ISOLATION, CHARACTERIZATION AND ANTIBIOTIC SUSCEPTIBILITY PATTERN OF DIARRHEAGENIC ESCHERICHIA COLI (DEC) AMONG CHILDREN ATTENDING SOME SELECTED HOSPITALS WITHIN KADUNA METROPOLIS

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ABSTRACT
Diarrhoea has been associated with significant morbidity and mortality, ranked the second cause of death in children aged 0 to 5 years. Bacteria, viruses, protozoa, and helminths have all been implicated in diarrhoea, however rotavirus and diarrhoeagenic E. coli (DEC) are the most common cause. This study aimed to determine the antibiotic susceptibility pattern of diarrhoeagenic Escherichia coli isolated from children 0-5 years attending selected hospitals in Kaduna metropolis. A total of 264 stool samples were collected from children attending four selected hospitals in Kaduna metropolis. Standard culture procedures and molecular techniques such as PCR and 16s rRNA were employed in isolation and characterization of diarrhoeagenic E. coli from the stool samples. The study established a prevalence of 24.2% for diarrhoeagenic Escherichia coli, and all the isolates demonstrated multiple antimicrobial resistance index (MAR) of 0.5 and above, and showed significant resistance against augmentin (100%), amoxicillin (100%), ampicillin-cloxacillin (ampiclox) (100%), erythromycin (100%), gentamycin (100%), cefoxitin (100%), ceftizoxime (95%), ceftriaxone (95%) and cefixime (85%). The least (60%) resistance was observed against imipenem. The study concluded that antibiotics have not been very effective in the treatment of E. coli-related diarrhoea. The study, therefore, recommends the implementation of programmes geared towards good hygiene, good nutrition and good health.

Keywords: Diarrhoea, Diarrhoeagenic E.coli (DEC), Kaduna, Multidrug resistance (MDR)

INTRODUCTION
Diarrhoeal diseases are still one of the leading causes of morbidity and mortality among children under five years in developing countries (WHO, 2017). Diarrhoea is the second leading cause of death in children aged 0 to 5, killing nearly 525,000 children each year (WHO, 2017). Nigeria is one of the five countries with a high childhood diarrhoea rate, with an estimated 150,000 deaths each year (Rehinde & Umar, 2018). The condition is common in developing countries, especially in areas with poor sanitary standard such as open defecation that results in the contamination of water sources. In developing countries, other factors such as malnutrition can increase the risk of diarrhoea. These factors may result in a serious disease problem and negative economic consequences such as higher medical costs, lower quality of life, and a high mortality rate (Zhang et al., 2018). Diarrhoea is caused by infectious organisms such as bacteria, viruses, protozoa, and helminths. These infectious agents are transmissible from person to person through faecal-oral route. However, the severity of disease depends on the portal of entry and size of inoculum necessary to induce illness (Zhang et al., 2018). The most common causes of diarrhoea are enteric infections; rotavirus and Diarrhoeagenic E. coli (DEC), with DEC, cited as the most important cause in developing countries (Agbla et al., 2018). The emergence of multidrug-resistant E. coli, has been observed in various countries over the past decades. With the increase in cephalosporins resistance, especially the parallel increasing frequency of multidrug-resistant E. coli, the treatment of E. coli-associated disease is becoming worrisome to expert. The predominant mechanism of resistance to β-lactam antibiotics in E. coli is the production of plasmid-borne extended-spectrum β-lactamas (ESBLs). Since the first report at the beginning of the 1980s, ESBL-producing organisms have become widespread throughout the world (Rawat & Nair, 2010). The ESBL genes are frequently encoded on transferable plasmids that encode resistance genes. Acquisition of such resistant genes by commensal or faecal isolates leads to multidrug resistant (MDR) pathogens. Multidrug-resistant (MDR) strains and strains that produce extended-spectrum-lactamas (ESBL) are also becoming more common in humans and animals (Zeighami et al., 2015). Due to the high prevalence of multidrug resistance, there is an urgent need for broad-based, local antimicrobial resistance surveillance and the development of successful approaches to reduce multidrug resistance in these pathogens (Olayinka et al., 2004). The presence of plasmids containing one or more resistance genes, each encoding a single antibiotic resistance phenotype, is most commonly associated with multiple antibiotic resistance (MAR) in bacteria (Nikaido, 2009). Multiple antibiotic resistance (MAR) indexing has proven to be a reliable and cost-effective method of tracking bacteria sources. The ratio of the number of resistant antibiotics to which an organism is resistant to the total number of antibiotics to which the organism is exposed is calculated as the multiple antibiotic resistance index (Afunwa et. al., 2020). MAR index values greater than 0.2 indicate the high-risk source of contamination where antibiotics are frequently used. This study was carried out to investigate the antibiotic susceptibility pattern, of Diarrhoeagenic Escherichia coli (DEC) isolated from children 0-5 years attending selected hospitals in Kaduna metropolis, in order to provide a proper basis for clinical treatment of DEC-based diarrhoea infections.
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**MATERIALS AND METHODS**

**Study area and Study Population**

The study was conducted in Kaduna metropolis. Kaduna metropolis is the capital of Kaduna state. It comprises of two local government areas: Kaduna South and Kaduna North and also extends to Chikun and Igabi Local Government areas. A cross-sectional study was conducted among children between the ages of 0-5 years, presenting with diarrhoea in selected hospitals in Kaduna metropolis. Ethical approval for the study was obtained from Kaduna State Ministry of Health, Kaduna (MOH/ADM/744/VOL.19/39).

**Sample Size**

The sample size was determined using the formula of (Chioma et al., 2019), which is as follows: $N = \frac{Z^2 \times \pi (1-\pi)}{q^2}$

Where $n$ is sample size, $Z$ is the standard normal distribution at 95% confidence interval $= 1.96$, $P$ is the prevalence rate, which is taken as 21.1% (Chioma et al., 2019), $q$ is $1-P$, $L$ is the allowable error, which is taken as $5 = 0.05$ Therefore $n = (1.96)^2 \times 0.217 \times (1-0.217) = 0.052n = 3.8416 \times 0.217 \times 0.783 / 0.0025n = 261$. The sample size calculated is 261 samples. A total of two hundred and sixty-four samples were collected from the diarrhoeic children for this study.

**Sample Collection and Processing**

Stool samples were collected from children aged 5 and below visiting four selected hospital presenting with diarrhoea. Stool samples were collected using sterile stool containers and transferred to the microbiology laboratory immediately for laboratory analysis.

**Preparation of Media**

Media used in this study included, MacConkey Agar, Nutrient Agar and Mueller-Hinton Agar were prepared according to the manufacturer’s instructions.

**Inoculation of Faecal Sample into Culture Media**

Approximately 10 μl volumes of a micropipette homogenate of faecal samples were inoculated directly onto MacConkey Agar and Sorbitol MacConkey agar (Chioma et al., 2019). The plates were incubated at 37°C, for 18 - 24 hrs. Lactose fermentation (pinkish colonies) on MacConkey Agar is presumptive for *E. coli*.

**Characterization of Diarrheagenic *E. coli***

Bacterial isolates suspected to be *E. coli* were identified according to the standard microbiological procedures as described (Gillespie & Hawkey, 2006), which includes: microscopy (e.g. gram stain), culture techniques and biochemical characterization (such as Indole, methyl red, voges-proskauer, citrate, catalase, coagulase, motility, sugar fermentation tests (glucose, lactose), mannitol salt agar Hydrogen sulphide production (H₂S), urease reaction and blood agar).

**Antibiotic Susceptibility Testing**

Antimicrobial resistance patterns of diarrheagenic *E. coli* isolates were determined by the standard disc diffusion method of Kirby-Bauer as described by (Clinical and Laboratory Standards Institute, 2012). The bacteria isolates were screened for resistance against 10 antibiotics belonging to different families of antimicrobials (Mast Diagnostics, United Kingdom). These included penicillins [ampicillin (10 μg)], amoxicillin (25 μg) and augmentin (30 μg); cephalosporins [ceftriazone (30 μg), cefazidime (30 μg), cefoxitin (30 μg) and cefuroxime (30 μg)]; carbapenems; [imipenem (10 μg)]; aminoglycosides [gentamicin (10 μg)]; macrolides [erythromycin (20μl)]. Pure isolates previously grown on sterile nutrient agar were inoculated on sterile physiological-buffered saline (PBS) solution (0.85% NaCl) to make up a bacterial suspension with a density equivalent to 0.5 McFarland standards. Sterile cotton swab-stick (Copan, Italy) was stroke into the suspension and spread uniformly onto the entire surface of the Mueller Hinton agar plates. Relevant antibiotic discs were placed on the surface of the inoculated plates using a disc dispenser (Mast Diagnostics, UK) and were incubated at 37°C for 18-24 hours. After incubation, the test plates were examined for confluent growth and zone of inhibition. The diameter of each zone of inhibition was measured in millimetre (mm) using a ruler on the underside of the plate. The interpretation of the measurement as sensitive, intermediate and resistant was made according to (Clinical Laboratory Standards Institute manual, 2012).

**Multiple Antimicrobial Resistance Index (MARI)**

The isolates which displayed resistance to three or more than three classes of antibiotics were designated as multi-drug resistant (MDR) bacteria. The Multiple Antibiotic Resistance (MAR) Index was calculated by using the formula as described by Afuwa et al. (2020) which is expressed as: MAR index = $a/b$, where “$a$” represents the number of antibiotics to which an individual isolate is resistant to and “$b$” is the sum of to which individual isolate was tested.

**Molecular Analysis**

Pure colonies of bacterial isolates were placed into appropriately labelled Eppendorf tubes for DNA extraction. A commercially available kits (Accu prep Genomic DNA extraction kit) from Bioneer was used to extract DNA of the bacterial isolates according to the manufacturer’s instructions. The sequences GGACTACAGGATCTATAAT (16s Primer Forward) and AGAGTTTGATCCTG (16s Primer reverse) were used to amplify the DNA of diarrhoeagenic *E. coli* (DEC) by PCR. The PCR amplicons were sequenced using a DNA sequence machine (ABI 3100). All the sequences were matched against nucleotide sequences present in GenBank using the BLAST of the NCBI program to identify the organism based on the most similar 16s rRNA gene.

**Statistical Analysis**

Results and data obtained from the study were entered into Microsoft Excel and analysed using statistical package for social sciences (SPSS) version 23. Chi-square analysis was used to determine association between the observed and expected frequencies and infection at 95% confidence interval and at 0.05 significant levels.

**RESULTS**

A total of 264 samples from four different hospitals were collected for the study. Eighty eight (33.3%) from hospital A, 60 (22.7%) from hospital B, 53 (13.3%) from hospital C and 81 (30.7%) from hospital D. Sixty four (64) out of the 264 samples were positive for *E. coli* culture yielding a prevalence of 24.2%. The study established a significant difference in the distribution of *E. coli* in relation to hospitals. Hospital B, had the highest percentage prevalence of 26.7%, while Hospital D had a prevalence of 25.9%. The prevalence of *E. coli* infection on patients in Hospital A was 22.7%, and Hospital C (20%) (Table 1).

Table 2 show the antibiotic susceptibility pattern of *E. coli* isolated from diarrhoeic stool samples of children less than five years in Kaduna state. Isolates were most resistant...
(100%) to Cefoxitin, Augmentin, Amoxacillin, Ampicillin-Cloxacillin (Ampiclox), Erythromycin and Gentamycin while the lowest rate of resistance was observed against Imipenem. Diarrheagenic E. coli was defined as multidrug resistant isolate when it was found non-susceptible to at least one agent in three or more different classes of antimicrobial agents. The multiple antimicrobial resistance indices of the isolates were found to be above the acceptable 0.2 threshold value. When the MAR indices of the isolates were calculated, all isolates (100%) were resistant to at least 5 of the 10 antibiotics screened having a MAR index of at least 0.5 (Table 3). E. coli presented high multidrug resistance, showing a MAR index of 1.00.

Table 1. Distribution of Escherichia coli isolates to in relation to hospital.

<table>
<thead>
<tr>
<th>Name of Hospital</th>
<th>Samples Collected</th>
<th>Culture Positive</th>
<th>Prevalence (%)</th>
<th>X2</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hospital A</td>
<td>88</td>
<td>20</td>
<td>22.7</td>
<td>192.0</td>
<td>0.000*</td>
</tr>
<tr>
<td>Hospital B</td>
<td>60</td>
<td>16</td>
<td>26.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hospital C</td>
<td>35</td>
<td>7</td>
<td>20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hospital D</td>
<td>81</td>
<td>21</td>
<td>25.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>264</td>
<td>64</td>
<td>24.2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

X2 = chi-square, p-value<0.05, (*) = statistically significant

Table 2: Susceptibility patterns of E. coli isolates to different antimicrobials

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Concentration (µg)</th>
<th>Sensitivity patterns</th>
<th>NT</th>
<th>NS</th>
<th>NI</th>
<th>NR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ceftriazone</td>
<td>30</td>
<td>64</td>
<td>0(0%)</td>
<td>0(0%)</td>
<td>3(5%)</td>
<td>61(95%)</td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>30</td>
<td>64</td>
<td>0(0%)</td>
<td>0(0%)</td>
<td>10(15%)</td>
<td>54(85%)</td>
</tr>
<tr>
<td>Cefoxitin</td>
<td>30</td>
<td>64</td>
<td>0(0%)</td>
<td>0(0%)</td>
<td>0(0%)</td>
<td>64(100%)</td>
</tr>
<tr>
<td>Cefuroxime</td>
<td>30</td>
<td>64</td>
<td>0(0%)</td>
<td>0(0%)</td>
<td>3(5%)</td>
<td>61(95%)</td>
</tr>
<tr>
<td>Augmentin</td>
<td>30</td>
<td>64</td>
<td>0(0%)</td>
<td>0(0%)</td>
<td>0(0%)</td>
<td>64(100%)</td>
</tr>
<tr>
<td>Amoxacillin</td>
<td>25</td>
<td>64</td>
<td>0(0%)</td>
<td>0(0%)</td>
<td>0(0%)</td>
<td>64(100%)</td>
</tr>
<tr>
<td>Ampicillin-Cloxacillin</td>
<td>10</td>
<td>64</td>
<td>0(0%)</td>
<td>0(0%)</td>
<td>0(0%)</td>
<td>64(100%)</td>
</tr>
<tr>
<td>Imipenem</td>
<td>10</td>
<td>64</td>
<td>6(10%)</td>
<td>19(30%)</td>
<td>38(60%)</td>
<td>64(100%)</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>20</td>
<td>64</td>
<td>0(0%)</td>
<td>0(0%)</td>
<td>0(0%)</td>
<td>64(100%)</td>
</tr>
<tr>
<td>Gentamycin</td>
<td>10</td>
<td>64</td>
<td>0(0%)</td>
<td>0(0%)</td>
<td>0(0%)</td>
<td>64(100%)</td>
</tr>
</tbody>
</table>

NT= Number tested, NR= Number resistant, NI= Number intermediate, NS=Number susceptible

Table 3: Showing Multiple Antibiotic Resistance (MAR) Index of Diarrhoeagenic E. coli

<table>
<thead>
<tr>
<th>MARI Value</th>
<th>Percentage of Isolates (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>0(0)</td>
</tr>
<tr>
<td>0.2</td>
<td>0(0)</td>
</tr>
<tr>
<td>0.3</td>
<td>0(0)</td>
</tr>
<tr>
<td>0.4</td>
<td>0(0)</td>
</tr>
<tr>
<td>0.5</td>
<td>0(0)</td>
</tr>
<tr>
<td>0.6</td>
<td>3(5%)</td>
</tr>
<tr>
<td>0.7</td>
<td>0(0)</td>
</tr>
<tr>
<td>0.8</td>
<td>22(35%)</td>
</tr>
<tr>
<td>0.9</td>
<td>29(45%)</td>
</tr>
<tr>
<td>1.0</td>
<td>10(15%)</td>
</tr>
</tbody>
</table>

Molecular Characterization of Diarrheagenic E. coli

Based on 16S rRNA gene sequencing, bacterial species were taxonomically confirmed as Diarrheagenic E. coli as shown in Plate 1.
A total of 264 diarrheal children aged 0-5 years participated in the study. Out of this number, 64 yielded cultures characterized as diarrheagenic *E. coli* giving a prevalence of 24.2% which corroborate with the findings of other studies (Chiomia et al., 2019; Franzolin, 2015 and Mandomando et al., 2009) which demonstrated a prevalence of 21.7%, 25.2% and 22.6% respectively. David et al. (2019) reported that 20% of diarrheagenic *E. coli* isolated from children with diarrhea in Ebonyi state, Nigeria. Isegbhi et al. (2021) in a study carried out in Minna reported a prevalence of 37.7% of diarrheagenic *E. coli* (DEC) obtained from stool samples evaluated. On the other hand, significant higher prevalence of 40% (Spano et al., 2017), 60% (Eseigbe et al. 2013) and 62.8% (Ifeanyi et al. 2010) were also reported in previous studies. David et al. (2020), reported the isolation of 54 out of 80 faecal samples of children, under five years, with 67.5% prevalence in a tertiary health centre in south-east Nigeria. Saka et al. (2019) also reported a 73.7% identification of *E. coli* from children with diarrhea in Kano.

In the present study, Diarrheagenic *E. coli* demonstrated a much higher resistance rate against augmentin, amoxicillin and ampicillin-cloxacillin (ampiclox) (100%). This study indicated that *E. coli* isolates presented high resistance rate to ampicillin. This finding is in agreement with reports from Bangladesh, Thailand, Iran and Kenya (Loha et al. 2021; Wilunda and Panza, 2009; Alizade, 2018 and Sang et al., 2011). A study by Adesoji et al. (2020) also reported a 100% resistance to ampicillin and high resistance of 80% to amoxicillin, in Katsina, North-west Nigeria. This substantiates the fact that; these penicillins used in treating a broader range of bacterial infections are the most administered antibiotic in children, thus suggesting an elevated use of this drug, and consequently resulting in high resistance rates and a severe threat to public health (Fair and Tor, 2014). The level of resistance observed in this study for ceftriazone, ceftazidime, cefoxitin and cefuroxime corroborates with results from several studies by other authors who demonstrated high rates of resistance of *E. coli* against these third generation cephalosporins. Ugwu et al. (2017) reported 100% resistance to cefuroxime; 91% for cefazidime and ceftiraxone, while, Zhou et al. (2018) reported 59% and 35.2% for cefuroxime and ceftazidime, respectively. One explanation for this could be the widespread use of these antibiotics in the treatment of infectious diarrrhoea because of their ready availability (Fair and Tor, 2014). The isolates were, however, least resistant to imipenem (60%), indicating that imipenem may still be the most effective antimicrobial against the isolates. Similar findings by Mahmoud et al. (2020) reported a 33% resistance of *Escherichia coli* isolates to imipenem, with the detection of carbapenemases resistant genes. However, Beshiru et al. (2022) reported a much higher sensitivity (98%) for imipenem against DEC isolated from ready to eat foods sold in Yenagoa, Nigeria. Another report, Adesoji et al. (2020) reported that the *E. coli* isolates were more sensitive to ceftiraxone than imipenem, and this disagrees with the level of sensitivity of imipenem observed in this study. The low sensitivity recorded (10%) in this study, corroborates with several research globally, indicating the steady rise in carbapenem resistant Enterobacteriaceae (CRE), which could be as a result of increased use of last resort/reserved antibiotics such as Carbapenems. Multiple antibiotic resistance index helps analyse health risks, as well as to check the extent of antibiotic resistance (Joseph et al., 2017). MAR index analysis has been used to differentiate isolates from different sources using antibiotics that are commonly used in the treatment of infectious cases. According to Thennozhi et al. (2014), MARI values higher than 0.2 indicates existence from high risk contaminated sources with frequent use of antibiotics. In this study, all the isolates of Diarrheagenic *E. coli* demonstrated a MARI index of 0.6 and above which should be a great cause of concern to

**DISCUSSION**

A study by Adesoji et al. (2020) also reported a 100% resistance to ampicillin and high resistance of 80% to amoxicillin, in Katsina, North-west Nigeria. This substantiates the fact that; these penicillins used in treating a broader range of bacterial infections are the most administered antibiotic in children, thus suggesting an elevated use of this drug, and consequently resulting in high resistance rates and a severe threat to public health (Fair and Tor, 2014). The level of resistance observed in this study for ceftriazone, ceftazidime, cefoxitin and cefuroxime corroborates with results from several studies by other authors who demonstrated high rates of resistance of *E. coli* against these third generation cephalosporins. Ugwu et al. (2017) reported 100% resistance to cefuroxime; 91% for cefazidime and ceftiraxone, while, Zhou et al. (2018) reported 59% and 35.2% for cefuroxime and ceftazidime, respectively. One explanation for this could be the widespread use of these antibiotics in the treatment of diseases associated with Gram- negative bacteria, especially in children under two years of age with acute infectious diarrhoea (Bruzzone et al. 2018; Sulaiman et al. 2020). Castro et al. (2019), in a study reported that the *E. coli* isolates were more sensitive to cefuroxime, ceftiraxone and this was in contrast to the level of sensitivity observed in this study. These antibiotics tested are widely used to treat diarrhoea because of their ready availability (Fair and Tor, 2014). The isolates were, however, least resistant to imipenem (60%), indicating that imipenem may still be the most effective antimicrobial against the isolates. Similar findings by Mahmoud et al. (2020) reported a 33% resistance of *Escherichia coli* isolates to imipenem, with the detection of several carbapenemases resistant genes. However, Beshiru et al. (2022) reported a much higher sensitivity (98%) for imipenem against DEC isolated from ready to eat foods sold in Yenagoa, Nigeria. Another report, Adesoji et al. (2020) reported that the *E. coli* isolates were more sensitive to ceftiraxone than imipenem, and this disagrees with the level of sensitivity of imipenem observed in this study. The low sensitivity recorded (10%) in this study, corroborates with several research globally, indicating the steady rise in carbapenem resistant Enterobacteriaceae (CRE), which could be as a result of increased use of last resort/reserved antibiotics such as Carbapenems. Multiple antibiotic resistance index helps analyse health risks, as well as to check the extent of antibiotic resistance (Joseph et al., 2017). MAR index analysis has been used to differentiate isolates from different sources using antibiotics that are commonly used in the treatment of infectious cases. According to Thennozhi et al. (2014), MARI values higher than 0.2 indicates existence from high risk contaminated sources with frequent use of antibiotics. In this study, all the isolates of Diarrheagenic *E. coli* demonstrated a MARI index of 0.6 and above which should be a great cause of concern to
health providers. In principle, these findings reveal inappropriate use of antimicrobials in the region which poses a significant therapeutic setback and consequently, public health burden. Similar results were obtained in a study conducted in Oyo, southwest, Nigeria (Ayandele et al., 2019). The high prevalence of multiple antibiotic resistance obtained in this study may be because E. coli acts as a reservoir for resistance available to enteric pathogens (Akingbade et al., 2013) or may be due to the fact that antimicrobial resistance in E. coli has increased worldwide and its susceptibility patterns show substantial geographic differences and variations (Rodrigo, 2020).

CONCLUSION

The dangers of diarrhoeal diseases in children under five years of age have long been established, to this end, the following conclusion has been drawn from this study. High levels of multidrug resistant Diarrheagenic Escherichia coli were isolated from the study population further confirming E. coli as an active causative agent of diarrhoeal diseases in Kaduna state, Nigeria. This study concluded that the diarrhoeic isolates presented multidrug resistance as many commonly prescribed antibiotics were no longer effective against it. This indicates the often unnecessary and uninformed use of these drugs in the treatment of most infantile diarrhoea cases. A significant number of the diarrhoeic isolates had a MAR index > 0.2 indicating previous exposure to antibiotics.

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