

# Molecular Characterisation of Bacteria in Diarrhoeic stool samples of Children less than five years in Port Harcourt, Nigeria

## ABSTRACT

**Introduction:** Diarrheal diseases remain a significant cause of morbidity and mortality among children under five years old, particularly in developing regions such as Port Harcourt, Nigeria.

**Aim:** This study aimed to molecularly characterize bacteria in diarrheic stool samples from children less than five years in Port Harcourt .

**Methodology:** Two hundred and seventy (270) Stool samples were collected from pediatric patients presenting with diarrhea at Rivers State University teaching hospital Port Harcourt from September 2023 to June 2024 for isolation and identification of bacteria causing childhood diarrhoea and ten (10) samples out of them was used for molecular analysis which involves 16S rRNA gene extraction, amplification using specific primers and sequencing. Data generated was used to create a phylogenetic tree to establish the origin and relationship between isolates.

**Results:** The results reveal that out of the 270 samples, bacteria causing childhood diarrhoea was present in 98 at varying frequencies. They include: *Escherichia coli* 46 (17.0%), *Salmonella species* 20 (7.4%), *Shigella species* 16(5.9%), *Yersinia species* 10(3.7%) and *Vibrio species* 6(2.2%). There was no statistically significant association between sex and the prevalence of diarrhoea-causing bacterial pathogens ( $p = 0.7981$ ) but there was a significant association found between age and the prevalence of bacterial pathogens ( $p < 0.0001$ ). There was high level of multi drug resistance found amongst the isolates with all isolates having a 100% resistance to septrin and augumentin having a better performance index. Genetic analysis highlighted the presence of multiple strains as compared to 5 bacteria isolated using the conventional method of bacteria identification. The phylogenetic analysis revealed that all isolates evolved from a common ancestral lineage.

**Conclusion:** This study shows the importance of comprehensive molecular surveillance and targeted intervention strategies to combat diarrheal diseases in young children in Port Harcourt. Understanding the genetic diversity and prevalence of pathogens can inform public health initiatives, including vaccination programs, sanitation improvements, and antimicrobial stewardship, aimed at reducing the burden of diarrheal illnesses and improving child health outcomes in the region.

**Key words:** Bacterial pathogens, diarrhoeic stool, public health, phylogenetic

## 1. Introduction

Gastroenteritis is a very common condition that causes diarrhoea and vomiting. The term diarrhoea depends on what is common for an individual but for the context of this study, it is seen as passing loose stool three or more times within a twenty-four hour

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Molecular Characterization of Enteric Bacterial Pathogens in Diarrhoeic Stool Samples of Children Under Five in Port Harcourt, Nigeria.

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**Commented [OS5]:** Suggested keywords: Diarrheal diseases, molecular characterization, 16S rRNA sequencing, pediatric bacterial infections, multidrug resistance (MDR), phylogenetic analysis.

period [1]. Most diarrhoeal cases are self-limiting mild infections that can (e.g., "on its own" → "on their own," "in such situations" → "in such cases"). can be resolved on its own but some can be acute, severe and life threatening particularly among children. In such situations, antibiotics therapy is recommended [2]. In developing countries, more than 10 million children less than 5 years die and diarrhoea is one of the major causes of these deaths [3]. Annually there are about 1.7 billion cases of diarrhoea worldwide and it is the most important cause of malnutrition in children less than 5 years [2].

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Microorganisms are very important in life and they are found everywhere naturally. They not only help in stabilising the environment that we live in, but they also contribute greatly in health and diseases. At birth, humans are made up of only their own eukaryotic cells but as the years go by, they become colonized by different forms of life especially on their skin, mouth and gut. These microorganisms in association with the host organism form a complex microbial community called the microbiota, where they carry out all their functions and this microbiota accounts for the major weight of the body [4]. Under normal circumstances, microorganisms are normal flora helping to digest our foods and maintain the immune system but when their composition and relative abundance changes, they are highly associated with disorders and diseases such as diarrhoea, bacterial vaginosis, cancer, colitis, chronic fatigue syndrome and inflammatory bowel diseases [5]. In bacterial diarrhoea, the microorganisms first adheres to the walls of the intestine, produce toxins that affect epithelial cell functions and also invade and destroy mucosal epithelial cells. It affects people of all ages but children are more at risk. Diarrhoea is a major cause of death of infants all over the world; unavailability of safe water, basic nutrition and hygiene are major prevailing conditions to the disease. Worldwide, the most commonly associated enteric pathogens responsible for acute diarrhoea include organisms of bacterial origin such as

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*Escherichia coli*, *Salmonella* species, *Shigella* species, *Camphylobacter jejuni*, *Vibrio* species, *Yersinia* species, *Aeromonas* species, *Clostridium difficile*, parasites such as *Cyclospora*, *Cryptosporidium* species, *Gardia lamblia*, *Entamoeba histolitica* and Viruses such as *Rotavirus*, *Calicivirus* and other enteric viruses [6].

The means of transmission of the microorganisms is by ingestion of food and drinking of water contaminated by faeces through the gastro intestinal tract. World Health Organisation in [7] revealed that water borne disease is a major contributor to the over 4 billion cases of diarrhoea disease annually and 1.8 billion people currently use sources of water contaminated with faeces.

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Symptoms usually appear up to a day after becoming infected, last less than a week and resolves on its own but sometimes last longer when complications occur and these symptoms include fever, abdominal cramps, nausea, headache, seizure, blood, mucus or foul smelling faeces [8].

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The incidence of diarrhoea varies greatly with seasons and age and can have major impact on infectious disease burden and quality of life of individuals in a locality. It is common even in Port Harcourt, Rivers State, Nigeria as in some other countries in sub

Saharan Africa. In 2009, it was said that 260 people died of cholera an acute form of diarrhoea in 4 northern states in Nigeria and in 2010, there was yet another outbreak of cholera and gastroenteritis in Port Harcourt and some other regions of the country such

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as Adamawa, Borno, Bauchi, Cross River, FCT, Gombe, Jigawa, Osun, Taraba and Yobe with 2,137 new cases and 95 deaths [13]. In 2018, 44,201 cases were reported in 20 States, by 2021, over 111,062 suspected cases and 3,604 deaths were recorded however, as of 29 May 2022, a total of 2,339 suspected cases of cholera infections with over 74 deaths were reported in 30 States across the country sparing Abia, Ebonyi, Edo, Enugu, Ogun, Yobe, and FCT [14, 15]. Data available in Africa shows that 106 children

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under five years die out of 1000 yearly and reports available in Nigeria reveals that over 315,000 children in nursery schools die yearly as a result of diarrhoea and there has been series of outbreaks in the country which Rivers State is part of [16, 17]. Poor sanitation and lack of hygiene are also responsible for these outbreaks. In Rivers State, various cholera outbreaks a major form of diarrhoea have occurred particularly in communities located within some riverine local government areas (LGAs) with poor hygiene and handwashing practices, and lack of potable water supply [18].

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Conventional methods of identification of these pathogens responsible for diarrhoea rely mostly on microscopy, routine cultures and biochemical testing. As a result of not being able to culture many of the microorganisms from a microbial community, it becomes difficult to interpret the structure of the community. A major challenge in studying the gut microbiota is the inability of culturing most of the gut micro organisms, using molecular genetics analysis, studies have shown that up to 60 – 80% of the human flora have not been isolated in the laboratory [19]. While it is very easy to get fresh faeces samples, the picture gotten from them using the conventional cultural method which has been the method for studying gut microbiota does not represent the complete picture within because the cultural method is limited and can only culture 10 – 30% of microorganisms in the gut [20]. Therefore, it now became necessary to try to understand the evolution, lifestyle and diversity of these microorganisms.

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The 16srRNA sequencing analysis is one useful DNA sequencing approaches used to study non culturable gut microbiota. Amplification of the 16S ribosomal RNA (rRNA) gene using universally conserved primers is presently the most frequently used technique to describe and classify microorganisms at high taxonomic levels in complex environments [21]. Using Basic Local Alignment Search Tool (BLAST) searching for genomic sequences, the number and relative abundance of the human gut microbiome,

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Suggested: While obtaining fresh fecal samples is relatively easy, the results obtained using the conventional culture method, which has traditionally been used to study gut microbiota, do not provide a complete representation of the microbial community. This limitation arises because culture-based methods can only grow 10–30% of the microorganisms present in the gut, as many gut microbes are anaerobic or have specific growth requirements. Molecular techniques such as 16S rRNA sequencing and metagenomics offer a more comprehensive approach to studying gut microbiota.

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new genes, microbial pathway and functional dysbiosis can be revealed. This study is particularly interested in the molecular characterisation of bacterial organisms in diarrhoeic stool samples of children less than five years in Port Harcourt, Rivers State Nigeria. The hyper variable regions of the extractable DNA of all the microorganisms from the faecal samples will be subjected to 16srRNA sequence analysis. The sequences generated will be used to create a phylogenetic tree and also BLAST searched against reference sequences to trace the origin of the microorganisms and check for their relatedness. This will reveal the microbial diversity and also detect the microorganisms associated with diarrhoea in children less than 5 years in Port Harcourt, Nigeria. The aim of this research work is to molecularly characterize bacteria isolates in diarrhoeic stool samples of children less than five years in Port Harcourt, Nigeria in order to identify prevalent pathogens and understand the genetic diversity of these bacteria

## 2. Materials and Methods

### 2.1 Study Area

The study was carried out at Rivers State University Teaching hospital Port Harcourt a city in the South-South Nigeria; it has a population of about 1,382,592 according to the 2006 Nigerian census. It is approximately 140 square miles (360 Km<sup>2</sup>) in area. It lies along the Bonny River and it is located in the Niger Delta. The area lies approximately between Longitude 7° E and Latitude 4.75°N. The climate is tropical monsoon with an annual rainfall of about 1600mm and average atmospheric temperature of 25°C - 28°C. Port Harcourt was selected as the study area because of it is one of the most populous cities in Nigeria.

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### 2.2 Study Design

This research work is a cross sectional study carried out among 270 randomly selected children under the age of 5 who presented with signs and symptoms of diarrhoea in the Rivers State University teaching hospital Port Harcourt. This hospital was chosen because it is among the busiest health facilities in the State and it is accessible to people of different ethnic groups that live in and around the state. The randomly selected children represented a sub group of children in the Port Harcourt.

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### 2.3 Ethical Approval and Informed Consent

Following ethical approval from the Rivers State University Teaching hospital health and research ethics committee Port Harcourt, the participants parents were informed about the study, questionnaires were administered and written informed consents obtained from each of the parents before stool specimen was collected from the children.

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### 2.4 Sample size determination

According to Daniel [22], the following formular was used.

$$n = \frac{Z^2 \times P (1-P)}{d^2}$$

Where

n = minimum sample size,

Z = standard value for the level of confidence of 95% which is 1.96 from normal distribution table

P = prevalence of diarrhoea = 18% [16]

d = degree of accuracy desired or maximum allowable margin of error, it was set at 5% (0.05).

The number of stool samples n for this study

$$n = \frac{(1.96)^2 \times 0.188 (1- 0.188)}{(0.05)^2}$$

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n =270

The number of sample collected in this study for conventional analysis was 270.

## 2.5. Inclusion Criteria

Children who are residents of Port Harcourt, whose parents consented to the study, and are within the age range of 5 and below with signs and symptoms of diarrhoea and must have had 3 or more loose and watery stool within 24 hours in the past week including the day of visit to the clinic and parents were ready for a home visit if the need arises were selected for this study.

## 2.6 Exclusion Criteria

Apparently healthy children within the age range, who though their parents consented to the study but they do not have any signs and symptoms of diarrhoea, those who had diarrhoea but their parents did not consent to study and were not ready for any home visit, were excluded.

## 2.7 Sample Collection

One hundred and twenty-four (124) males and 146 female participants who met the inclusion criteria were given sterile clean dry wide mouthed screw-top stool specimen containers and unique identification patient numbers for specimen collection, processing and retrieval of results.

## 2.8. Sample Analysis

### 2.8.1. Conventional Method

The colour, consistency of the stool, presence of blood and mucus were examined. Following the manufacturer's instructions, MacConkey agar, Xylose Lysine deoxycholate, Salmonella Shigella agar, Deoxycholate Citrate agar, Nutrient agar, peptone water, Thioglycholate Citrate Bile Salt agar and Selenite F broth were prepared

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Suggested: Who had three or more loose and watery stools in a 24-hour period at least once in the past week, including the clinic visit day.

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and used for isolation of bacteria present in the stool samples. Culture plates were inoculated with a loop full of fresh stool samples and incubated at 37°C for 24 hours for the growth of pure single colonies. After 24 hours incubation, Selenite F broth was sub-cultured on DCA/SSA for possible growth of *Yersinia species* and growth on Peptone water with 3% NaCl was sub-cultured on TCBS to look for *Vibrio species*. Apperance of the colonies was observed after 24 hours incubation, gram staining and other biochemical test were also done for proper identification and characterisation using standard methods as described by[23].

#### **2.8.1.1 Antimicrobial susceptibility testing.**

Using the Kirby –Bauer disk diffusion procedure [24], antimicrobial susceptibility test was done using the following antimicrobials Gentamycin 10µg, Amoxil 20µg, Azithromycin 10µg, Erythromycin 30µg, Ampiclox 20µg, Augumentin 30µg, Septrin 30µg, Amplicin 30µg and Ciprofloxacin 10µg to assess the susceptibility patterns of the isolates. Bacterial Suspension were prepared by inoculating 3-5 colonies of the pure culture of the isolates in normal saline adjusting to match the standardized turbidity of a 0.5 McFarland standard (approximately  $1 \times 10^8$  CFU/mL). Sterile swab was used to evenly spread the bacterial suspension across the surface of Mueller-Hinton agar plate prepared, allowed to dry for 5 minutes at room temperature before antibiotic discs were placed on the agar surface with sterile forceps giving 15-20 mm distance between disc. The plates were incubated at 37°C for 24 hours [24]. The growth of bacteria around the disc were observed, measured using a ruler and results compared to standard interpretive criteria and written as resistance (R) or sensitive (S) [25].

#### **2.8.1.2 Statistical Analysis**

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The study examines the effectiveness of antibacterial agents against bacterial pathogens."



The data generated was statistically analyzed using graph pad prism 9 to check for association between the sociodemographic characteristics of the infants and the prevalence of diarrhoea-causing bacteria.

## **2.8.2 Molecular Methods**

### **2.8.2.1 Extraction of DNA**

DNA was extracted from pure colonies of 10 isolated bacteria using ZR Fungal/Bacterial DNA MiniPrep™ 50 extraction kit model D6005 (Zymo Research, California, USA). In a ZR Bashing Bead Lysis tube the colonies were mixed with isotonic buffer (200 µl) and lysis solution (750 µl) and spun at 10,000g for 5 minutes. Four hundred (400) µl of the supernatant was re-spun at 7000 xg for 1 minute and 1200 µl of binding buffer added. Eight hundred (800) µl of the new supernatant was now moved to another collection tube and spun at 10,000xg for 1 minute. The remaining volume was moved to the same Zymo-spin and spun. Two hundred (200) µl of the DNA Pre-Wash buffer and 500 µl of Wash Buffer added to a new collection tube containing the Zymo-spin IIC column and spun for 2 minute at 10,000xg. The Zymo-spin IIC column was moved to a clean 1.5µl of fungal/bacterial DNA Wash Buffer centrifuge tube, 100 µl of DNA elution buffer was put into the column matrix and spun 30 seconds at 10,000xg to elude the DNA. The ultra pure DNA was then stored at -20 degree for further reactions.

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### **2.8.2.2 Amplification of 16S rRNA and sequencing**

The 16S rRNA genes of the isolates was amplified using specific primers on a thermal cycler (ABI 9700 Applied Biosystems) for 35 cycles at a volume of 50 µl. The PCR conditions were 95°C for 5 minutes for the Initial denaturation; 95°C for 30 seconds for denaturation; 52°C for 30 seconds for annealing, 72°C for 30 seconds for

extension, and 72°C for 5 minutes for final extension and this was done for 35 cycles.

Sequencing was done using the BigDye Terminator kit on a 3510 ABI sequencer.

## 2.9. Phylogenetic Analysis

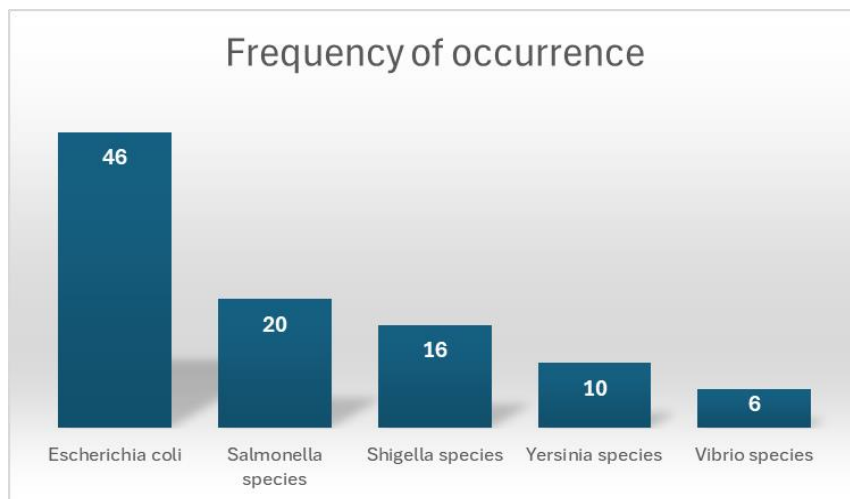
Using ClustalX, Sequences generated were aligned with similar sequences downloaded from the data bank using BLASTN and their origins, inter relatedness and evolutionary history were identified in MEGA 6.0 using Neighbor-Joining method [26].

## 3. Results

### 3.1. Enteric Bacteria associated with infantile diarrhea in Port Harcourt Using Conventional Methods of Isolation.

The result showed that out of the 270 paediatric diarrhoeic samples, 98 had bacteria isolates causing childhood diarrhoea at varying frequencies. The bacteria isolated were *Escherichia coli* 46 (17.0%), *Salmonella species* 20 (7.4%), *Shigella species* 16(5.9%), *Yersinia species* 10(3.7%) and *Vibrio species* 6(2.2%). Figure 1 shows a pie chart of the different species of bacteria isolated and their frequencies of occurrence. *Escherichia coli* was found to be highest in prevalence amongst the bacteria isolated followed by *Salmonella species* then *Shigella species*, *Yersinia species* while *Vibrio species* was the organism that was least present.

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**Figure 1:** Occurrence of Enteric bacteria isolated from Infantile Diarrhoeic Stool using Conventional Methods

### 3.2 Sociodemographic Characteristics of Infants with Infantile diarrhoea in Port Harcourt

The sociodemographic characteristics described in terms of age and sex revealed that most subjects were aged between 1 and 3 years, accounting for 112 (41.5%). Subjects younger than 1 year made up 53 (19.6%) of the sample, while those older than 3 years constituted 105 (38.9%). The mean age of the subjects was 2.5 years. In terms of sex distribution, there were slightly more females in the sample. Out of the total, 146 (54.1%) were female, while 124 (45.9%) were male. This shows a nearly equal representation between the sexes as shown in Table 1 below.

**Table 1:** Sociodemographic Characteristics of Infants with Infantile diarrhoea in Port Harcourt

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Suggested: Sociodemographic Characteristics of Infants with diarrhea in Port Harcourt

Variable	Frequency (N)	Percentage (%)
<b>Age</b>		
<1	53	19.6
1 to 3	112	41.5
> 3	105	38.9
Total	270	100
Mean	2.5	-
<b>Sex</b>		
Female	146	54.1
Male	124	45.9
Total	270	100

The findings in Table 2 present the association between the sociodemographic characteristics of the subjects and the prevalence of diarrhoea-causing bacterial pathogens. A total of 270 subjects were examined, with 98 (36.3%) testing positive for bacterial pathogens and 172 (63.7%) testing negative.

When considering age, a significant association was found between age and the prevalence of bacterial pathogens ( $p < 0.0001$ ). Infants younger than 1 year had the highest prevalence, with 44 (83.0%) testing positive and 9 (17.0%) testing negative. In contrast, infants aged 1 to 3 years showed a lower prevalence, with 48 (42.9%) testing positive and 64 (57.1%) testing negative. The prevalence was lowest in infants older than 3 years, where only 6 (5.7%) tested positive, while 99 (94.3%) were negative.

In terms of sex, there was no statistically significant association between sex and the prevalence of diarrhoea-causing bacterial pathogens ( $p = 0.7981$ ). Among female

infants, 54 (37.0%) were positive for bacterial pathogens, while 92 (63.0%) were negative. Similarly, 44 (35.5%) male infants tested positive, and 80 (64.5%) tested negative.

**Table 2:** Association between the sociodemographic characteristics of the subjects with the prevalence of diarrhoea-causing bacterial pathogen

Variable	N	Positive (%)	$\chi^2$ , df	p-value
<b>Age</b>				
<1	53	44 (83.0)	94.59, 2	<0.0001*
1 - 3	112	48 (42.9)		
> 3	105	6 (5.7)		
Total	270	98 (36.3)		
<b>Sex</b>				
Female	146	54 (37.0)	0.06546, 1	0.7981
Male	124	44 (35.5)		
Total	270	98 (36.3)		

\* = Significant at  $p < 0.05$

### 3.3 Antimicrobial Susceptibility Pattern of Bacteria associated with Infantile diarrhea in Port Harcourt

Antibiotics susceptibility testing of the isolates to commonly used antimicrobial agents revealed different levels of resistance to the various antimicrobials used. All isolates showed some level of resistance to all antibiotics used in this study with 100% resistance to Septrin. *Escherichia coli*, *Shigella species*, *Yersinia species* and *Vibrio species* showed 100% resistance to Augumentin while *Yersinia species* and *Vibrio species* showed 100% resistance to ampicillin. All 5 isolates were not less than 80% sensitive to ciprofloxacin and gentamicin with *Shigella species*, *Yersinia species* and *Vibrio cholera* showing 100% sensitivity to Gentamicin. A significantly high level of resistance to more than three of the antimicrobial agents used was detected in *Yersinia species* and *Vibrio species* (Table 3).

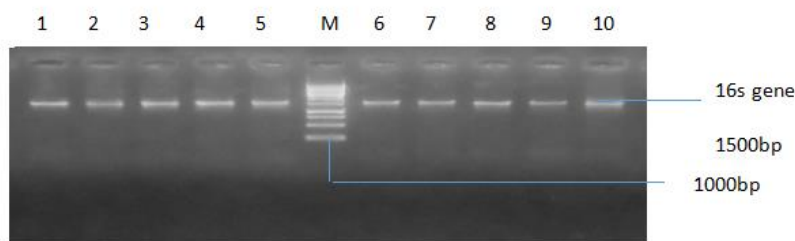
**Table 3:** Antimicrobial susceptibility testing of the bacterial pathogens associated with infantile diarrhea in Port Harcourt.

Antimicrobial agent	Percentage (%) susceptibility of bacteria isolates				
	<i>Escherichia coli</i> (n=46)	<i>Salmonella species</i> (n=20)	<i>Shigella species</i> (n=16)	<i>Yersinia species</i> (n=10)	<i>Vibrio species</i> (n=6)
Gentamicin	37(80)	18(90)	16(100)	10(100)	6(100)
Amoxicillin	18(39.1)	2(10)	4(25)	0(0)	0(0)
Azithromycin	9(19.6)	4(20)	2(12.5)	0(0)	1(16.7)
Erythromycin	9(19.6)	2(10)	2(12.5)	0(0)	0(0)
Ampicillin	5(10.9)	6(30)	7(43.8)	0(0)	0(0)
Augumentin	0(0)	4(20)	0(0)	0(0)	0(0)
Septin	0(0)	0(0)	0(0)	0(0)	0(0)
Ampiclox	18(39.1)	2(10)	4(25)	0(0)	0(0)
Ciprofloxacin	41(89.1)	16(80)	14(87.5)	8(80)	5(83.3)

### 3.4 Enteric Bacteria associated with infantile diarrhea in Port Harcourt Isolated Using Molecular Methods

#### 3.4.1 Genomic analysis (16S Genomics DNA extraction).

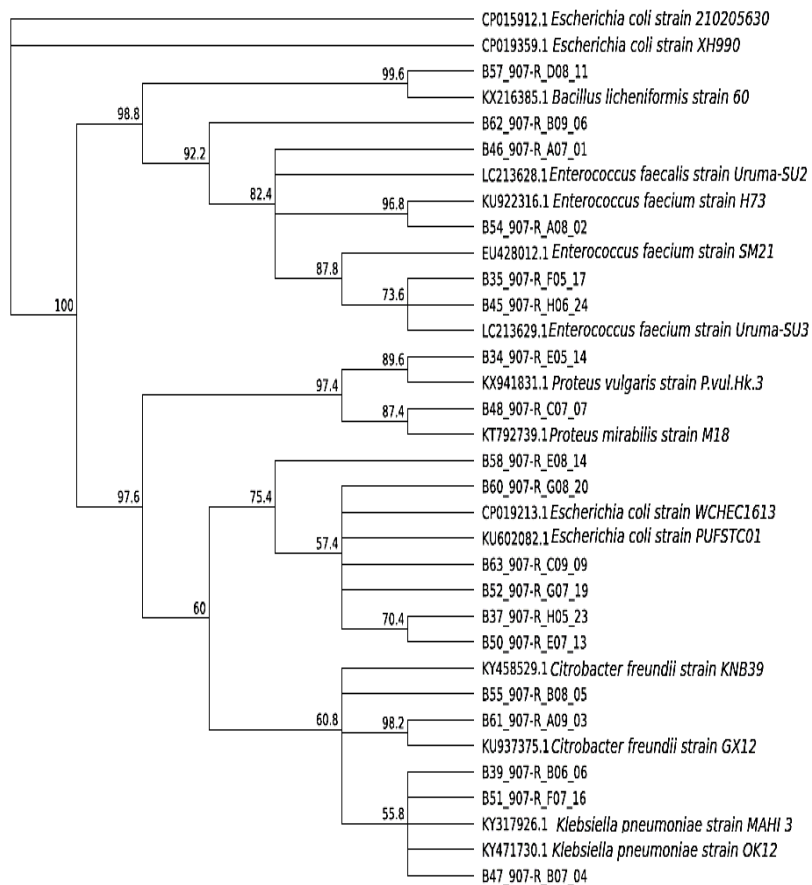
The extraction of 16S genomic DNA is represented in plate 1. Lane 1-10 represents the samples while lane M represents the 1000bp Quick-Load DNA molecular ladder. Figure 2 shows neighbour-joining phylogenetic tree of some of the bacterial isolates showing their evolutionary relationship.



**Plate 1:** Agarose gel electrophoresis showing amplified 16S Genomic DNA (1500bp) isolated from diarrhoeic stool samples.

### 3.4.2 Phylogenetic Analysis

The 16S rRNA sequences obtained were aligned with similar sequences downloaded from National Centre for Biotechnology Information (NCBI) data base using clustal X and a phylogenetic tree was created to establish the relationships between the isolates (Fig 2). The phylogenetic analysis in this study revealed that all isolates were divided into 3 clusters evolving from a common root which 100% similarity showing that they are from a common ancestral lineage. The first cluster is *Escherichia coli* strain 210205630, *Escherichia coli* strain XH990 formed the second cluster and the closest relatives of the third cluster are *Bacillus licheniformis* strain 60, *Enterococcus faecalis* strain Uruma-SU2, *Enterococcus faecium* strain H73, *Enterococcus faecium* strain SM21, *Enterococcus faecium* strain Uruma-SU3, *Proteus vulgaris* strain P. vul.Hk.3, *Proteus mirabilis* strain M18, *Escherichia coli* strain WCHEC1613, *Escherichia coli* strain PUFSTC01 and *Citrobacter freundii* strain KNB39, *Citrobacter freundii* strain GX12, *Klebsiella Pneumoniae* strain MAHI 3, *Klebsiella Pneumoniae* strain OK 12 and 19 unassigned bacteria which is further grouped into two clusters with 98.9% and 97.6% similarity.



**Figure 2: Neighbour-joining phylogenetic tree of bacteria associated with infantile diarrhea in Port Harcourt**

#### 4. Discussion

There are some microorganisms in the gut that cannot be detectable using the conventional methods of bacteria isolation but they are responsible for a lot of infections such as diarrhoea. The aim of this research work is to molecularly characterize diarrhoeic stool samples of children less than five years in Port Harcourt, Nigeria. Two(2) approaches were used to characterise the microorganisms present in



diarrhoeic stool samples of children below 5 years in Port Harcourt, Nigeria. The conventional method and the molecular (16S rRNA sequence analysis) method. and there were major differences using both approaches.

#### **4.1. Bacteria isolated using conventional method.**

In this study using the conventional identification method it was established that there are 98(36.2%) cases of diarrhoea in children less than 5 years in Port Harcourt. Similar studies have been carried out by Duru and his colleagues in [27] that reported a 49% case of infantile diarrhoea in Port Harcourt.

In this study, *Escherichia coli*, *Salmonella* species, *Shigella* species, *Yersinia* species and *Vibrio* species were identified with *Escherichia coli* found to be the highest in prevalence followed by *Salmonella* species then *Shigella* species, *Yersinia* species while *Vibrio* species was the least prevalent. Other studies by Cajetan *et al.*, [28] in Abuja and Mbuthia *et al.*, [29] in Kenya have reported similar bacteria in infantile diarrhoea with *Escherichia coli* as the highest prevalent and *Vibrio* species the least prevalent. *E coli* is known as an important cause of epidemics and endemic diarrhea worldwide.

Belina and colleagues in [30] did a study on the occurrence of diarrheagenic pathogens and their coinfection profiles in diarrheic children under 5 years and tracked human contacts in urban and rural settings of Eastern Ethiopia and reported cases of *Escherichia coli*, *Salmonella* species, *Shigella* species, *Yersinia* species and *Vibrio* species. In a study of in vitro sensitivity profiles of enteric bacteria associated with diarrheic patients within Katsina metropolis, Katsina state, Nigeria carried out by Odewade, Fasogbon and Onyekachi in [31] similar bacteria were also identified.

Other studies have revealed *Shigella* species and *Salmonella* species in diarrhoeic stool samples of children less than 5 years. Tosisa, Mihret, Ararsa, Eguale and Abebe in [32] in their study of prevalence and antimicrobial susceptibility of *Salmonella* and *Shigella* species isolated from diarrheic children in Ambo town revealed *Shigella* species and *Salmonella* species in diarrhoeic stool samples of children less than 5 years.

The result of this work also agrees with a study in Nigeria in 2014, which reveals *Escherichia coli*, *Salmonella species*, *Shigella species*, *Yersinia species* and *Vibrio species* as prevalence bacteria isolated from childhood diarrhoeic samples [27]. The high occurrence of *Escherichia coli* could be due to the fact that it is very easy to identify to species level and it is the major facultative flora of the human intestine, which corroborates the study by Martinson and Walk, [33].

Different researchers have reported diarrhoea episodes to lack of food and water safety. Studies by Birhan, *et al.*, [34] have revealed diarrhoea episodes were caused by contaminated food and water. Agi *et al.*, [35] revealed that some drinking water sources in Port Harcourt did not meet the approved acceptable limits of the World Health Organization (WHO) for drinking water. Joseph and Curtis [36] have also stated likewise that there is a relationship between transmission of enteric bacterial pathogens and food vending practices. In this study the diarrhoea episodes could be as a result of the introduction of solid foods and increased exploration of their environment such as daycare centers or communal settings and the frequent handling of various objects and surfaces that can be contaminated. can make the children more susceptible to diarrhoea infection. Additionally, young children are more likely to ingest contaminated food or water, they may not have also fully mastered handwashing and hygiene practices with their immune system that is still developing makes them more susceptible to infections.

Studies by Nuraeni *et al.*, [37] have also showed important positive relationship between the reduction of childhood diarrhoea and availability of hand washing facilities.

In this study, a significant association was found between age and the prevalence of bacterial pathogens. Infants younger than 1 year had the highest prevalence, while infants aged 1 to 3 years showed a lower prevalence as compared to those younger than 1 year but prevalence was lowest in infants older than 3 years. This is in agreement with a similar study by Duru *et al.*, [38] carried on bacterial agents associated with infantile diarrhea and their antibiotics susceptibility pattern in Port Harcourt, South- South, Nigeria where they discovered that the prevalence of the bacterial infection was found to be more in infants of 0-12 months old than in the older infants. There was no statistically significant association between sex and the prevalence of diarrhoea-causing bacterial pathogens in this study. Female infants, 54 (37.0%) were positive for bacterial pathogens, while 92 (63.0%) were negative and 44 (35.5%) male infants tested positive while 80 (64.5%) tested negative as opposed to 25(49.0%) Females and 28(48.9%) in males in a similar study by Duru *et al.*,[38] . This might be due to the number of infants sampled; male infants sampled were 49 infants and 51 females in their study while in this study, male infants sampled was 124 and 146 for females.

#### **4.2 Antibiotic Susceptibility Pattern of the Isolates.**

In this study, the antibiotics sensitivity testing of the isolates to commonly used antibiotics revealed that the isolates showed different levels of antibiotic resistance (Table 1). All isolates showed some level of resistance to all antibiotics used in this study with 100% resistance to Septrin. *Escherichia coli*, *Shigella* species, *Yersinia* species and *Vibrio* species showed 100% resistance to Augumentin while *Yersinia*

*species* and *Vibrio* species showed 100% resistance to ampicillin. *Shigella* species was found to be resistant to septrin and augmentin and this is in agreement with studies by Okebugwu and his colleagues in [39] that carried out a study on types of bacteria associated with diarrhoeal cases among children in Akure, Nigeria and their antibiogram profile. *Salmonella* species in this study had varying forms of resistance to almost all the antibiotics used with 100% resistance to septrin in agreement with Okebugwu *et al.*, [39] who carried out a study on the types of bacteria associated with diarrhoeal cases among children in Akure, Nigeria and their antibiogram Profile.

A significantly high level of resistance to more than three of the antimicrobial agents used was detected especially in *Yersinia* species and *Vibrio* species and this agrees with the study by Odewade *et al.*, [31] which reveals a high level of antibiotic resistance among bacterial isolates obtained from stool samples of diarrheic patients as quite alarming and requiring urgent public health attention. All 5 isolates were not less than 80% sensitive to ciprofloxacin and gentamicin with *Shigella* species, *Yersinia* species and *Vibrio cholera* showing 100% sensitivity to gentamicin. There has been similar report of high level of sensitivity to Chloramphenicol and gentamicin in a study carried out by Duru and his colleagues in [38]. The highest level of resistance in all isolates was seen in septrin however, gentamicin displayed a better performance index to all isolates followed closely by ciprofloxacin. When a microorganism is resistant to 3 or more classes of antimicrobial agents, it is said to be multidrug resistant. The findings in this study revealed a significantly high level of multidrug resistance to antimicrobials used frequently for diarrhoea treatment in Port Harcourt. The high multidrug resistance found among the isolates may be as a result of the hospital's unnecessary prescription of antibiotics leading to over use. Children, especially those under 5, may be prescribed

antibiotics without proper consideration of age-specific dosing and potential impacts on their developing microbiome. Inadequate tailoring of antibiotic therapy to the specific pathogen and its resistance profile can also contribute to resistance development. Hospitals can also harbor resistant strains of bacteria, which may colonize or infect patients, particularly those with weakened immune systems or chronic conditions and these strains can spread from patient to patient, especially if infection control measures such as hand hygiene, inadequate disinfection of surfaces and medical equipment are not strictly followed. Not educating parents and caregivers about the importance of following prescribed treatments and the risks associated with antibiotic misuse can also be a factor as some parents may use leftover antibiotics from previous prescriptions for their children, which is not only inappropriate but can also contribute to resistance.

Resistance to antibiotics is a public health challenge worldwide because new resistance methods are coming up worldwide and affecting how common infections are treated and this can lead to death. It also increases the cost of health care and makes therapy ineffective.

#### **4.3 Phylogenetic analysis**

Of all the identified bacteria, only *Escherichia coli* was able to be isolated using the conventional culture based method of bacteria identification. A negative result may have been either true, because the organism was not present, or false, because the organism did not grow as a result of inadvertent inhibition by the growth medium used, specimen transport conditions compromising the organism, laboratory inefficiencies or other factors. The result in this suggests that several unknown species inhabit the human intestinal tract and that traditional culture-based methods underestimate the numbers of

stool samples in which specific enteric pathogens can be detected compared with those by the alternative molecular methods and this is in agreement with a study by Lindsay and colleagues [40] who compared four distinct detection technologies for the identification of pathogens in stools from children under 5 years of age in Gambia, Mali, Kenya, and Bangladesh and agreed that molecular technologies have a high potential for highly sensitive identification of bacterial diarrheal pathogens.

## 5. Conclusion

The high level of antibiotic resistance and inability of isolating a large number of microorganisms using the conventional method of bacteria identification makes it difficult to know the microorganisms present in a particular environment, their individual roles in causing infections and the actual treatment required for such infections. The aim of this research was to molecularly characterize diarrhoeic stool samples from children less than 5 years in Port Harcourt Nigeria.

Bacteria associated with childhood diarrhoea in Port Harcourt and their susceptibility pattern was determined. From this study, it can be concluded that *Escherichia coli*, *Salmonella* species, *Shigella* species, *Yersinia* species and *Vibrio* species are bacteria responsible for diarrhoea in children less than 5 years in Port Harcourt with *Escherichia coli* more common in our environment.

Our findings also demonstrated increase in multi drug resistance to the frequently used antibiotics in diarrhoea treatment in Port Harcourt. The high level of multidrug resistance found among the isolates may be as a result of lack of antibiotics stewardship, educational and behavioural factors and lack of hygiene and infection control practices in healthcare settings and communities. This study was able to isolate and identify

bacteria in diarrhoeic stool samples of children less than 5 years in Port Harcourt and also show their evolutionary inter relatedness. It is therefore recommended that the use of molecular diagnostic tools should be encouraged, there should be implementation of programs that promote the appropriate use of antibiotics, including guidelines for prescribing and monitoring. The Ministry of Health should review its Standard Treatment Guideline for diarrhoea treatment, and the use of septrin should be discouraged. Ciprofloxacin should be reserved for cases where other antibiotics are not effective or suitable due to potential risks in children under 5 years. Decisions about its use should be guided by careful consideration of the potential benefits and risks, with close monitoring of the child's response to treatment. The authorities concerned should promote health education campaigns to raise awareness about transmission of diarrhoea and dangers of poor hygienic practices.

**Conflict of Interest:** Not available

## References

- [1] Ratnakar, B. B., Patel, Y. R., & Rajabhushan, B. (2022). Feeding Practice during Diarrheal Episode among Children Aged between Six to Twenty four Months in Suryapet, Telangana, South India. *International Journal of Early Childhood Special Education*, 14(7).
- [2] Ugboko, H. U., Nwinyi, O. C., Oranusi, S. U., & Oyewale, J. O. (2020). Childhood diarrhoeal diseases in developing countries. *Heliyon*, 6(4), 1-12.
- [3] Hodge, J., Chang, H.H., Boisson, S., Collin, S. M., Peletz, R., & Clasen, T(2016). Assessing the association between thermotolerant coliforms in drinking water and diarrhea: an analysis of individual-level data from multiple studies. *Environmental Health Perspectives*, 2016;124(10):1560–1567.
- [4] Dekaboruah, E., Suryavanshi, M. V., Chettri, D., & Verma, A. K. (2020). Human microbiome: an academic update on human body site specific surveillance and its possible role. *Archives of microbiology*, 202, 2147- 2167.

- [5] Yu, D., Meng, X., de Vos, W. M., Wu, H., Fang, X., & Maiti, A. K. (2021). Implications of gut microbiota in complex human diseases. *International journal of molecular sciences*, 22(23), 12661.
- [6] Siddiqui, F. J., Belayneh, G., & Bhutta, Z. A. (2021). Nutrition and diarrheal disease and enteric pathogens. *Nutrition and infectious diseases: Shifting the clinical paradigm*, 219-241.
- [7] World Health Organization, (2015). *Progress on sanitation and drinking water: 2015 update and MDG assessment*. World Health Organization. Retrieved from [https://books.google.co.uk/books?hl=en&lr=&id=KFA0DgAAQBAJ&oi=fnd&pg=PA1&dq=World+Health+Organization.+\(2015\).](https://books.google.co.uk/books?hl=en&lr=&id=KFA0DgAAQBAJ&oi=fnd&pg=PA1&dq=World+Health+Organization.+(2015).) [Retrieved 20 August 2023].
- [8] Solijon o'g'li, A. S. (2024, May). Diarrhoea in Children, Causes and Symptoms. In *Interdisciplinary Conference of Young Scholars in Social Sciences (USA)* (Vol. 7, pp. 12-15).
- [9] Zhang, Y., Chen, T., Zhang, Y., Hu, Q., Wang, X., Chang, H., ... & Xia, Y. (2021). Contribution of trace element exposure to gestational diabetes mellitus through disturbing the gut microbiome. *Environment international*, 153, 106520.
- [10] Rogers, G. B., Keating, D. J., Young, R. L., Wong, M. L., Licinio, J. & Wesselingh, S. (2016). From gut dysbiosis to altered brain function and mental illness: mechanisms and pathways. *Molecular Psychiatry*, 21(6), 738-748.
- [11] Liu, Z., Li, N., Dang, Q., Liu, L., Wang, L., Li, H., & Han, X. (2023). Exploring the roles of intestinal flora in enhanced recovery after surgery. *Iscience*, 26(2).
- [12] Ohkusa, T., Nishikawa, Y., & Sato, N. (2023). Gastrointestinal disorders and intestinal bacteria: advances in research and applications in therapy. *Frontiers in Medicine*, 9, 935676.
- [13] Adagbada, A. O., Adesida, S. A., Nwaokorie, F. O., Niemogha, M. T. & Coker, A. O. (2012). Cholera epidemiology in Nigeria: an overview. *Pan African Medical Journal*, 12(1), 23-29.
- [14] Elimian KO, Musah A, Mezue S, Oyeibanji O, Yennan S, Jinadu A, et al. Descriptive epidemiology of cholera outbreak in Nigeria, January- November 2018: Implications for the global roadmap strategy. *BMC Public Health*. 2019;19(1264):11.
- [15] Elimian KO, Musah A, Ochu CL, Onwah SS, Oyeibanji O, Yennan S, et al. Identifying and quantifying the factors associated with cholera-related death during the 2018 outbreak in Nigeria. *Pan Afr Med J*. 2020;37(368):13.
- [16] Odo, C., Onalu, C., Nwatu, U., Nwafor, N., & Ebimbo, S. (2023). Factors associated with the prevalence of diarrhoea among children in rural areas of Enugu



State, Nigeria: Practice considerations for social workers. *International Social Work*, 66(2), 518-533

[17] World Health Organization, (2024). Diarrhoeal disease. Retrieved from <https://www.who.int/news-room/fact-sheets/detail/diarrhoeal-disease> [Retrieved 21 July 2024].

[18] Kanu N, Osinubi M, Nwadiuto I, Okeke C. Cholera outbreak in Andoni local government area, Rivers State, Nigeria; January 2015: The role of hand washing with soap. *Niger J Med*. 2018;27(2):140-7.

[19] Hitch, T. C., Afrizal, A., Riedel, T., Kioukis, A., Haller, D., Lagkouvardos, I., ... & Clavel, T. (2021). Recent advances in culture-based gut microbiome research. *International Journal of Medical Microbiology*, 311(3), 151485.

[20] Ito, T., Sekizuka, T., Kishi, N., Yamashita, A., & Kuroda, M. (2019). Conventional culture methods with commercially available media unveil the presence of novel culturable bacteria. *Gut Microbes*, 10(1), 77-91.

[21] Qian, X., Liu, A., Liang, C., He, L., Xu, Z., & Tang, S. (2022). Analysis of gut microbiota in patients with acute myocardial infarction by 16S rRNA sequencing. *Annals of translational medicine*, 10(24).

[22] Daniel, W. W. (1999). *Biostatistics: A Foundation for Analysis in the Health Sciences*. 7th edition. New York: John Wiley & Sons.

[23] Cowan, S. T. & Steel, K. J. (2003). *Cowan and Steel's manual for the identification of medical bacteria*. England: Cambridge University Press

[24] Bauer, A. W., Kirby, W. M. M., Sherris, J. C., & Turck, M. (1966). "Antibiotic susceptibility testing by a standardized single disk method." *American Journal of Clinical Pathology*, 45(4), 493-496.

[25] Clinical and Laboratory Standards Institute. (2020). *Performance standards for antimicrobial susceptibility testing* (30th ed.; M100-Ed30). CLSI. Available [https://clsi.org/media/2704/m100ed30\\_sample.pdf](https://clsi.org/media/2704/m100ed30_sample.pdf). Retrived 18 July, 2024.

[26] Saitou, N., & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular biology and evolution*, 4(4), 406-425.

[27] Duru, C., Olanipekun, G., Odili, V., Kocmich, N., Rezac, A., Ajose, T. O., ... & Obaro, S. (2020). Molecular characterization of invasive Enterobacteriaceae from pediatric patients in Central and Northwestern Nigeria. *PLoS One*, 15(10), 1-11.

[28] Cajetan, I. C. I., Nnennaya, I. R., Casmir, A. A., & Florence, I. N. (2010). Enteric bacteria pathogens associated with diarrhoea of children in the federal capital territory Abuja, Nigeria. *New York Science Journal*, 3(1), 62-69.

- [29] Mbuthia, O. W., Mathenge, S. G., Oyaro, M. O., & Ng'ayo, M. O. (2018). Etiology and pathogenicity of bacterial isolates: a cross sectional study among diarrheal children below five years in central regions of Kenya. *Pan African Medical Journal*, 31(1), 1-15.
- [30] Belina, D., Gobena, T., Kebede, A., Chimdessa, M., Hailu, Y., & Hald, T. (2023). Occurrence of Diarrheagenic Pathogens and Their Coinfection Profiles in Diarrheic Under Five Children and Tracked Human Contacts in Urban and Rural Settings of Eastern Ethiopia. *Microbiology Insights*, 16.
- [31] Odewade, J., Fasogbon, A., & Onyekachi, F. (2020). In Vitro Sensitivity Profiles Of Enteric Bacteria associated with diarrheic patients Within Katsina Metropolis, Katsina State, Nigeria. *Fudma Journal of Sciences*, 4(2), 178-182.
- [32] Tosisa, W., Mihret, A., Ararsa, A., Eguale, T., & Abebe, T. (2020). Prevalence and antimicrobial susceptibility of Salmonella and Shigella species isolated from diarrheic children in Ambo town. *BMC pediatrics*, 20, 1-8.
- [33] Martinson, J. N., & Walk, S. T. (2020). Escherichia coli residency in the gut of healthy human adults. *EcoSal plus*, 9(1), 10-1128.
- [34] Birhan, T. A., Bitew, B. D., Dagne, H., Amare, D. E., Azanaw, J., Genet, M., ... & Maru, T. (2023). Prevalence of diarrheal disease and associated factors among under-five children in flood-prone settlements of Northwest Ethiopia: A cross-sectional community-based study. *Frontiers in pediatrics*, 11, 1056129.
- [35] Agi, V. N., Ollor, A. O., Azike, C. A., & Ifeakandu, N. C. (2022). Comparative analysis of the microbial content of some drinking water sources in Port Harcourt Metropolis, Rivers State, Nigeria. *JAMB*, 22(12), 53-64.
- [36] Joseph, S., & Curtis, M. A. (2021). Microbial transitions from health to disease. *Periodontology 2000*, 86(1), 201-209.
- [37] Nuraeni, A., Rosiah, R., Supendi, M. P. K., & Efendi, A. (2022). the Relationship of Hand Washing Behavior Towards Diarrhea Cases in School-Age Children. *Journal of Vocational Nursing*, 3(2), 105-109.
- [38] Duru, E. E., Agbagwa, O. E., & Umoren, F. E. (2014). Bacterial agents associated with infantile diarrhea and their antibiotics susceptibility pattern in Port Harcourt, South-South, Nigeria. *J Medical Sci Public Health*, 3(1), 1-12
- [39] Okebugwu, Q. C., Adebolu, T. T., & Ojo, B. A. (2017). Types of Bacteria Associated with Diarrhoeal Cases among Children in Akure, Nigeria and Their Antibigram Profile. *Journal of Advances in Microbiology*, 2(2), 1-10.
- [40] Lindsay, B., Pop, M., Antonio, M., Walker, A. W., Mai, V., Ahmed, D., ... & Nataro, J. P. (2013). Survey of culture, goldengate assay, universal biosensor assay,

and 16S rRNA gene sequencing as alternative methods of bacterial pathogen detection. *Journal of Clinical Microbiology*, 51(10), 3263-3269.

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