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Determination of Extended Spectrum Beta-lactamase (ESBL) Producing Enterobacteriaceae from Children infected with Diarrhea in Kano, Northern Nigeria

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ABSTRACT: Diseases caused by microorganisms are currently causes of morbidity and motility worldwide. The study was aimed to determine the prevalence of extended spectrum beta lactamase producing Enterobacteriaceae isolate from children infected with diarrhea in Kano, Northern Nigeria. Two hundred and fifty (250) stool samples of the infected children were obtained from Microbiology department of Murtala Muhammad Specialist Hospital. Multidrug resistant (MDR) isolates were determined using disc diffusion method. The multidrug resistant isolates were evaluated using antibiotic disc of ceftriaxone and cefotaxime respectively. The ESBL producing isolates were confirmed by means of double disc synergy test. Molecular method was employed to determine the resistant gene. A sum of five hundred and twenty three (523) enteric bacteria were recovered from stool 250 samples which include *E. coli* as the predominant species (36.9%), *Salmonella* (17.6%), *Shigella* (12.0%), *Klebsiella* (8.4%), *Proteus* (7.5%), *Enterobacter* (8.2%), *Citrobacter* (5.4%) and *Serratia* (4.0%). From the result, the isolates resistant to antibiotics in the presence is considerably low as only 5.2%

(27 out of 523) isolates were multi drug resistant (MDR) isolates and only 11 (40.7 %) out of the 27 MDR isolates were ESBLs positive and 16 (59.3%) were non ESBLs producing isolates. However, 9 isolates out of the 11 of the isolates were positively confirmed as ESBL producing isolates confirmed to be ESBLs producers. Molecular identification confirmed the presence of DNA bands of the expected size for CTX-M, TEM and SHV resistant genes. It is concluded that the multidrug resistant bacteria were present in diarrhea of infected children.

Keywords: *Children, diarrhea, Enterobacteriaceae, Extended spectrum beta lactamase*

Introduction

The resistant to antibiotics by bacteria especially Gram negative bacillus are mostly created by widespread and often indiscriminate use of antibiotics. In addition, the development of health support technology, new surgical instrument as well as antibiotic regiment have also provided the new portal of entry which comprises host defenses. Diseases caused by microorganisms are currently causes of morbidity and motility worldwide [1]. The onset of resistance to antimicrobial agents has increased significantly to the influence of diseases in the number of infectious disease and the amount of infection and increase health care cost [2].

Since its first description in 1983, extended spectrum beta lactamase have become endemic in several countries and as well as spread worldwide [3,4]. The distribution of the resistance was mainly due to ESBL encoding genes that are transmitted by mobile genetic elements such as plasmid that make it easier for their dissemination [5]. Enterobacteriaceae family such as *Escherichia coli* are mostly commensal in nature and are present in the gut of human and various elements and are important reservoir of resistance genes leading to ESBL producing isolates among community members [6]. This resulted in both community and hospital infections of ESBL responding genes were acquired by infectious bacterial, or if not pathogenic producing isolates became infectious [7].

Enterobacteriaceae members are widely distributed in nature and most of them were living in the intestinal tract of human and other animals where they act as commensal organism or cause enteric diseases such as diarrhea. However, this organisms play

vital role biotechnological microorganisms, plants pathogens and for production of proteins [8]. This indicated that not all Enterobacteriaceae are pathogen, only a small group of species are considered strict pathogens [9]. The Gram-negative bacilli of the genera *Escherichia, Klebsiella, Enterobacter, Serratia, Salmonella, Shigella*, *Proteus* and others, they are members of the normal intestinal flora of humans and animals, including insects and may be isolated from a variety of environmental sources [10]. With the exception of *Proteus,* they are sometimes collectively referred to as the coliform bacilli because of some shared properties, particularly the ability of most species to ferment sugar lactose. Some microorganism seems to be harmless and commensals are today known to be responsible for some diseases worldwide [10]. Certain numbers of species such as *Klebsiella pneumoniae, Proteus mirabilis, Enterobacter cloacae*, *S. marcescens* and *Escherichia coli* are considered to be responsible for most of the infections produced by Gram negative bacilli [11].

However, colonization of host by extended spectrum beta lactamase (ESBL) producing isolates such as *Escherichia coli, Klebsiella pneumoniae* and *Enterobacter* is one of the major risk factor for infection with antibiotic resistant [12]. This menace represent a great challenge due to limited antibiotic option including increase morbidity and mortality rates as well as increasing prolonged hospitalization [13]. In view of the above reason, the study aimed to determine the prevalence of extended spectrum beta lactamase (ESBL) producing Enterobacteriaceae from diarrhea infected children in Kano State, Nigeria.

Methodology

Study sites

The stool samples of the study subjects were obtained from Microbiology department of Murtala Muhammad Specialist Hospital Kano while the screening of ESBL producing isolates was conducted at department of Microbiology of Aminu Kano Teaching Hospital, Kano.

Ethical approval for the study

The ethical clearance of this study was obtained from ethical committee of Kano State Ministry of Health Kano, Kano State in conjunction with ethical committee of MMSH. The reference number for the approval is NHREC/17/03/2050

Samples collection

Two hundred and fifty (250) stools sample from gastrointestinal patients attending Murtala Muhammad Specialist Hospital (MMSH) were used in this study. Samples were collected using a sterile universal container. Patients' diarrhea samples were collected, and using conventional microbiological procedures, they were processed for bacterial isolation and identification according to methods described by Cheesbrough [14].

Bacteria Isolation and Identification

Isolation of bacteria was conducted according to method of Cheesbrough [14]. During the process MacConkey and *Salmonella-Shigella* agar plates was used. A loop full of the stool sample was streaked and incubated in the presence of air for 24 hours at 37^0 C. Bacterial growth was monitored for colony appearance and morphology following incubation. Until a pure colony was achieved, each colony was re-inoculated into newly made agar plates. Identification of the isolates was done by Gram staining it and then undergoing other biochemical tests like indole, methyl-red, Voges Proskauer, citrate utilization, and motility test [15].

Multi Drug Resistance (MDR) Screening

The identified bacteria isolates were tested for multidrug resistance using the method of Bauer et al. [16] by means of agar disc diffusion method. The isolates were inoculated on Mueller Hinton agar plates. Plates were incubated with some commonly used antibiotics at 37^0C for 24 hours. Susceptibility to the antibiotics by the isolates was recorded based the Clinical and Laboratory Standard Institute (CLSI) [17]. Resistance of isolates to three or more classes of antibiotics was used as the criteria to be qualified as multidrug resistant (MDR) isolate [18].

Screening for Extended Spectrum Beta Lactamase

For the screening of ESBL among MDR isolates, method of Wayne [19] was adopted using antibiotic sensitivity disc of 30 µg of cefotaxime and ceftriaxone respectively. Based on the guidelines, isolates showing zone of inhibitions of < 22 mm and < 27 mm for cefotaxime and ceftriaxone discs respectively were considered possible ESBL producers. Isolates were confirmed using double disc synergy test a described by Harwalkar *et al*. [20]. In the process, amoxicillin/clavulanic acid disc was placed

adjacent to cefotaxime and ceftriaxone discs and observe for synergy between the amoxicillin/clavulanic acid disc and the cephalosporins [21]

Molecular Characterization of resistance gene of the Isolates

Specific primers for the CTX-M (F: TTTGCGATGTGCAGTACCAGTAA, R: CGATATCGTTGGTGGTGCCATA), SHV (F: CTTTATCGGCCCTCACTCAA, R: AGGTGCTCATCATGGGAAAG) and TEM (F: CGCCGCATACACTATTCTCAGAATGA, R: ACGCTCACCGGCTCCAGATTTAT) resistance genes were designed using NCBI Primer-BLAST and was used in the study [22].

Results

Distribution of Bacteria Isolated from Study Subjects

The distribution of the bacteria isolated from the stool samples of the subjects is presented in Table 1. The result showed that *E. coli* has the highest frequency with total of 193 occurrence (36.9%), followed by *Salmonella* with total frequency of 92 (17.6%), then *Shigella* with frequency of 63 (12%). The number of isolates recorded by *Citrobacter* and *Serratia* in this study were 28 (5.4%) and 21 (4%)

Table 1 Distribution of bacteria isolated from the study subjects

S/N	Isolates	No. of isolates	Percentage (%)	*P*-value
1	*E. coli*	193	36.9	0.00001*
2	*Salmonella* spp	92	17.6	
3	*Shigella* spp	63	12.0	
4	*Klebsiella* spp	44	8.4	
5	*Proteus* spp	39	7.5	
6	*Enterobacter* spp	43	8.2	
7	*Citrobacter*	28	5.4	
8	*Serratia*	21	4.0	
	Total	**523**	**100**	

Key: * = The result is significant at $p<0.05$

Distribution of Multi-drug Resistant Isolates from Study Subjects

The determination of Multi Drug Resistant (MDR) isolates is presented in Table 2. The result showed that 523 Enterobacteriaceae isolates identified were subjected to multi drug resistance (MDR) test, of which 27 (5.2%) isolates were resistant to four

or more antibiotics tested while 496 (94.8%) of the isolates were resistant to less than four antibiotics.

Table 2: Distribution of Multi Drug Resistant (MDR) Isolates from Study Subjects

Isolates	**No. identified**	**Percentage (%)**	***P*-value**
MDR	27	05.2	0.00001*
NMDR	496	94.8	
Total	**523**	**100**	

Key: * = The result is significant at p<0.05 MDR = Multi-drug Resistant, NMDR = Non Multi-drug Resistant

Phenotypic Detection of Extended Spectrum Beta Lactamase producing Isolates

The phenotypic determination of Extended Spectrum Beta Lactamase (ESBLs) producing Isolates is presented in the Table 3. The Multidrug resistant isolates (n=27) were further subjected to Extended Spectrum Beta Lactamases detection using Clinical Laboratory Standard Institute (CLSI) break point for screening test, where only 11 (40.7%) were found to be ESBLs positive and 16 (59.3%) were non ESBLs producing isolates. After the confirmatory test for the ESBLs detection, only 9 isolates were positively confirmed to be ESBLs producers by DDST

Table 3 Phenotypic Determination Extended Spectrum Beta Lactamase producing Isolates

Isolates	**No. screened**	**No. positive**	**No. confirmed**	**Prevalence (%)**	***P*-Value**
E. coli	11	5	3	18.5	0.9594*
Klebsiella spp	1	1	1	3.7	
Salmonella spp	8	2	2	7.4	
Shigella spp	7	3	3	11.1	
Total	**27**	**11**	**9**	**40.7**	

Key: * = There is no statistically significant difference in the number of ESBL producing isolates confirmed in the study. Hence, the result is statistically significant at p<0.05

Molecular Characterization of ESBL producing Genes

The presence of DNA bands of the expected size for SHV, TEM and CTX-M indicated the presence of these resistance genes in the bacterial isolates. Lane 1 is 3000bp ladder; lanes 1, 2 and 3 are for CTX-M, lanes 4, 5 and 6 are for TEM while 7, 8 and 9 are for SHV. Sample 1, 4 and 7 are *E. coli*, 2, 5 and 8 are *Shigella*, 3, 6 are *Salmonella* and 9 is *Klebsiella* as shown in plate 1.

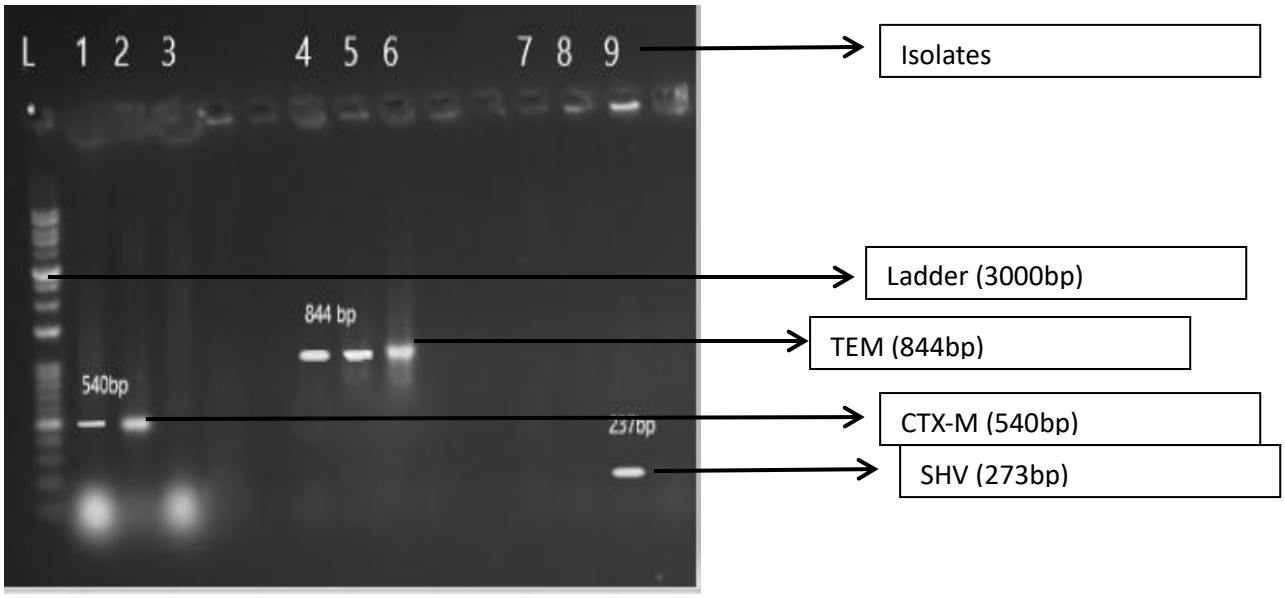

Plate 1: Molecular detection of the resistant gene.

Discussion

A total of 523 enteric bacteria were isolated from stool 250 samples of the subjects collected in the present study. The result shows eight bacterial genera belonging to Enterobacteriaceae family were identified. They include *E. coli, Salmonella, Shigella, Klebsiella, Proteus, Enterobacter, Citrobacter* and *Serratia*. In the present study, *E. coli* was found as the predominant isolate with (36.9%) followed by *Salmonella* with total frequency of 92 (17.6%), then *Shigella* with frequency of 63 (12%). The number of isolates recorded by *Citrobacter* and *Serratia* in this study were 28 (5.4%) and 21 (4%) respectively. Finding of this study was in conformity with that of Sang *et al*. [23]; Amukoshi [24] and Adeh *et al*. [25] who all found *E. coli, Salmonella* and *Shigella* as major enteric bacteria associated with diarrhea in children. The present study also justifies the finding of Abdullahi *et al*. [26] in Kano, Nigeria.

In the present study, 193 samples out of 250 were positive for *E. coli* and this is accounted for 77.2%. This finding is lower than the prevalent rate of 83.21% reported from similar study conducted in Abakaliki in South eastern Nigeria [27]. On the other hand, the finding is higher than 34% recorded by Sule *et al*. [28] in Kaduna, Northwestern Nigeria. In the study, E. coli was the most prevalent isolate identify. This finding correlate with the finding of Akingbde *et al*. [29] and that of Clarke [30] in which both reported E. coli as the most dominant bacterial pathogen associated

with diarrhea among children. According to Bahl *et al*. [31], enteropathogenic *Escherichia coli, Campylobacter jejuni, Shigella* spp, *Salmonella* spp and *rotavirus* are the most common cause of diarrhea among children in Nigeria. This statement justified the finding of the present study

The isolation and identification of bacteria in stool of diarrhea patient may not directly implies that the infection is related to that particular isolate, but can provide useful information on the control of the infection and can complement case-control studies on diarrhea etiology [15]. The present study reveals that *E. coli* (36.9%) was observed to be the most prevalent among the isolates within the study sites followed by *Salmonella* spp (34.3%), *Shigella* (12%), *Klebsiella* (8.4%) with *Serratia* (4%) being the least occurring isolate. The result of this study was in conformity with the finding of Bonkoungou *et al*. [32] who found *Escherichia coli* (24%) as the most prevalent enteric bacteria among those causing diarrhea among children in Ougadougou, Burkina Faso, *Salmonella* (9%), and *Shigella* spp. (6%). This result disagree with the finding of Ansari *et al*. (2012) who found *Shigella* (4.6%) was the most prevalent species among enteric bacteria causing diarrhea in children followed by *Escherichia coli* 12 (2.3%) and *Salmonella* spp 10 (1.9%).

The overall resistance of the isolates to antibiotics in the presence is considerably low as only 5.2% (27 out of 523) isolates were multi drug resistant (MDR) isolates i.e. resistant to four or more antibiotics tested while 496 (94.8%) of the isolates were resistant to less than four antibiotics. This figure was less than that found in Bangladesh [33] who reported 15% and in Iran [34] who reported less than 30% MDR among enteric bacteria associated with diarrhea in children. Lower prevalence of MDR among enteric bacteria in this study may be attributed to the fact that most of the children lack history of using antibiotics. It can be deduce that the prevalence of multi-drug resistant bacteria among children infected with diarrhea might be a significant cause of severe and prolonged hospital stay [35]

From the study, the 27 multidrug resistant enteric isolates obtained were further subjected to Extended Spectrum Beta Lactamases detection using Clinical Laboratory Standard Institute (CLSI) break point for screening test, only 11 (40.7 %) were found to be ESBLs positive and 16 (59.3%) were non ESBLs producing isolates. After the confirmatory test for the ESBLs detection, only 9 isolates out of

the 11 isolates screened were positively confirmed to be ESBLs producers based on double disc synergy test (DDST) while 2 were non ESBLs producers based on DDST. The result of the present study is higher than that of Yahaya *et al*., [36], which reported that out of 439 isolates screened for ESBL production, the results based on CLSI breakpoint test showed that 147 (33.5%) were screened to be ESBL producers but only 121 (23.6%) were confirmed by double disk synergy test. Also, a report by Yusha'u *et al*. [37], which reported that out of the Enterobacteriaceae isolates screened for ESBL, the results showed that 76 (66.7%) were ESBLs producers while only 47 (41.2%) were confirmed to be ESBLs producers which is higher to the findings of the present study. The prevalence of ESBLs producers in this study could be as result of the patients having prior exposure of antibiotics which could be a predictor factor for development of multi drug resistance as well as ESBL production. In the present study, the majority of the ESBL producers were *E. coli* and *Shigella* spp (3 isolates each) followed by *Salmonella* spp (2 isolates) while *Klebsiella* spp has 1 isolate. This finding correlates with findings of other studies by Yusuf [38] where *E. coli* was the common ESBL producer. Increase in ESBL producers may be attributed to increase in resistance to beta lactam antibiotics. On the other hand, increasing resistance to third-generation cephalosporins has become a cause for concern about *Enterobacteriaceae* [39].

Conclusion

Fromm the study, a total of 523 enteric bacteria were isolated from stool 250 samples which include *E. coli* as the predominant species (36.9%), *Salmonella* (17.6%), *Shigella* (12.0%), *Klebsiella* (8.4%), *Proteus* (7.5%), *Enterobacter* (8.2%), *Citrobacter* (5.4%) and *Serratia* (4.0%). The overall resistance of the isolates to antibiotics in the presence is considerably low as only 5.2% (27 out of 523) isolates were multi drug resistant (MDR) isolates and only 11 (40.7 %) out of the 27 MDR isolates were ESBLs positive and 16 (59.3%) were non ESBLs producing isolates. However, 9 isolates out of the 11 isolates were positively confirmed to be ESBLs producers. Molecular identification confirmed the presence of DNA bands of the expected size for CTX-M, TEM, and SHV resistance genes in the bacterial isolates.

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