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A study of bacterial pathogens associated with diarrhoea in children under 2 years in Ile-Ife, Nigeria

Akinwumi F. O.[1*], Igbeneghu O. A.[1], Oyelami O. A.[2] and Lamikanra A.[1]

[1]Department of Pharmaceutics, Faculty of Pharmacy, Obafemi Awolowo University, Ile-Ife, Osun State, Nigeria.
[2]Department of Paediatrics, Faculty of Clinical Sciences, College of Health Sciences Obafemi Awolowo University, Ile-Ife, Osun State, Nigeria.

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Diarrhoea, a major contributor to high mortality in children may be caused by an array of bacterial, viral or parasitic pathogens acting individually or synergistically. This study identified the bacterial pathogens associated with diarrhoea in children aged 0-24 months, established the frequency of diarrhoea caused by each pathogen encountered in diarrhoea and determined the antibiotic resistance pattern of the children's faecal isolates obtained to some commonly used antibiotics in the study environment. A total of 107 children within the age range 0-24 months presenting with diarrhoea and 115 apparently healthy children were recruited into this study. Stool samples or rectal swabs were collected and cultured using standard microbiological procedures. Recovered isolates were tested against commonly used antibiotics using disc diffusion method. The isolation of enteric pathogens was found to be significantly ($p<0.0001$) higher among diarrhoeal children. *Campylobacter* spp. was the most frequently isolated enteric pathogen among diarrhoeal (17.8%) and apparently healthy (16.5%) children. There was no significant ($p > 0.05$) difference in the isolates recovered from both diarrhoeal and control subjects. The isolates were found to be multiply resistant to all the antibiotics tested. However, the incidence of resistance to commonly-used antibiotics was found to be less than 50%.

Key words: Diarrhoea, children, bacterial pathogens, antibiotic resistance, Ile-Ife.

INTRODUCTION

As defined by the WHO (2017) diarrhoea is a condition in which the patient passes watery stools which take the shape of its container on three occasions within a period of twenty-four hours. The danger in this condition lies in its ability to cause severe dehydration as failure to reverse the loss of fluids results in death, especially in children under two years, who are particularly vulnerable to uncontrolled fluid loss. It is only to be expected therefore that UNICEF/WHO (2009) and Fischer et al. (2012) have reported that nearly half of the children dying from all causes in Africa die of diarrhoea and reports by Babaniyi (1991), Alabi et al. (1998), Akinnibosun and Nwafor (2015) and the Centre for Disease Control, CDC (2016) from Nigeria indicate that more than 315,000 of the 525,000 children of pre-school age who die, die of diarrhoea disease. This is because the conditions which

*Corresponding author. E-mail: florenceakinwumi@gmail.com.

enhance the spread of microbial contaminants in food and drinks consumed are prevalent within communities in developing countries such as Nigeria. For this reason, it is important that various aspects of diarrhoeal disease should be the subject of continued investigation.

Whilst it is true that diarrhoea may be caused by conditions which are not related to underlying disease such as malnutrition, taking a liquid diet, food intolerance, stress, anxiety and even the liberal use of laxatives (WHO, 2017), by far the most frequent causes of diarrhoea are viruses, bacteria and parasites which are present in food and drinks including untreated water. Various studies that have been carried out in Nigeria by Ayolabi et al. (2012); Imade and Eghafona (2015) and Junaid et al. (2011) have shown that viruses are the most frequently encountered diarrhoea pathogens within their study environment. Parasites have also been shown by Farthing et al. (2013) to be significant diarrhoea pathogens. The most frequently studied diarrhoea pathogens in Nigeria are however bacteria. At various times, Lamikanra et al. (1989a); Ogunsanya et al. (1994); Okeke et al. (2003); Akinkunmi and Lamikanra (2010); and Oloruntoba et al. (2014) have identified various species of bacteria as being involved in cases of diarrhoea in Nigeria. Over the years the bacterial agents associated with childhood diarrhoea in Ile-Ife where the present study was carried out include various diarrheogenic strains of *Eschcericia coli* by Lamikanra et al. (1996), Okeke et al. (2000) as well as Igbeneghu (2009); *Salmonella* species by Omololu-Aso et al. (2010); *Shigella* species by Abdu et al. (2014); and *Staphylococcus aureus* by Akinkunmi and Lamikanra (2012). Olusanya et al. (1983) as well as Aboderin et al. (2002) have also reported the isolation of *Campylobacter* from the faeces of children with diarrhoea within the study environment.

In all the studies mentioned above, the workers have investigated the presence of only one pathogen in their samples but it is not unlikely that faecal samples collected from children with diarrhoea would contain more than one pathogen and that the outcome of infestation may depend on the interaction between the pathogens that are present at any given time within the patient. In the present study therefore, an attempt was made to study the identity of all bacterial and fungal entities associated with diarrhoea in children under the age of two years in Ile-Ife.

The problem of antibiotic resistance within communities in developing countries such as Nigeria is huge and growing making it imperative to study this phenomenon at every opportunity. The study of antibiotic resistance in these diarrhoeal organisms of bacterial origin is important from the point of view of the fact that at least, such organisms may act as a reservoir for antibiotic resistance genes within the gastrointestinal tract and these genes may be passed on horizontally to more virulent and invasive organisms in the course of an infection. Previous studies by Okeke et al. (1999); Onanuga et al. (2014) and Akinnibosun and Nwafor, (2015) report the high prevalence of antibiotic resistances within communities in Nigeria and other developing countries, hence the importance of assessing the incidence of antibiotic-resistant organisms in this study.

MATERIALS AND METHODS

Ethical approval

Approval (ERC/2012/10/04) to undertake the study was obtained from the Ethical Research Committee of the Obafemi Awolowo University Teaching Hospitals Complex, Ile-Ife, Nigeria and informed consent was obtained from the children's parents or guardians before enrollment into the study.

Sampling

The study was conducted in Ile-Ife, Osun state, Nigeria, a town which lies between latitudes 7°-8° north of the equator and longitudes 4°-5° east of Greenwich. Administratively, it consists of two local government areas (LGAs); Ife central and Ife east LGAs. Subjects recruited attended one of two Health Centres in Ile-Ife, Osun state, Nigeria: The Obafemi Awolowo University Teaching Hospital (Urban Comprehensive Health Centre) Eleyele, in Ife Central Local Government Area and State Hospital, Okeogbo in Ife East Local Government Area. Stool or rectal swabs were collected from a total of 107 children with diarrhoea and 115 healthy controls within a period of three months using sterile cotton applicators. All samples were processed immediately after collection in the laboratory. All children up to 2 years of age were included in the study if they had acute diarrhoea characterized by frequent watery stools with or without blood or mucus (Baqui et al., 1991). Diarrhoea was diagnosed by nurses and/or doctors attending to the children in the health facilities and control subjects were apparently healthy children of the same age attending immunization clinics at the same health centres.

Isolation and identification of bacteria

The samples were cultured on routine, differential and selective media for bacterial cultivation in order to isolate bacterial enteropathogens. MacConkey agar, Eosin methylene blue agar, Mannitol Salt agar, *Campylobacter* selective agar (Butzler), and *Salmonella*-*Shigella* agar were used for the isolation of bacterial pathogens. Sabouraud Dextrose agar was used for the isolation of fungi (All from Oxoid Ltd, UK). Organisms producing colonies after 24 or 48 h incubation were sub-cultured onto Nutrient agar for further identification by standard biochemical tests and their characteristics were compared with those of known taxa, as described by Jolt et al. (1994); Cheesebrough (2006), and Oyeleke and Manga (2008).

Antibiotic susceptibility test

Bacterial isolates were examined for their susceptibility to 8 commonly prescribed and available antibiotics (Oxoid Ltd, UK) in the study environment namely: ampicillin/sulbactam (20 µg), erythromycin (15 µg), ciprofloxacin (5 µg) tetracycline (30 µg), chloramphenicol (30 µg), ceftriaxone (30 µg), trimethoprim (5 µg) and gentamicin (10 µg). The standard agar diffusion method (CLSI,

Table 1. Organisms isolated from stool samples.

Bacterial isolates	No recovered n=107 ill subjects	No recovered n=115 healthy subjects	p-value
Campylobacter spp.	69	46	0.0003*
Staphylococcus aureus	64	42	0.0007*
Escherichia coli	36	47	0.3326
Salmonella spp.	32	24	0.1218
Shigella spp.	27	19	0.1001
Coagulase negative *Staphylococcus*	31	15	0.0029*
Klebsiella spp.	15	12	0.5383
Enterobacter spp.	15	8	0.1217
Citrobacter spp.	10	6	0.3015
Proteus mirabilis	12	4	0.1395
Pseudomonas aeruginosa	6	3	0.5014
Serratia marcescens	7	0	0.0054*
Fungal isolates			
Candida spp.	54	47	0.1367
Aspergillus niger.	10	6	0.3015
Total	388	279	P<0.0001*
Total Bacterial Isolates	**324**	**226**	**P<0.0001***

*- significant p- value.

2012) was employed. A distinct colony of each test organism taken from a nutrient agar culture was inoculated into 2ml of sterile water using a sterile loop. The suspension was thoroughly mixed with a spin mixer. The resulting suspension was applied to the surface of over-dried Mueller Hinton agar (Oxoid Ltd, UK) and spread evenly with a sterile cotton-tipped applicator (Sterilin Ltd, Middlesex, UK). The inoculated plates were incubated at 37°C for 20 min for acclimatization and growth of the inocula. Selected antibiotic discs were then lightly but firmly pressed unto the surface of the agar using a multi-antibiotic disc dispenser. The plates were then refrigerated at 4°C for 30 min to ensure proper diffusion of the antibiotics. All plates were incubated at 37°C for 18 h. The diameters of inhibition zones were measured in millimeters and interpreted according to CLSI (2012) manual. *E. coli* ATCC 25922 was used as control strain.

Statistical analysis

The Statistical Package for Social Scientists (SPSS, version 16.0) was used for the analysis of the data obtained. Two-way ANOVA test was used to determine the level of significance of the test organisms at 95% confidence limits or 5% level of significance.

RESULTS

Incidence of diarrhoea

During the study, a total of 222 children between the ages of 0 to 24 months were recruited from two health facilities, one from Ife Central Local Government Area and the other from Ife East Local Government Area, both in Osun East Senatorial District. Of the 222 children (79 from OAUTHC and 143 from Okeogbo) involved, 107 were diagnosed with diarrhoea and 115 were healthy children receiving immunization at the health facilities.

In this study, 30.8% of the children with diarrhoea were within the age range of 13 to 18 months. Of the diarrhoeal subjects, 60.7% were male and 39.3% of them were female and of the healthy subjects, 43.5% were male and 56.5% were female. The age distribution of the subjects was as follows: 27 (25.2%) aged 0 to 6 months, 24 (22.4%) aged 7 to 12 months, 33 (30.8%) aged 13 to 18 months and 23 (21.5%) aged 19 to 24 months. The ratio of males to females was approximately 3:2. Fifty-two (48.6%) of those with diarrhoea had mucous in their stool while 5 (4.7%) had visible blood in their stool samples.

Recovery of enteric isolates from stool specimens

As shown in Table 1, 388 isolates were recovered from diarrhoea subjects and 279 isolates from control (apparently healthy) subjects. Although, there was no significant difference in the types of organisms isolated, there was a significant difference in the number of organisms isolated from subjects in either group ($p < 0.0001$). (Table) 1, shows the different organisms that were isolated. As shown in the table, *Campylobacter* spp. with a total of 115 (17.2%) isolates was the most commonly encountered organism. Other isolates identified were *Staphylococcus aureus* (15.9%), *Candida* spp. (15.1%), *Escherichia coli* (12.4%), *Salmonella* spp. (8.4%), *Shigella* spp. (6.9%), coagulase-negative *Staphylococcus*

Table 2. Age and Sex distribution of subjects with diarrhoea.

Age (months)	No. (%) of female	No. (%) of male	Total
0-6	9 (21.4)	18 (27.7)	27 (25.2)
7-12	10 (23.8)	14 (21.5)	24 (22.4)
13-18	12 (28.6)	21 (32.3)	33 (30.8)
19-24	11 (26.2)	12 (18.5)	23 (21.5)
Total	**42 (39.3)**	**65 (60.7)**	**107**

Table 3. Frequency of isolation of pathogens from subjects.

No isolates	Case	Control	p-value
	No of subjects	No of subjects	
0	4	14	0.0022
1	12	22	0.0283
2	14	14	1.0000
3*	15	42	<0.0001
4	16	12	0.4230
5*	37	11	<0.0001
6*	9	0	<0.0001
Total	107	115	
Average number of isolates per child	**3.6**	**2.4**	

*- significant p- value.

(6.9%), *Klebsiella* spp. (4%), *Enterobacter* spp. (3.4%), *Citrobacter* spp. (2.4%), *Aspergillus* spp. (2.4%) and *Proteus* spp. (2.4%), *Pseudomonas* spp. (1.3%) and *Serratia* spp. (1.0%).

Table 2 shows the distribution of diarrhoeal children by age with presentation of diarrhoea occurring more frequently in males than females. It shows that diarrhoea is associated with age and majority of cases occurring in children between 13 months and 18 months of age. The frequency of isolating pathogens varied between infected children and apparently healthy ones. The tendency of not finding any associated bacterial or fungal pathogen was significantly associated with being apparently healthy ($p<0.05$) while the likelihood of isolating three or more pathogens from any one subject was significantly associated with diarrhoea as shown in Table 3. Stool samples obtained from seventy-seven infected children were found to contain more than two diarrhoea pathogens (Figure 1).

DISCUSSION

Diarrhoea is a common cause of illness and death among children in developing countries including Nigeria (CDC, 2013). Various studies within the study environment have implicated various bacteria in diarrhoea diseases but all these studies were focused on individual organisms as aetiologic components of diarrhoea (Olusanya et al., 1983; Lamikanra et al., 1996; Aboderin et al., 2002; Igbeneghu, 2009; and Akinkunmi and Lamikanra, 2012).

Results showed that more (60.7%) male children presented with diarrhoea than their female (39.3%) counterparts. This difference was found to be statistically significant with $p < 0.05$. This conforms to results obtained by Abdullahi et al. (2010) who reported that there was a statistically significant difference between the number of male (22.3%) and female (18.3%) children who presented with diarrhoea as at the time of their study. The reason for this difference may be that male children are more prone to infections than their female counterparty as reported by Muenchhoff and Goulder (2014). Boys have also been observed to be more active than girls at the age of study, hence they could be more exposed to more sources of infections than girls (Abdullahi et al., 2010). This result is also in conformity with results from Bangladesh (Begum et al., 2013) where boys (11%) were observed to have presented with diarrhoea than the girls (8.5%). The observation in this study was found to be statistically significant with $p = 0.00001$.

It was also observed from the results of this study that 31(54.8%) of children aged 13-24 months were affected by the diarrhoea disease ($p < 0.05$). This could be attributed to the mobility of children in this age group. The propensity of these children to explore their environment

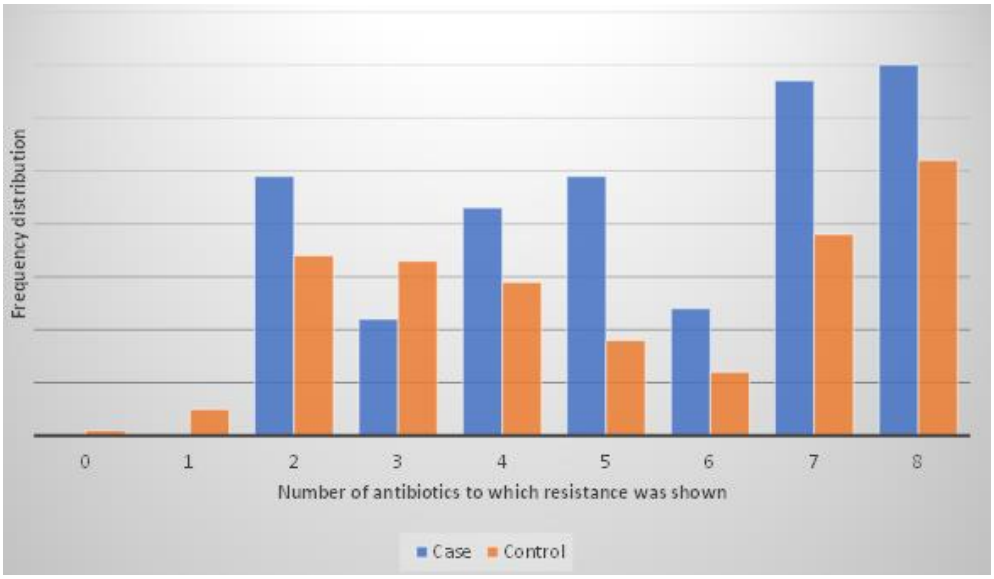

Figure 1. Distribution of resistant isolates.

extensively could mean that they are able to grasp and put contaminated items into their mouths. There is also the issue of weaning- these children are very likely to have just been introduced to family diet after they are no longer breast-fed hence, the pattern of age presentation observed in this study.

Our results show that 12 genera of bacteria namely: *Campylobacter* spp., *Staphylococcus* spp., *Escherichia coli, Salmonella* spp., *Shigella* spp., *Klebsiella* spp., *Enterobacter* spp., *Citrobacter* spp., *Proteus* spp., *Pseudomonas* spp., *Serratia* spp. and various species of *Candida* and *Aspergillus* were recovered from stool samples that were processed. *Campylobacter* species (17.8%) were the most commonly isolated species from diarrhoeal stool samples. This suggests that as with results obtained in related studies in other parts of the world including Tanzania (Lindblom et al., 1995), Thailand (Taylor et al., 1993), Bangladesh (Haq and Rahman, 1991) and Egypt (Rao et al., 2001), *Campylobacter* spp. are the most commonly isolated bacterial pathogen from children aged less than 2 years presenting with diarrhoea. *Campylobacter* species of organisms (16.5%) were also recovered from apparently healthy children from the control group. This strongly suggests that being infected with a species of *Campylobacter* may not lead to diarrhoea in children further suggesting that there are other factors which are related to the development of diarrhoea besides the presence of the organism within the patient.

The recovery of *Campylobacter* spp. from children without diarrhoea is common in developing countries, with some reports showing no statistically significant difference in the isolation rates for symptomatic and asymptomatic children (Akitoye et al., 2002). There was however a significant association (p= 0.0003) of the isolation of *Campylobacter* with the presentation of diarrhoea in children aged 0-24 months in this study. Other studies including those that were carried out by Rathaur et al. (2014) and Somily et al. (2014) have shown that *Campylobacter* species are often isolated alongside other enteric pathogens. This study further corroborates these reports as other bacterial pathogens were co-isolated with *Campylobacter* in every case in which this organism was associated with diarrhoea.

A total of 667 pathogens were isolated from stool samples collected in this study, with 388 and 279 microorganisms from diarrhoeal and apparently healthy children respectively. There was no significant difference in the types of bacterial pathogens isolated from both infected and healthy children but the frequency of occurrence of bacterial pathogens was significantly associated with diarrhoea with a p-value of <0.0001. It was observed that more individual pathogens were recovered from stool samples obtained from children with diarrhoea. It was further observed that recovering four pathogens or more, from any stool sample was significantly associated ($p < 0.0001$) with diarrhoea disease. On the average, about four different organisms were isolated from infected children as opposed to two different organisms from apparently healthy children. This implies that diarrhoea diseases are caused by the joint assaults carried out by multiple enteric pathogens numbering up to four or more. Hence, the concept of one pathogen, one disease might need to be re-evaluated. This could suggest that there could be synergistic or additive effects on the coexistence of the bacterial pathogens isolated as was reported by Sinha et al. (2013).

Recovered Campylobacter spp. from diarrhoea samples showed resistance to all antibiotics against which they were tested for susceptibility except to Ciprofloxacin (29%). The low percentage resistance shown could be attributed to the fact that ciprofloxacin is not often administered to paediatric patients in the study environment. Campylobacter isolates from healthy controls also showed resistance but at a reduced rate of 21.7%. This means that healthy subjects could also be carriers of resistant strains of Campylobacter, hence, serving as a reservoir of antibiotic resistance which could further be transferred amongst man and his animals.

Isolates recovered from both diarrhoea and control samples were multiply-resistant to tested antibiotics. This suggests that the number of antibiotic-resistance genes carried by enteropathogens and not just the number of pathogens isolated from the patient may be responsible for the diseased state observed in the study environment. The high frequency of isolation of multiply-antibiotic resistant enteropathogens in this study reflects the abuse of these antibiotics within this environment but more than that, it also suggests that people living within our study environment have been rendered more susceptible to diarrhoea and maybe, other infections that are endemic to this region. The recovery of so many multiply-antibiotic resistant pathogens in both healthy subjects and those with diarrhoea suggests that there is a large reservoir of antibiotic-resistant organisms in children living within the study environment and this can be attributed to the high abuse of antibiotics in Nigeria due to an absence of policy regulating the sale of antibiotics in open markets. The high incidence of quinolone-resistant organisms in this group of subjects is particularly noteworthy since the quinolones are not frequently prescribed for children and suggests that there is a repository of quinolone resistant-organisms in the study area even though the use of this group of antimicrobial agents and the incidence of resistance to them are recent phenomena (Lamikanra, 2011).

A study of the resistance pattern of the isolates recovered showed that the isolates from the diarrhoeal samples were resistant to a higher number of antibiotics than those isolated from the control samples. This difference is statistically significant ($p < 0.0001$) therefore, it suggests that diarrhoeal patients tend to carry more multiply-resistant pathogens and as such may be able to spread these arsenals better in their diseased state than their apparently healthy counterparty. This could be related to the extensive misuse of antibiotics within the study environment.

Conclusion

This study gives an insight into the identity of bacterial and fungal pathogens associated with childhood diarrhoea amongst children aged 0-24 months in Ile-Ife. The assessment of the bacterial load of stool samples and antimicrobial susceptibility patterns of isolates from stool samples could serve as a basis for further studies on the management of childhood diarrhoea. The isolation of antibiotic-resistant enteropathogens from apparently healthy children shows an increased number of reservoirs for antibiotic resistance within the study environment, therefore sensitization of the public and policy makers on the dangers of irrational use of antibiotics is important. Physicians should be aware of the increasing antibiotic resistance of enteric pathogens. Since resistance varies geographically, continuous local studies should be conducted to determine drug resistance patterns of enteric pathogens.

Ceftriaxone should be used in the treatment of these infections as the other agents with demonstrable activities against these pathogens are clinically contraindicated for use in children. The search for effective antimicrobials active against multidrug resistant diarrhoeal pathogens should be intensified. The number of pathogens isolated from children with diarrhoea was almost double the number recovered from apparently healthy children and antibiotic resistance was also more associated with children with diarrhoea.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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