

➢ *Data Collection*

To collect demographic and clinical data from the participants, we employed a standardized questionnaire. In addition to geographic nursery hospital demographics such as age, gender and so forth; we assessed their clinical symptoms, history of antibiotics used and what type of water source they normally consume. Animal exposure will also be included in the demographics collected by these participants.

➢ *Sample Collection and Processing*

During sample collection for stool samples sterile faecal swab was utilized to capture samples and transported directly from the point of collection to the laboratory setting. After inoculation of the samples onto MacConkey agar plates, the plates were aerobically incubated at 37 degrees C for 1-4 days. The identification of E. coli isolates was achieved through the use of colony morphology, Gram staining, and standard biochemical testing.

➢ *Antimicrobial Susceptibility Testing*

The MicroScan WalkAway system, produced by Siemens Healthcare Diagnostics, USA, was utilized for antimicrobial susceptibility testing in this experiment. The antibiotics tested in this study include: cefepime, cefixime, cefotaxime, ceftazidime, cefoxitin and cefuroxime; and results were evaluated according to CLSI standards [9].

➢ *DNA Extraction*

DNA extracted from the cultured bacteria was obtained following Promega's method for isolating genomic DNA from microbial cultures according to the manufacturer's specifications (Promega, USA). Genomic DNA purity and concentration was determined by spectrophotometry.

➢ *Molecular Detection of Virulence and ESBL Genes*

Moreover, the primers used during the polymerase chain reaction (PCR) amplification process were specifically as follows in accordance to the information that was published in other locations [10], [11]:

- *Shiga Toxin Genes:*

✓ *stx1*-F: 5'-ACACTGGATGATCTCAGTGG-3'
✓ *stx1*-R: 5'-CTGAATCCCCCTCCATTATG-3' (product size: 130 bp)
✓ *stx2*-F: 5'-CCATGACAACGGACAGCAGTT-3'
✓ *stx2*-R: 5'-CCTGTCAACTGAGCAGCACTTTG-3' (product size: 510 bp)

- *ESBL Genes [12]:*

✓ *bla*TEM-F: 5'-AGTGCTGCCATAACCATGAGTG-3'
✓ *bla*TEM-R: 5'-CTGACTCCCCGTCGTGTAGATA-3' (product size: 431 bp)
✓ *bla*SHV-F: 5'-GATGAACGCTTTCCCATGATG-3'
✓ *bla*SHV-R: 5'-CGCTGTTATCGCTCATGGTAA-3' (product size: 214 bp)
✓ *bla*CTX-F: 5'-ATGTGCAGYACCAGTAARGT-3'
✓ *bla*CTX-R: 5'-TGGGTRAARTRAGTSACCAGA-3' (product size: 593 bp)

The thermocycling conditions for stx1 were an initial denaturation of 95°C for 5 minutes, followed by 33 cycles consisting of 1-minute denaturation at 94°C; 1-minute annealing at 60°C; and 1-minute extension at 72°C and then 7-minute final extension at 72°C. For stx2, the conditions were initial denaturation at 95°C for 4.5 minutes, with 35 cycles included consisting of 1-minute denaturation at 95°C; 1-minute annealing at 62°C; and 1-minute extension at 72°C and then 5-minute final extension at 72°C. The multiplex PCR to detect ESBL genes included an initial denaturation at 94°C

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for 5 minutes and consisted of 30 cycles each consisting of 20 seconds denaturation at 94°C; 30 seconds annealing at 61°C; and 60 seconds extension at 72°C along with a final extension at 72°C for 5 minutes. The PCR products were visualized under UV light after staining with ethidium bromide and running them on 1.5% agarose gels.

➢ *Statistical Analysis*

The data were analyzed with SPSS v23.0 (IBM Corp., USA) and the descriptive statistics were presented as percentages and frequencies. The chi-square ($\chi 2$) test determined the relationships between gene carriage and antibiotic resistance by comparing the frequency of antibiotic-resistant strains to the gene carriage in the"); %' group of isolates. A p-value < 0. 05 was found to be statistically significant.

➢ *Ethical Considerations*

The study was approved to proceed with ethical considerations by the Institutional Review Board of the University of Kassala. The children involved in the study had been adequately informed and written consent obtained from their parents or legal guardians.

III. RESULTS

➢ *Distribution of Detected Pathogens*

A total of 404 stool samples were collected. Of these, 123 (30.4%) yielded E. coli isolates, while 281 (69.6%) were positive for other pathogenic agents as shown in Fig 1.

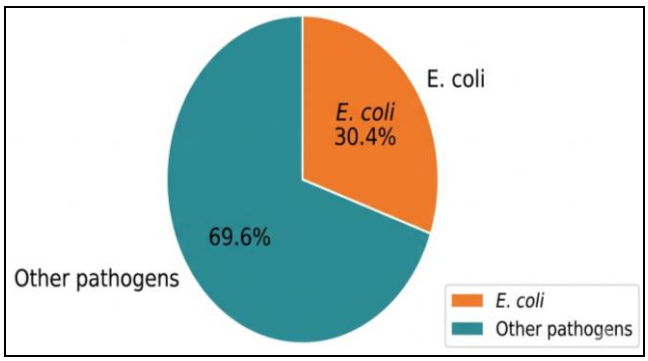

Fig 1 Distribution of Detected Pathogens in Stool Samples

➢ *Demographic Characteristics*

Of the 123 children who had E. coli isolates, 72% were male and 49% were female. More than half of the children were less than one year old (63, 51.2%) followed by children between the age range of one to two years old (40,3.5%). Table 1 shows that the majority of women had completed elementary school (67, 54.5%).

Table 1 Sociodemographic Characteristics of Participants

Variable	Category	Frequency (n)	Percent (%)
Gender	Male	74	60.2%
	Female	49	39.8%
Age (years)	< 1 year	63	51.2%
	1-2 years	40	32.5%
	2-3 years	8	6.5%
	3-4 years	9	7.3%
	4-5 years	3	2.4%
Mother's Education	No education	6	4.9%
	Primary	67	54.5%
	Secondary	47	38.2%
	University	3	2.4%
Total Participants	---	**123**	**100%**

➢ *Behavioral and Environmental Factors*

An Analysis was performed and revealed that 42 (42.3%) of the children had a previous use of antibiotics. There were 92 homes (74.8%) that used municipal water and only 18 (14.6%) children had been exposed to animal products.

Table 2 Behavioral and Environmental Factors

Variable	Category	Frequency (n)	Percent (%)
Antibiotic use	Yes	52	42.3%
	No	71	57.7%
Water source	Municipality	92	74.8%
	Wells	31	25.2%
Animal exposure	Yes	18	14.6%
	No	105	85.4%

➢ *Detection of Shiga Toxin Genes*

Molecular assessment showed 25 isolates had the stx2 gene (20.3%) and 15 isolates had stx1 gene (12.2%) After stx2 was identified as more prevalent than stx1, clinical expression associated with stx2 is severe; therefore, stx2 should be monitored. (Refer to Table 3 and Fig 2)

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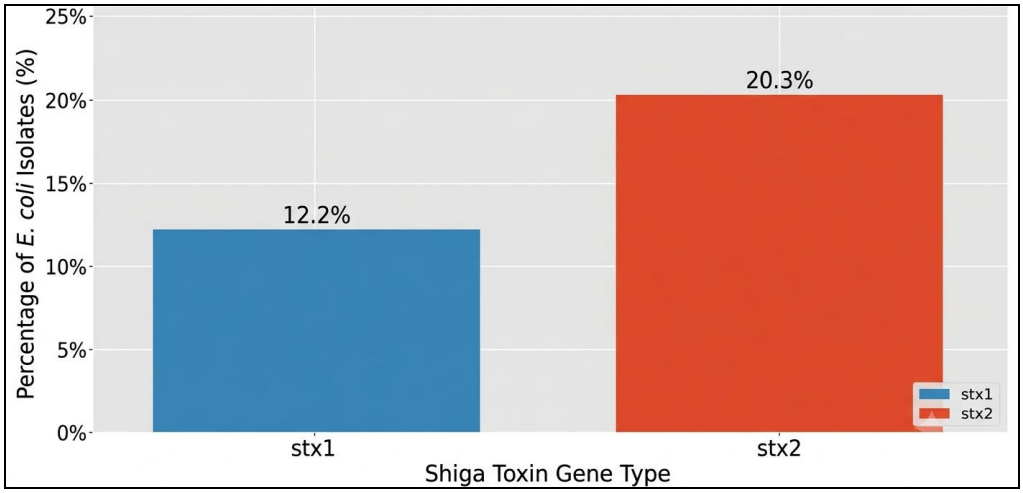

Fig 2 Distribution of Shiga Toxin Genes Among E. Coli Isolates

Table 3 Distribution of Shiga Toxin Genes

Gene	Positive (+VE)	Negative (-VE)	Prevalence (%)
stx1	15	108	12.2%
stx2	25	98	20.3%

➢ *Detection of ESBL Genes*

88 (72.4%) of the tested isolates were positive for blaCTX, and 85 (69.1%) tested positive for blaTEM. 37 of the tested isolates (30.1%) were found to contain blaSHV. Table 4 and Fig 3 illustrate that the ESBL gene frequency was demonstrated to be significant across all 3 genera.

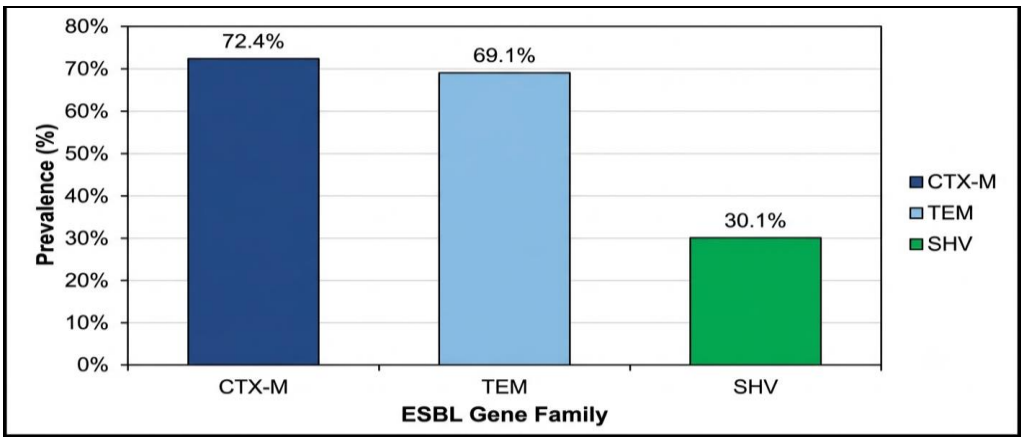

Fig 3 Distribution of ESBL Genes Among E. Coli Isolates

Table 4 Distribution of ESBL Genes

Gene	Positive (+VE)	Negative (-VE)	Prevalence (%)
blaCTX	89	34	72.4%
blaTEM	85	38	69.1%
blaSHV	37	86	30.1%

➢ *Antimicrobial Susceptibility Patterns*

Antimicrobial resistance rates were alarmingly high, exceeding 85% for all tested cephalosporins. Resistance to cefixime was highest (108 isolates, 87.8%), followed by cefuroxime (110 isolates, 89.4%), cefotaxime (105 isolates, 85.4%), cefepime (104 isolates, 84.6%), ceftazidime (104 isolates, 84.6%), and cefoxitin (70 isolates, 56.9%) (Table 5, Figs 4,5 and 6).

Table 5 Antimicrobial Susceptibility of *E. coli* Isolates

Antibiotic	Sensitive (n)	Resistant (n)	Resistance Rate (%)
Cefepime	19	104	84.6%
Cefixime	15	108	87.8%
Cefotaxime	18	105	85.4%
Ceftazidime	19	104	84.6%
Cefuroxime	13	110	89.4%
Cefoxitin	53	70	56.9%

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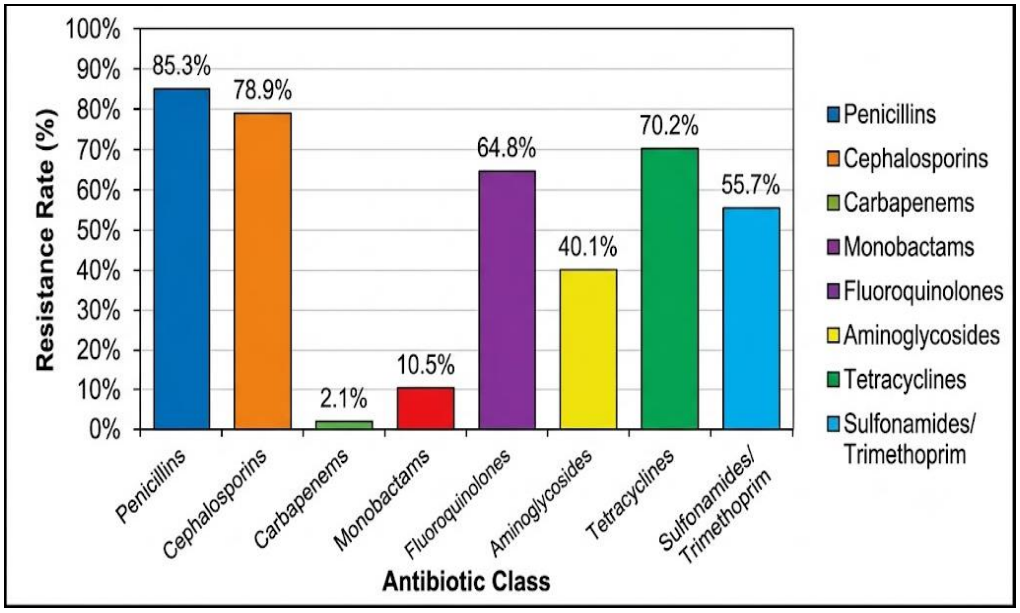

Fig 4 Antimicrobial Resistance Patterns of E. coli Isolates

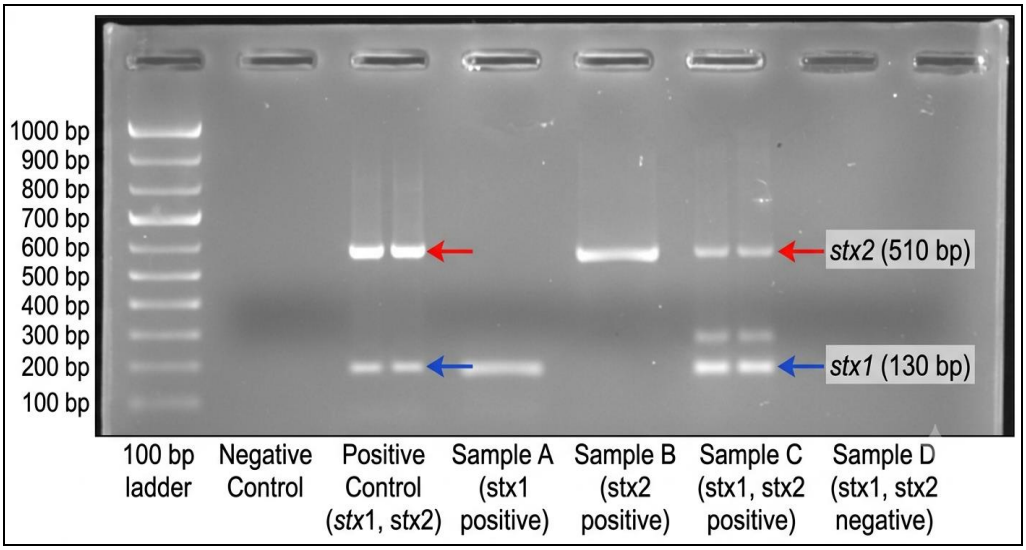

Fig 5 Gel Electrophoresis Image of PCR Amplification of Stx1

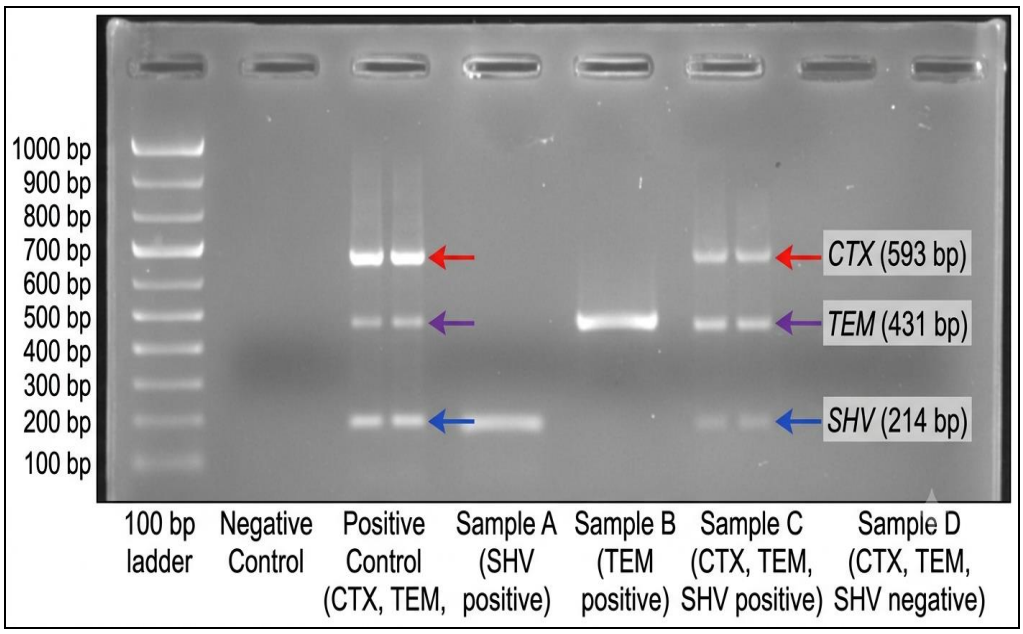

Fig 6 Gel Electrophoresis Image Multiplex of PCR amplification of ESBL Genes

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➢ *Association Between Shiga Toxin Genes and Antibiotic Resistance*

To identify any relationships between Shiga toxin genetic composition (stx1 and stx2) and resistance to cefotaxime (CTX), ceftriaxone (CRO) and ceftazidime (CAD) the chi-square test was performed on the 164 isolates. No statistically significant correlation was observed between either type of shiga toxin's presence or absence with all three cephalosporins tested. The p-values (0.562-1.000) and the chi-square values (0.000-0.336) were each above the significance level (>0.05) (Table 6).

Table 6 Association of Shiga Toxin Genes with Cephalosporin Resistance

Antibiotic	stx1 (χ^2, p-value)	stx2 (χ^2, p-value)	Interpretation
Ceftazidime (CAZ)	0.044, 0.834	0.004, 0.953	No significant association
Ceftriaxone (CRO)	0.130, 0.718	0.336, 0.562	No significant association
Cefotaxime (CTX)	0.198, 0.656	0.043, 0.836	No significant association
Cefepime (FEP)	0.000, 1.000	0.129, 0.720	No significant association

➢ *Association Between ESBL Genes and Antibiotic Resistance*

Statistical analysis. Did the analysis identify definite significant results? There was no statistical difference identified among the phenotypically resistant organisms to the cephalosporins and the ESBL genotype carriers (blaCTX, blaTEM, and blaSHV). The p-value for the statistical relationship was between 0.453 - 1.00; the chi-square ($\chi 2$) results were between 0.000 - 0.563. The data was far above any level of statistical significance ($p > 0.05$). (refer to Table 7)

Table 7 Association of ESBL Genes with Cephalosporin Resistance

Antibiotic	blaCTX (χ^2, p-value)	blaTEM (χ^2, p-value)	blaSHV (χ^2, p-value)	Interpretation
Ceftazidime	0.299, 0.584	0.389, 0.533	0.030, 0.861	No significant association
Ceftriaxone	0.082, 0.774	0.006, 0.941	0.217, 0.641	No significant association
Cefotaxime	0.000, 1.000	0.000, 1.000	0.043, 0.836	No significant association
Cefepime	0.004, 0.953	0.563, 0.453	0.144, 0.704	No significant association

IV. DISCUSSION

This research's findings are useful to understanding the molecular epidemiology of Shiga toxin-producing and ESBL-producing Escherichia coli infection of Kassala's children. The data supports that, in addition to significant numbers of virulence genes, many resistance-associated DNA sequences also exist. The relevance of these data to clinical decisions must be determined.

➢ *Prevalence of Shiga Toxin Genes*

The frequency of the strains stx2 (20.3%) and stx1 (12.2%) detected in this investigation is similar to those reported in many regions throughout Africa. Research in Ethiopia has shown that the prevalence rate of STEC detected in young children with diarrhea was between 15%-20% [13] and there are comparable findings from Tanzania [14]. The elavation of the prevalence of type 2 (stx2) relative to type 1 (stx1) is concerning because of the increasing clinical associations between stx2 and increased severity of disease as well as HUS [15]. Additional studies have concluded that an association with stx2 was an independent risk factor for severe disease because stx2 was shown to occur more frequently than stx1 in those with both hemorrhagic colitis and/or HUS as displayed in Fig 7 [16].

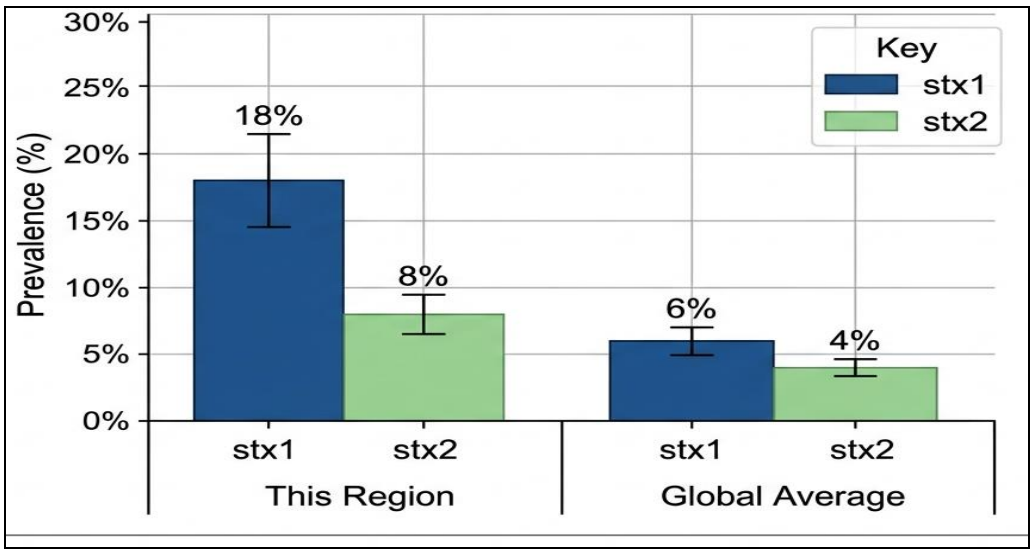

Fig 7 Comparative Analysis of stx1 and stx2 Prevalence in the Region and Globally

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A few isolate types showed that there were genes associated with both Shiga toxin and ESBL (Extended Spectrum Beta Lactamase), which raises the issue of possible emergence of drug-resistant pathogenic strains of bacteria in general. Recent research shows how Common Resident STEC Isolation Type populations display these ESBL type genes (i.e. where people live), showing how there is an urgent need to have some method of doing prolonged surveillance on both virulence and resistant determinants that exist within isolate(s) [17].

➢ *High Prevalence of ESBL Genes*

The prevalence of blaCTX (72.4%) over blaTEM (69.1%) and blaSHV (30.1%) is in accordance with the global epidemiological trend of CTX-M-type ESBLs replacing TEM and SHV enzymes as the predominant types of ESBLs [18]. The prevalence of blaCTX in our study is higher than that reported in many studies in Africa. In Ethiopia, the CTX-M-type ESBLs comprised 78.9% of the ESBL-positive isolates [19]. In Tanzania, 94.7% of the ESBL-producing isolates carried a blaCTX-M-15-like gene [20]. In Egypt, bla CTX-M was the most common ESBL gene (73%) among E. coli isolates according to a meta-analysis as shown in Figure 8 [21].

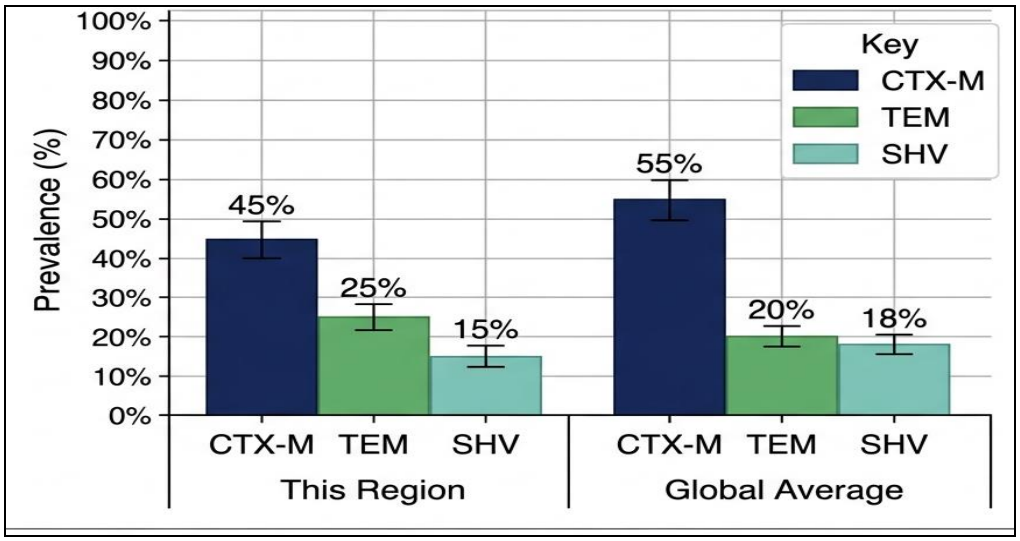

Fig 8 Comparative Analysis of ESBL Gene Prevalence in the Region and Globally

The significant number of CTXM genes that have been identified as prevalent raises an alarm since many of these genes have been associated with mobile genetic elements and can easily spread from one host or species of bacteria to another. For example, the CTX-M-15 ESBL (Extended Spectrum Beta-Lactamase) gene has been reported to be the most frequently isolated ESBL gene in the world's population when associated with community-acquired infections (reference) [22]. The high prevalence of CTXM genes in children suggests a greater degree of community spread, further emphasizing the need to require greater monitoring of surveillance within this population. italic) in addition to the style provided by the drop-down menu to differentiate the head from the text.

➢ *Antimicrobial Resistance Patterns*

The findings from this study demonstrate that the level of resistance identified is alarmingly higher then would be expected based on other studies done in many areas of Africa. For cephalosporin antibiotics, for most of the studied antibiotics, resistance rates exceeded those reported by any other sources in continental Africa by 85% or greater. In support of this, a study in Ethiopia revealed a 48% resistance rate for ceftriaxone among ESBL-producing E. coli [23], while studies conducted in Tanzania showed similar resistance levels of approximately 50-60% [20]. The excessive levels of antibiotic resistance observed in Kassala appear to be related to some of the following factors: frequent misuse of antibiotics, limited access to quality-assured medicines, and the use of highly resistant clones.

Cefoxitin resistance is relatively low (56.9%) indicating that some isolates may still be susceptible, and cephamycins are likely to retain activity against E. coli in this region. However, the more than 50% of the population that has presented evidence for the resistance expressed against cefoxitin suggests that there is a significant level of resistance present and the population has shown the ability to utilize other agents as well.

➢ *Discordance Between Gene Carriage and Phenotypic Resistance*

Evidence of no statistical significance between the genes responsible for carrying Shiga toxins and extended spectrum beta-lactamase (ESBL) and the phenotypical resistance of bacteria carrying these genes is an interesting coincidence as reported on multiple occasions [24],[25]. This lack of correlation could possibly be attributed to several different factors.

To begin with, gene expression does not always equate gene carriage. There are different regulatory systems that may inhibit gene expression [26]. Additionally, there are alternative resistance mechanisms (e.g. porin mutations,

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AmpC β-lactamases, efflux pumps, etc) that confer phenotypic resistance in addition to ESBL genes [27]. Phenotyping limitations may also account for differences noted [9].

As recent genomic research has shown, a majority of β-lactamase genes are often present together in isolates that produce ESBL. From a genomic research study in Ethiopia in the year 2024, 57.9% of E. coli produced from ESBL sequencing contained more than one β-lactamase gene. Additionally, 52.6% of these had blaCTX-M-15 and blaTEM-1B at the same time [19]. This genetic nightmare may explain in part the lack of concordance between the gene possession and the phenotypic resistance.

- *Clinical and Public Health Implications*

This paediatric group is at high risk of STEC and ESBL-producing E. coli with major consequences for clinical practice. Children colonized with STEC strains are at a greater risk of developing serious sequelae, such as hemorrhagic colitis and HUS [2]. The higher incidence of stx2 compared to stx1 is of particular concern, because stx2 has been associated with severe disease [15].

Moreover, colonization with ESBL-producing organisms also places children at a higher risk of acquiring infections that are difficult to treat, as only a few treatment options are still working well [28]. Because treatment options are limited, sometimes carbapenems must be used, which are not only expensive but also less available in low-resource settings [29].

Children in homes and communities are reservoirs for the dissemination of virulent and treatment-resistant germs [30]. High carriage rates observed in this study are consistent with widespread community dissemination that may result in outbreaks and ongoing transmission in the absence of health care exposure.

- *Comparison with Regional and Global Data*

Our results on the prevalence of blaCTX are in line with findings reported from other African countries. The CTX-M type of ESBLs, more specifically CTX-M-15, was reported to be the dominant ESBL type in Ethiopia, accounting for 78.9% of all ESBL positive isolates [19]. In Tanzania, there was a blaCTX-M-15-like gene that was present in 94.7% of the ESBL-producing isolates [20]. Similarly, in a meta-analysis in Egypt blaCTX-M was the most common ESBL gene (73%) among E. coli isolates [21]. The data that are compatible to each other indicate that CTX-M type ESBLs have emerged as the predominant form of ESBLs across Africa, which is indicative of the worldwide epidemiological trends [18].

Conversely, the rates of prevalence in Kassala are higher than those reported in a number of studies which demonstrates that ESBL-producing strains circulate in a particularly intensive manner in eastern Sudan. The geographical diversity encountered [31] emphasizes the importance of local monitoring to guide empirical treatment and infection control measures.

V. RECOMMENDATIONS

Based on our findings, we recommend to enhanced surveillance by continuous monitoring of STEC and ESBL-producing E. coli in pediatric populations is essential to track trends and guide interventions, antimicrobial stewardship applying strengthening antibiotic prescribing practices is crucial to reduce selection pressure favoring resistant strains, infection prevention and control for improving water quality, sanitation, and hygiene practices can reduce transmission of both virulent and resistant bacteria, molecular surveillance by incorporating molecular methods (e.g., PCR, sequencing) into routine surveillance can provide valuable data on circulating virulence and resistance genes, and further research by doing longitudinal studies are needed to understand the dynamics of STEC and ESBL colonization and transmission in this population.

VI. CONCLUSION

In Kassala, Sudan, a study has shown that a large percentage of children five years of age or younger were found to be infected with Escherichia coli that produce Shiga toxins and produce Extended Spectrum β-lactamases (ESBLs). Stx2 was found to be far more common than Stx1 (≥12.2%); in addition, the only pathogenic strains of E. coli known to produce Stx2 were associated with poor clinical outcomes. Almost 85% of the E. coli strains tested are resistant to cephalosporins and 72.4% of the E. coli strains tested have one of the many genotypes of blaCTX. Collectively, this data indicates a poor outlook on medical care currently being provided to the population in Kassala, as well as pose an urgent challenge for public health in Sudan.

This indicates that there is a breakdown between gene presence and resistance to E. coli based infections. For this reason it is important to monitor both pheno- and genotypic forms of antibiotic resistance to understand all aspects of antibiotic resistance as it relates to these patients, given that multidrug resistant and virulent E. coli is a growing threat to this group of patients; therefore increased awareness of the need for improved antibiotic stewardship, regular surveillance, and improved infection control in order to mitigate the risk of infection from multidrug resistant E. coli.

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