Original article

Evaluation of bacterial pathogens in paediatric poliovirus-positive faecal specimens

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Abstract

Objective: To evaluate the *in vitro* inhibitory potential of commonly available antibiotic (discs) and paediatric suspensions against bacterial species from polio-positive faecal specimens. **Methods**: Commonly available antibiotic (discs) and oral, paediatric suspensions were screened for *in vitro* inhibitory activities against bacterial species from infantile polio-positive faecal specimens, using agar disc-diffusion and modified agar well-diffusion methods. **Results**: Isolated bacteria were *Bacillus cereus*, *B. subtilis*, *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Aeromonas hydrophila*, *Citrobacter aerogenes*, *Escherichia coli*, *Enterobacter aerogenes*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Pr. vulgaris*, *Shigella dysenteriae*, *Sh. flexneri*, *Sh. sonnei and Vibrio parahaemolyticus*. Overall phenotypic antibiotic susceptibility rates among Gram-positive bacterial species were between 33.3% (augmentin) and 75.0% (chloramphenicol, erythromycin and gentamicin); higher susceptibility rates (48.6%-100.0%) were recorded among Gram-negative bacterial species, while between 7.8% / 10.1% (metronidazole / ampicillin) and 25.2% /28.1 % (cotrimoxazole / septrin) were recorded towards paediatric antibiotics. **Conclusions**: Bacterial species from polio-positive fecal specimens are minimally susceptible to commonly available oral paediatric antibiotic suspensions in Nigeria.

Keywords: Antibiotics; Co-infection; Diarrhoea; Infant mortality; Paediatric; Polio

INTRODUCTION

Poliomyelitis, which is often referred to as infantile paralysis, is an acute viral infectious disease of the nerves in the spine that usually results in permanent paralysis. It is an infection in which the anterior horn of the spinal cord and sometimes the motor cranial nerves of the brain stem are destroyed, with consequent muscle paralysis and atrophy, which is often regarded as a form of degeneration[1]. Polio has remained a predominantly childhood illness with 80% to 90% of cases occurring in children less than five years old, and currently, Africa has been a continent where polio epidemic has been very difficult to control[2]. In 1988, W. H. O launched a global initiative to eradicate poliomyelitis by the year 2000, however, by 2003, six countries, India, Niger, Pakistan, Afghanistan, Egypt and Nigeria were still reporting new polio cases[3]. Till today, polio has not been totally eradicated in Nigeria due to various factors, ranging from lack of social infrastructures and updated primary health facilities to religion, as well as illiteracy non-conformation to government policies[4].

Poliovirus is spread by the faecal-oral route[5], which is also a common route of most microbial infections. The virus can be isolated from human faeces and sewage and in areas where raw sewage enters

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a watershed without treatment. In addition to untreated drinking water, the virus appears to spread through contact, especially among children, whose hands are often contaminated. The major illness associated with poliovirus often follows a mild illness which has symptoms of fever, nausea and vomiting. In most cases, there is a transient, self-limiting gastroenteritis, or completely asymptomatic condition[6,7]. While diarrhoeal illness stands, as an important cause of infectious morbidity in children[8,9], because infants have proven to be particularly vulnerable[10], the aetiology and pathogenesis of persistent diarrhoea are usually multifactorial[11].

Polymicrobial infections have been found to occur more frequently in paediatric patients[12,13], and several examples support the concept of co-infections in clinical cases. Although it could not be affirmed that there were concurrent viral-bacteria infections in polio cases, and in spite of the caution raised in the use of antibiotics prior to the onset of disease symptoms, which is considered to be a risk factor[14], most parents´first line of domestic treatment in cases of infectious conditions is antibiotic therapy, especially in the developing countries like Nigeria, where inadequate health services, inadequate drug supplies, non-adherence to treatment strategies and dubious drug quality all favour the emergence of microbial resistance. Other numerous interconnected factors include misuse of antimicrobials, inappropriate antimicrobial use, self-medication, counterfeit drugs, prescribers´perceptions etc.[15]. Even in cases where antibiotic susceptibility testing is carried out, before the results are transmitted to the physician, the patient would have already started antibiotic therapy. These lead to over prescription and favours resistance to antibiotics[16]. It may not be possible to propose that bacterial pathogens can be implicated as opportunistic pathogens in polio co-infections but the major aim of this research finding is to evaluate the therapeutic effects of antibiotics in bacterial co-pathogens obtained from polio-positive faecal specimens of children with associated diarrhoea.

MATERIALS AND METHODS

Collection of faecal specimens

Two separate faecal specimens of each subject were collected and sent for analyses at the National Poliovirus Reference Laboratory (NPRL), Department of Virology, University of Ibadan. It was ensured that appropriate infection control procedures were followed in the collection, transfer and analyses of all the faecal specimens from patients with suspected polio.

Preparation and extraction of faecal specimens

To each faecal specimen was added 1 mL of chloroform, 10 mL of sterile phosphate buffer saline (PBS) and 1 mg sterile glass beads to enhance thorough mixing. A portion of each specimen was processed while the remaining was kept as stock and stored at about 20℃. Tubes containing the faecal specimen, PBS and chloroform were separately placed on a mechanical shaker for 20 minutes and subsequently centrifuged at 3 500 rpm for 10 minutes in a refrigerated centrifuge, after which the supernatants were transferred into sterile screw capped storage vials. The first vial was processed for viral isolation on tissue culture, while the second vial was kept until further use.

Isolation and identification of polioviruses from faecal specimens

The isolation and identification of the polio viruses from the faecal specimens were according to the polio laboratory routine diagnostic methods[17]. The specimens for the selective isolation of bacterial pathogens were prepared from the stock prior to the addition of antibiotics (200 units penicillin and 200 g streptomycin). A certain portion of each untreated, original specimen was retained and kept at -20℃ for possible future transfer to a specialised reference laboratory.

Recommended cell lines for the isolation of polioviruses were derived from a human rhabdomyosarcoma and L20B cells, a genetically engineered mouse cell line expressing the human poliovirus receptor. The selection of these two cell lines permits the standardisation of the techniques and comparability of the results among different virus laboratories, while providing sensitivity for polio virus detection. Inoculated cell lines showing characteristic cytopathic effect (CPE) which features visible round, shrinking nuclear pycnosis, refractility and degeneration with detachment from the glass base were identified as positive for polio virus isolation.

Identification of the polio virus was according to the WHO[17] antibody micro-neutralisation assay

method using the serotype specific antisera supplied by the RIVM. The samples of diluted isolates (10^3-10^7) were mixed with equal volumes (50μL) of selected sets of polyclonal antisera against the polio virus types 1, 2 and 3. The serum / virus mixtures were incubated for one hour at 36℃ for binding of the antibodies and polio virus. Subsequently, 100 μL of 1.5 x 10^5 cells mL-1 added to the mixtures. The culture plates were then incubated at 36℃ cfu for 7 days but checked daily for CPE. The polio virus serotype was inferred from the antiserum that prevented the development of CPE. (Antibody, 1:128 consistent dilution; Antigen, 10^3-10^7 varied dilution).

Isolation and identification of bacterial pathogens from the faecal specimens

The media used for isolation of the bacterial pathogens were nutrient agar (NA), tryptone soy agar (TSA), McConkey agar (MCC), cystein lactose electrolyte deficient agar (CLED), eosin methylene blue agar (EMB), mannitol salt agar (MSA) and brain-heart infusion agar (BHI). All these media obtained from (Lab M, Ltd. Topley House, Lancashire, UK) were prepared for use according to manufacturer's specifications. Pure cultures of the bacterial pathogens were kept on BHI slants as working and bench cultures at 4℃, while identification of the bacterial isolates was according to standard phenotypic bacterial taxonomic tools[18-21].

Antibiotic Susceptibility patterns of the bacterial pathogens

Agar disc-diffusion method: The bacterial isolates were screened against the most commonly used routine antibiotic discs (manufactured by ABTEK Biologicals, England), using the agar disc-diffusion methods of Valladao and Sandine[22]. The antibiotic discs consist of eight antibiotic lobes [ampicillin (AMP 10μg); chloramphenicol (CHL 30μg); erythromycin (ERY 5 μg); gentamicin (GEN 10μg); ofloxacin (OFX 25μg); penicillin (PEN 15μg); streptomycin (S 10μg); tetracycline (T 30μg)]. Each inhibition zone size was interpreted by reference to the Performance Standards for Antimicrobial Susceptibility Testing[23].

Agar well-diffusion method: The antibiotic susceptibility determination of the bacterial isolates to 10 groups of 28 oral paediatric antibiotic suspensions (ampicillin/ampicillin-cloxacillin, cotrimoxazole, metronidazole, chloramphenicol, cephalexin and erythromycin) was carried out using the modified agar disc-diffusion and agar well-diffusion methods of Tagg and McGiven[24]. The antibiotic suspensions mostly with different compositions and trade names were in two forms; dry powder and liquid suspensions. The assay was carried out using sterile Mueller-Hinton agar.

Seeded Mueller-Hinton agar plates were prepared by transferring and streaking 500 μL of each 24h bacterial culture broth unto the sterile agar plate surface. The seeded agar plates were then left for about 15 min before aseptically dispensing the paediatric antibiotic suspensions (already incorporated into sterile, plain semi-solid agar) into the agar wells already bored in the agar plates. The plates were then incubated at 35℃ for 18-24 h. Zones of inhibition were measured and recorded in millimeter diameter according to the methods of NCCLS[25, 26]. Each inhibition zone size was interpreted by reference to the Performance Standards for Antimicrobial Susceptibility Testing[23].

RESULTS

One hundred and fifteen bacterial isolates obtained from polio-positive infantile faecal specimens were characterised as *Bacillus cereus* 2(1.7%), *Bacillus subtilis* 3 (2.6%), *Staphylococcus aureus* 5 (4.3%), *Streptococcus pneumoniae* 2(1.7%), *Aeromonas hydrophila* 3(2.6%), *Citrobacter aerogenes* 1 (0.9%), *Escherichia coli* 11(9.6%), *Enterobacter aerogenes* 10(8.7%), *Klebsiella pneumoniae* 10(8.7%), *Pseudomonas aeruginosa* 5(4.3%), *Proteus mirabilis* 11(9.6%), *Proteus vulgaris* 13(11.3%) *and Shigella dysenteriae* 17(14.8%), *Shigella flexneri* 7(6.1%), *Shigella sonnei* 3(2.6%) *and Vibrio parahaemolyticus* 12(10.4%).

The Gram-positive bacterial pathogens from the polio-positive faecal specimens were mostly phenotypically susceptible to chloramphenicol (88.9%), gentamicin (77.8%), cotrimoxazole (66.7%) and erythromycin (66.7%); moderate (44.4%) resistance was displayed against tetracycline, while very low susceptibility rates were exhibited towards amoxicillin (33.3%), cloxacillin (33.3%) and augmentin (22.2%) respectively. The Gram-negative bacterial pathogens were mostly phenotypically sus-

ceptible to the test antibiotics (discs) except nalidixic acid and tetracycline, against which moderate susceptibility (48.7%) were exhibited. Most of the Gram-positive and Gram-negative bacteria species showed moderate zones of inhibition of between 15.0-24.0 mm to the test antibiotic discs (Table 1). Nine and twenty antibiotic susceptibility patterns were exhibited by the Gram-positive bacterial species and by the Gram-negative bacterial species (Table 2).

The antibiotic susceptibility results using paediatric antibiotic suspensions indicated total rates of between 7.8% and 28.1%, while susceptibility rates of 0.0-3.88% were recorded among the Gram-positive bacterial species and 7.77% - 26.2% among the Gram-negative bacterial species (Table 3).

DISCUSSION

Polymicrobial diseases involve multiple infectious agents and are referred to as complex, complicated, mixed, dual, secondary, synergistic, concurrent, coinfections[27], and there is increasing evidence in the literature of the importance of polymicrobial infections, in which microorganisms interact in a synergistic or inhibitory fashion, impacting on pathogenesis or the maintenance of health[28, 29]. It is conceivable that a viral infection, for instance, may facilitate bacterial colonisation and enhance adherence to host cells, paving the way for invasive disease. For example, a virus infection may induce host cell membrane changes such as the expression of viral glycoproteins that may serve as receptors for bacteria, or up-regulate platelet activating factor receptor thereby promoting increased bacterial adherence. Likewise, it is equally conceivable that a bacterial infection may pave way for increased viral disease[30-32]. Pacheco-Gil *et al*.[31] as an example, reported a life-threatening, severe dysentery case due to enteroinvasive *E. coli* in a malnourished 4-month-old, Indian native, male infant co-infected with rotavirus, which led to severe gastrointestinal bleeding anaemia and hypovolemic shock.

According to Netty[33] and some other workers, polio infection is frequently accompanied only by minor symptoms. The major illness associated with poliovirus often follows a mild illness, which has symptoms of fever, nausea vomiting, diarrhoea, sore throat, cough and a mild flu[34], which may suggest synergistic effect of onset of polio infection on some bacterial pathogens implicated in infantile gastroenteritis. Eighteen out of twenty three (78.2%) randomly interviewed mothers of polio children claimed that diarrhoea and fever were associated with onset of polio infection in their children. In this present study, forty faecal specimens that were positive for poliovirus showed presence of certain bacterial pathogens. The pathogenic potentials of the bacterial species identified in this study are uncertain, but they are of similar species to those that have been implicated in infantile and children diarrhoea-like infections from different parts of the world[30, 31, 32, 35-41].

Infections are the major causes of severe morbidity and mortality among children worldwide[42, 43] but for decades, the focus of international public health concern has been on reducing child mortality[44], and rightly so. In as much as this study is not extensively on the coinfection of polio and gastroenteritis, the major concern is in evaluating the antibiotic effects on the associated bacterial pathogens, in order to justify or debunk the parental administration of antibiotic therapy in cases of onset of polio. Specific antimicrobial treatments are usually required to supplement supportive anti-dehydration treatment, which is the cornerstone of therapy of acute infant diarrhoea. Selective use of antimicrobial agents therefore, cannot be overemphasised. It was observed in this study that the Gram-positive bacterial pathogens from the polio-positive faecal specimens were phenotypically susceptible to cotrimoxazole, erythromycin (66.7%), gentamicin (77.8%) and chloramphenicol (88.9%), while the Gram-negative bacteria were mostly phenotypically susceptible to all the test antibiotic (discs) except nalidixic acid and tetracycline, to which moderate susceptibility of 48.7% were recorded.

Although prevalence of resistance varies greatly in different countries, an increase in antibiotic resistance has been shown in a large number of studies in recent years[12, 45-48]. Children are however, an easily defined population in which to determine if resistance to certain classes of antimicrobial agents can occur, especially in unexposed individuals[49]. The overall susceptibility values of the bacterial isolates to various paediatric oral antibiotic suspensions of less than 30.0% among the 8 classes of antibiotics indicated that the bacterial strains were more resistant to the paediatric oral suspensions, especially am-

picillin, cephalexin and metronidazole. The resistance values recorded against the paediatric suspensions, amoxicillin (80.0% - 91.2%); ampicillin (71.8% - 89.9%); cephalexin (86.4%); cotrimoxazole (74.8%); erythromycin (75.8%); rancotrim (76.7%); septrin (71.9%-77.7%); metronidazole (79.7% - 92.2%) were quite higher than expected in paediatric chemotherapy, which shows the great danger these commonly prescribed and consumed antibiotics may pose in paediatric infectious conditions, more especially in cases of high infant mortality rates recorded in developing countries.

In the study of Larson *et al*[50] it was reported that the most commonly used antibiotics in children were ampicillin (74%), penicillin (12%), amoxicillin (11%), erythromycin (5%), tetracycline (4%) and streptomycin (2%), still 74% of bacterial isolates from children were found to carry resistant pathogens. The study of Kaper *et al*[51], which also focused on children, found ciprofloxacin-resistant Gram-negative bacilli in antibiotic-naïve children. Although it is not possible from the present data to determine the source of acquisition of the resistant enteric bacteria, it is possible that they could have been from family members or due to unrestricted usage of antibiotics. In fact, according to Reves *et al*[52] and Fornasini *et al*[53], daycare studies have demonstrated non-familial transmission of *E. coli* isolates resistant to trimethoprim. The results of the study of Lamikanra *et al*[54] indicated multiple resistant strains, while that of Shebib *et al*[14] also indicated a large reservoir of antibiotic resistance within the community, and that the resistance genes were easily transferable to other strains even without direct exposure to antibiotics.

In spite of the fact that the limitations of the current study include low population size of cases, probable non isolation of some other viable but non-culturable bacterial species due to non-availability of some selective isolation culture media or kits and the limited number of antibiotics used in the sensitivity test, the present study has provided insight into the possibility of co-infection in polio cases, especially in cases of associated infantile diarrhoea and the very minimal antibiotic susceptibility rates among both the Gram-positive and Gram-negative bacterial pathogens, using the paediatric antibiotic suspensions available in Nigeria, indicating very high resistance towards the paediatric antibiotic suspension, which are the choice of drugs in paediatric cases. These microorganisms should not be dismissed as merely harmless members of the commensal microflora, more so, since they have potential clinical implications due to their high resistance towards the paediatric antibiotic suspensions.

Nigerian government is currently, strongly fighting an yet to be won battle against the menace of adulterated, clinical drugs in the country, it is therefore, advised that children and infants suspected of having bacterial infections should not only be limited to the antibiotics susceptibility tests using discs alone but the oral paediatric suspensions should also be bio-amazed and the differences contrasted as a way of prescribing an effective chemotherapeutic control against paediatric infections. Parents should also be advised and enlightened against drug abuse, especially the indiscriminate antibiotic therapy in paediatric clinical cases. For example, in the study of Mahalanabis *et al*[55], folic acid was administered by parents in the treatment of acute watery diarrhoea in children.

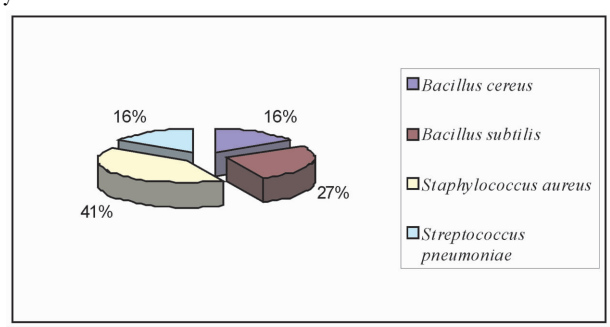

Figure 1 Percentage recovery rates of the Gram-positive and Gram-negative bacterial species isolated from polio-positive faecal specimens of children.

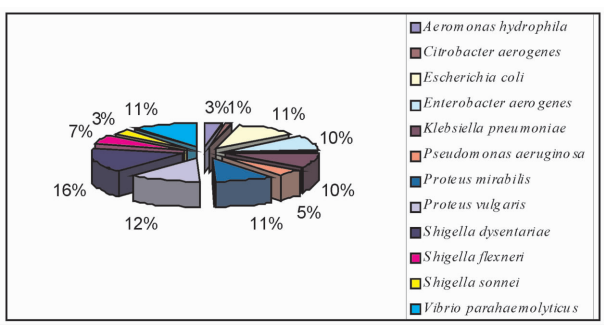

Figure 2 Percentage recovery rates of the Gram-negative bacterial species isolated from polio-positive faecal specimens of children.

Table 1 Percentage *in vitro* antibiotic susceptibility patterns of Gram-positive and Gram-negative bacterial pathogens from polio-positive faecal specimens (Discs).

Antibiotics		Antibiotic codes /conc. (Discs) μg/	Susceptibility patterns			
			Total % Susceptibility	High	Moderate	Low
Gram-positive	Amoxicillin *	AMX (25μg)	58.3	–	50.0	8.3
	Augmentin	AUG (30μg)	33.3	–	33.3	–
	Chloramphenicol	CHL (25μg)	75.0	50.0	25.0	–
	Cloxacillin	CXC (5μg)	58.3	–	41.6	16.7
	Cotrimoxazole *	COT (25μg)	66.7	25.1	41.6	–
	Erythromycin *	ERY (25μg)	75.0	–	75.0	–
	Gentamicin	GEN (10μg)	75.0	–	75.0	–
	Tetracycline	TET (30μg)	66.7	–	66.7	–
Gram-positive	Amoxicillin	AMX (25μg)	68.6	17.1	48.6	2.7
	Augmentin	AUG (30μg)	71.4	31.4	40.0	–
	Cotrimoxazole	COT (25μg)	88.6	28.6	51.4	8.6
	Gentamicin	GEN (10μg)	100.0	14.3	85.7	–
	Nalidixic acid	NAL (30μg)	48.6	5.7	34.2	8.6
	Nitrofurantoin	NIT (300μg)	88.6	8.6	71.4	8.6
	Ofloxacillin	OFL (30μg)	91.4	28.5	62.9	–
	Tetracycline	TET (10μg)	48.6	2.9	37.1	8.6

Interpretation of zones of inhibition: Highly susceptible: ≥25 mm; moderately susceptible:15-24 mm; slightly susceptible: ≤14 mm; * :Corresponding antibiotics in both discc and paediatric suspensions

Table 2 *In vitro* antibiotic susceptibility patterns of Gram-positive and Gram-negative bacterial pathogens from polio-positive faecal specimens.

Gram-positive		Gram-negative	
Antibiotic susceptibility patterns	No. of patterns	Antibiotic susceptibility patterns	No. of patterns
	1		1
Chl	2	Gen, Ofl	2
Gen, Cot, Chl	3	Cot, Nit, Gen	3
Amx, Ery, Tet, Gen, Chl	4	Gen, Ofl, Tet	4
Ery, Tet, Gen, Cot, Chl	5	Cot, Nit, Gen, Ofl	5
Aug, Amx, Ery, Tet, Clx	6	Cot, Gen, Nal, Ofl	6
Ery, Clx, Gen, Cot, Chl	7	Cot, Gen, Ofl, Tet	7
Amx, Ery, Tet, Clx, Gen, Cot	8	Nit, Gen, Ofl, Aug	8
Aug, Amx, Ery, Tet, Clx, Gen, Cot, Chl	9	Cot, Nit, Gen, Nal, Ofl	9
		Cot, Nit, Gen, Ofl, Aug	10
		Amx, Cot, Nit, Gen, Ofl, Aug	11
		Amx, Cot, Nit, Gen, Ofl, Tet	12
		Cot, Nit, Gen, Nal, Ofl, Tet	13
		Amx, Nit, Gen, Nal, Ofl, Aug	14
		Amx, Cot, Nit, Gen, Ofl, Aug	15
		Amx, Cot, Nit, Gen, Ofl, Aug, Tet	16
		Amx, Cot, Nit, Gen, Nal, Ofl, Aug	17
		Amx, Cot, Nit, Gen, Ofl, Aug, Tet	18
		Amx, Cot, Nit, Gen, Nal, Aug, Ofl	19
		Amx, Cot, Nit, Gen, Nal, Ofl, Aug, Tet	20

Table 3 Percentage *in vitro* antibiotic susceptibility profiles of the bacterial pathogens from polio-positive faecal specimens using paediatric antibiotic suspensions.

Paediatric antibiotic Suspensions	% Total antibiotic susceptibility patterns	% Antibiotic susceptibility patterns	
		Gram-positive	Gram-negative
Amoxicillin *	8.74-20.0	0.97-1.94	7.77-18.4
Ampicillin	10.1-28.1	0.97-1.94	9.9-26.2
Cephalexin	13.6	0.97	12.6
Cotrimoxazole *	25.2	2.91	22.3
Erythromycin *	24.2	1.94	22.3
Rancotrim	23.3	0.97	22.3
Septrin	22.3-28.1	1.94-3.88	20.4-24.2
Metronidazole	7.8-20.3	0.0-1.94	7.77-18.4

* Corresponding antibiotics in both discs and paediatric suspensions

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