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Substrate profile variation and drug resistance patterns of â – lactamase producing *Shigella* species isolated from diarrhoeal patients in Lagos, Nigeria

BA Iwalokun*[1]; GO Gbenle[1]; EO Akinrinmisi[1]; SI Smith[2] and A Ogunledun[3]

Derpartment of Biochemistry[1], College of Medicine, University of Lagos, Nigerian Institute of Medical Reseach[2], PMB 2013, Lagos and Department of Medical Microbiology and Histopathology[3], College of Health Sciences, Ogun State University, Sagamu, Nigeria.

Summary

The number and trend of antibiotic resistance by *Shigella* species recovered from food and diarrhoeal stools are on the increase in Nigeria and has resulted in a high frequency of hospitalisation. Increased cost of disease management, and higher mortality in children. This study exposes 51 â-lactamase producing *Shigella* isolates from Lagos to some newly introduced drugs in the country. The drugs include â – lactam – â -lactamase inhibitor antibiotics. â - lactam substrate hydrolysis and inhibitory effects of clavulanate were also investigated in-vitro. Results obtained revealed that all the isolates showed high level resistance to tetracycline, ampicillin, streptomycin, co-trimoxazole and amoxicillin with an MIC range of 128 – 1024 µg/ml. The isolates were susceptible to piperacillin, tobramycin, aztreonam and ofloxacin (0.03 – 8µg/ml). 18.2 – 40.9% of *S. flexneri* and *S. dysenteriae* showed low level resistance to cefuroxime and cefotaxime (MIC = 4 – 16µg/ml). Among the â-lactam – â- lactamase inhibitors tested, only piperacillin-tazobactam showed 100% resistance. Hydrolysis of â - lactam substrate was found to be species dependent in decreasing order of *S. flexneri, S. dysenteriae, S. sonnei* and *S.boydii*. An IC50 range of 0.8 – 2.4 mM was also observed in these isolates. Our data indicate that the incidence of multidrug resistance is high among â - lactamase producing *Shigella* isolates in Lagos, Nigeria. While the third generation cephalosporins should be used with cautions, some of the newly introduced drugs have the prospects of being used in the future control and management of shigellosis in the country.

Keywords: *â –lactamase, Shigellosis, Shigella species, drug susceptibility.*

Résumé

L'incidence de la résistance au spéces de *Shigella* contenu dans les nourritues et les selles de diarrhée aux antibiotiques sont en augmentation au Nigeria. Ceci resulte a l'augmentation de la frequence d'hospitalisation et le cout du ménagement de la maladie associée a une grande mortilité enfantile. Cette étude in vitro exposait et investiguait 51 B-lactamase aux isolats de shigella de Lagos a certains nouveaux medicaments dans le pays. Les résultats montraient que les isolats avaient un certain degrée de résistance au tetracycline, amplicillin, streptomycin, co-trimoxazole et amoxicillin avec un MIC variant entre 128-1024 µg/ml. Les isolats étaient susceptible a la piperacillin, totramycin, aztreonam et ofloxacin (0.03-8µg/ml). 18.2-40% de *S. flexneri* et *S. dysenteriae* montraient un faible degrée de résistance au cefuroxine et cefotaxine (MIC= 4 - 16µg/ml). Parmi les ant-B-lactamases testés ,seule la piperacillin, tazobactam montraient 100% de résistance. L'hydrolyse du substrate de B-lactamase était dependent des spéces et décroissait dans l'ordre du *S.fleneri*, S. *dysenteriae,S sonnei* et S. *boydui*. Le IC50 variait entre 0.8-24 mM dans tous les isolates. Ces resultats indiquent que l'impact de la multi-résistance est élevé parmi les B-lactamases produisant la shigellose a Lagos. Cependant la 3iéme generation des céphalosporines doivent etre utilisés avec attention et certain nouveaux antibiotiques nouvellement introduit pour le controle et le ménagement des shigellose dans ce pays.

Introduction

Shigellosis still ravages most communities of the world where it is endemic with occasional outbreaks [1] The disease causes great morbidity and mortality especially in under five-year old children and immunocompromised patients [2]. Longer stays in hospitals and high frequencies of complications among patients have been attributed to virulence, multidrug resistance and expression of â - lactamases by *Shigellae* [3,4]. In Lagos, where shigellosis is also endemic with *S. flexneri* as the sero-dorminant group, isolates identified have been found to be multi-drug resistant, virulent and show dynamics of plasmid transferability over a 10-year study [5, 6]. However, the mechanisms of resistance to drugs by these isolates have not been studied. â - lactam drugs, which constitute over 50% drugs used in the country are one of the antibiotics to which *Shigellae* have shown resistance.

In a recent study in Lagos (unpublished), we found expression of â-lactamase enzymes among isolates that displayed resistance to more than three antibiotics. Resistance to ampicillin and amoxicillin was found to be very high but the susceptibility profile of the isolates when these drugs are tested in combination with â - lactamase inhibitors such as clavulanate, sulbactam and tazobactam were not conducted.

The present study was designed to determine the susceptibility and resistance to drugs by â - lactamase producing *Shigella* species isolated in Lagos, Nigeria. Attempts will also be made to study utilization of â - lactam substrates and the level of inhibition by clavulanate.

Correspondence Dr. B.A Iwalokun, Department of Biochemistry, College of Medicine, University of Lagos, Idi Araba, Lagos, Nigeria.

Email bamwal@yahoo.com

Materials and methods

Shigella isolates

51 *Shigella* species out of 74 isolates obtained from patients with shigellosis in Lagos, Nigeria were screened by iodometric method [7] as â lactamase producers and were selected for study. The species distribution of the isolates was *S. flexneri* (n = 22), *S. dysenteriae* (n = 16), *S. sonnei* (n = 8) and *S. boydii* (n = 5). A standard strain expressing plasmid mediated SHV-1 - lactamase was used as a positive control.

Determination of minimum inhibitory concentration (MIC).

The susceptibilities of the â lactamase producing *Shigellae* to antibiotics were quantitatively determined by microbroth dilution technique according to NCCLS [8] and CEQA – AGAR [9] guidelines. The isolates were seeded into wells at a standardized inoculum size of 5 x 10^4 cfu/well. The antibiotics employed for study were ampicillin, piperacillin, gentamicin, tobramycin, amikamicin, tetracycline, nalidixic acid, ofloxacin, ciprofloxacin, ceftazidime, ceftriaxone, cephalothin, streptomycin, trimethoprim-sulphamethoxazole and chloramphenicol from Sigma (St Louis. USA). â -lactam – â -lactamase inhibitor combination drugs, which included amoxicillin-clavulanate, piperacillin-tazobactam and ampicillin-sulbactam were tested at a fixed ratio of 2:1 and were obtained from SmithKline Beecham, Nigeria. *Escherichia coli* ATCC 25922 was used as the control strain for each test run. In each test set, the well having the least antibiotic concentration was inoculated first and the well without antibiotic was inoculated last. MICs were defined as the lowest drug concentration at which no visible growth of organisms occurred. MIC_{50} and MIC_{90} defined as concentrations that inhibited visible growth of the isolates by 50% and 90% were also determined for the â-lactam – â - lactamase inhibitor combination drugs used.

â -lactamase substrate utilization and inhibition profile

Young cultures of *Shigella* isolates were used as sources of â -lactamase. To obtain a crude extract, five pure and discrete colonies of an isolate were inoculated in 10ml of trypticase soy broth and incubated in air for 18 hours. Then the broth was centrifuged at 250rpm at 4°C for 10 minutes and the resulting supernatant was filter sterilized through a 0.25µm Millipore filtration unit. The assay for substrate hydrolysis by â – lactamase was carried out spectrophotometrically according to Bush and Sykes [10]. Absorbance changes associated with the hydrolysis of â -lactam substrates by crude â -lactamase extract of *Shigella* were monitored at different wavelengths based on substrate specificity. Benzyl penicillin G, ampicillin and carbenicillin were measured at 240nm, cephalothin was measured at 260nm, oxacillin was measured at 265nm and nitrocefin at 500nm. Each assay contained 1ml each of crude extract and 0.1mM substrate in 10mM phosphate buffer, pH 7.0 except for ampicillin, which was assayed as a 1mM substrate. Each set of reactions was accompanied by a substrate blank. The assay mixture was incubated in a shaker (200rpm) at 37°C for 1 hour. Absorbance was immediately measured against substrate blank at appropriate wavelengths using spectrophotometer D700 (Beckman, USA). One unit of activity was defined as the amount of enzyme that hydrolyzed 1mol of benzyl penicillin G per min per mg total protein at room temperature. The rate of hydrolysis of each substrate was calculated relative to 100% hydrolysis for benzyl penicillin G. A standard strain expressing plasmid mediated SHV-1 â -lactamase was used as control.

Total protein determination

The total amount of protein in each of the filtered crude extracts was determined by Lowry method [11] using bovine serum albumin as the substrate (50 –150µg/ml).

Clavulanate inhibitory assay

Equal amount of enzyme from each isolate was pre-incubated with clavulanate at 0.05 – 2.8mM for 10 minutes. Thereafter, hydrolytic activities were measured using nitrocefin as the substrate. IC_{50} was defined as the concentration of clavulanate required to inhibit enzyme activity by 50% [10].

Statistical analysis

Data presented in Table 1 represent range of MIC values of antibiotics against the â - lactamase enzymes producing *Shigella* isolates studied. The range of MIC values of â -lactam – â -lactamase inhibitor drug combination represents MIC_{50} and MIC_{90} values respectively. Data in Tables 2 and 3 were presented as mean ± SD of the number of *Shigella* species.

Results

The â -lactamase producing *Shigella* isolates displayed diverse antibiotic susceptibility profiles. 10 of the 22 *Shigella flexneri* and 9 of the 16 *S. dysenteriae* isolates had a low resistance to nalidixic acid (MIC = 16 – 64µg/ml), while all the isolates of *S. boydii* and *S. sonnei* were susceptible (MIC = 1-4µg/ml). Similarly, 18.8 – 37.5% of the total *S. dysenteriae* and 18.2 – 40.9% of total *S. flexneri* displayed intermediate resistance against cefuroxime (8 – 16µg/ml) and cefotaxime (MIC = 4 – 16µg/ml), while other isolates were sensitive to these antibiotics (MIC = 0.015 – 1µg/ml). In *S. sonnei* and *S. boydii*, the MICs of ampicillin – sulbactam were either in the intermediate or susceptible range. Other isolates had intermediate resistance to this combination (16/8 – 64/16µg/ml) (Table 1).

The data presented in Table 2 demonstrated hydrolytic activity of the â-lactamases using ampicillin (AMP), carbenicillin (CAR), oxacillin (OXA), cephalothin (CEF) and benzyl penicillin G (PEN) as substrates. With the hydrolysis of benzyl penicillin G taken as 100, highest enzyme activity occurred with ampicillin as the substrate in all the *Shigella* isolates tested However, in a species dependent manner, the â lactamases of *S. flexneri* produced the highest ampicillin hydrolysis with mean relative activity of 661.3 ± 4.5%. The enzymes expressed by *S. boydii* isolates gave the least activity (relative activity, 157.6 ± 1.5%). The mean relative activity of â lactamase enzymes of *S. dysenteriae* was found to be 626.2 ± 2.3%, while in *S. sonnei*, the mean enzyme activity was 355.3 ± 3.4%. The â - lactamases of *S. boydii* used oxacillin poorly as a substrate with a mean activity 0.5 ± 0.1%. The activity of â -lactamases of S. *flexneri* on OXA was 161.7 ± 1.6%, while those of *S. dysenteriae* and *S. boydii* were 135.3 ± 1.2% and 120 ± 1.7% respectively. Cephalothin was generally a poor â -lactam substrate for the â -lactamases of all the isolates. The mean enzyme activity ranged from 1.0 - 5.2%.

Table 1: Comparisons of MICs (µg/ml) among â – lactamase producing *Shigella* species from Lagos, Nigeria.

Antibiotic	*S.dysenteriae* (N = 16) n(%)	MIC	*S. flexneri* (N =22) n(%)	MIC	*S.boydii* (N = 5) n(%)	MIC	*S. sonnei* (N = 8) N(%)	MIC
Tetracycline	16(100)	128 ≥ 512	22(100)	128 ≥ 256	5(100)	128 ≥ 256	8 (100)	128 ≥ 256
Streptomycin	16 (100)	64 ≥ 256	22 (100)	64 ≥ 256	5 (100)	32 ≥ 256	8 (100)	32 ≥ 256
Ampicillin	16 (100)	128 ≥ 256	22 (100)	512 ≥2048	5(100)	128 ≥ 512	8(100)	128 ≥ 256
Amoxicillin	15 (93.8)	128 ≥ 256	19 (83.4)	128 ≥ 256	5(100)	64 - 128	6 (75)	64 – 256
Co-trimoxazole	16(100)	64 - 256	22 (100)	64 – 256	5 (100)	64 - 128	8 (100)	64 – 128
Nalidixic acid	10 (62.5)	16 – 064	13 (59.1)	16 – 64	5(100)	1 - 4	8 (100)	1 – 4
Chloramphenicol	16 (100)	128 ≥256	22 (100)	128 ≥ 256	5 (100)	64 - 256	8 (100)	64 – 128
Gentamicin	16 (100)	1 - 2	22 (100)	1 – 2	5 (100)	1 - 2	8 (100)	1 – 2
Tobramycin	16 (100)	0.5 - 2	22 (100)	0.5 - 2	5 (100)	0.5 - 2	8 (100)	0.5 – 2
Piperacillin	16 (100)	4 - 8	22 (100)	2 – 8	5 (100)	2 - 8	8 (100)	2 – 8
Cephalothin	7 (43.8)	1 - 2	8 (36.4)	8 – 16	5 (100)	4 - 8	8 (100)	4 – 16
Cefotaxime	6 (37.5)	4 - 16	9(40.9)	4 – 16	5 (100)	0.015 – 0.06	8 (100)	0.03 – 0.06
Cefuroxime	3 (18.8)	8 - 16	4(18.2)	4 – 16	5 (100)	0.5 - 2	8 (100)	0.5 – 1
Aztreonam	16 (100)	1 - 2	22 (100)	0.5 – 2	5 (100)	1 - 2	8 (100)	0.5 – 2
Ofloxacin	16 (100)	0.03 – 1	22 (100)	0.03 – 0.06	5 (100)	0.03 - 0.06	8 (100)	0.02 – 0.04
Ciprofloxacin	16 (100)	0.0075 – 0.25	22 (100)	0.0075 – 0.25	5 (100)	0.0075 - 0.25	8 (100)	0.0075 – 0.25
Piperacillin-tazobactam	16 (100)	8/4 – 16/8	22 (100)	8/4 – 32/8	5 (100)	8/4 – 16/8	8 (100)	8/4 – 16/8
Amoxicillin-clavulanate	16 (100)	16/8 – 64/16	22 (100)	16/8 – 64/16	5 (100)	16/8 – 32/16	8 (100)	16/8 – 32/16
Ampicillin-sulbactam	16 (100)	16/8 – 32/16	22 (100)	16/8 – 32/16	5 (100)	8/4 – 16/8	8 (100)	8/4 – 16/8

N = Total number of isolates tested: n(%) = number (percentage) of isolates with MIC range. MIC range for â-lactam-â lactamase inhibitor combination represents MIC_{50} and MIC_{90} values.

Table 2: Comparison of â- lactamase hydrolytic activities among *Shigella* isolates from Lagos, Nigeria.

â – Lactamase producers	N	Relative activity (mean ± SD)% PEN	AMP	CAR	OXA	CEF
S. dysenteriae	16	100	626.2 ± 2.3	51.3 ± 1.7	135.3 ± 1.2	1.8 ± 0.2
S. flexneri	22	100	661.3 ± 4.5	70 ± 1.3	161.7 ± 1.6	5.2 ± 0.3
S boydii	5	100	157.6 ± 1.5	5.2 ± 0.6	0.5 ± 0.1	1.0 ± 0.2

At the same enzyme concentration, the IC_{50}s of clavulanate were 2.1 ± 0.3mM for *S. dysenteriae*, 2.4 ± 0.2mM for *S. flexneri*, 1.4 ± 0.5mM for *S. boydii* and 1.2 ± 0.6mM for *S. sonnei*. A mean IC_{50} value of 2.5 ± 0.6mM was produced by the â -lactamase of the control strain (Table 3).

Table 3: Comparison of IC_{50} among â – lactamase producing *Shigella* isolates from Lagos, Nigeria.

	IC_{50} (mean ± SD) mM
S dysenteriae	2.1 ± 0.3
S. flexneri	2.4 ± 0.2
S. boydii	1.4 ± 0.5
S. sonnei	1.2 ± 0.6
SHV – 1	2.5 ± 0.6

Discussion

Multidrug resistance among â -lactamase producing *Enterobacteriaceae* has been demonstrated in *Escherichia coli* , *Klebsiella pneumoniae* [12], *Salmonella spp* [13] and *Shigella* isolates from several endemic countries [14,15]. In this study, the â -lactamase producing *Shigella* isolates from Lagos, demonstrated high level resistance to chloramphenicol, tetracycline, co-trimoxazole and streptomycin. This observation is in accord with our previous findings [5] in which â -lactamase expression was not investigated. Resistance of our isolates to amoxicillin – clavulanate is similar to the report of Siu *et al*, [16] and Chu *et al*, [17]. Livermore [18] and Ahamed *et al*, [19] also attributed resistance of *Enterobacteriaceae* to â -lactam – â lactamase inhibitor combinations to acquisition of mutated TEM 1, TEM2 and SHV – 1 genes, which are borne on transferable plasmids. The emergence of *Shigella* isolates resistant to amoxicillin – clavulanate (i.e. augmentin) in Lagos, Nigeria is of great concern and might be a reflection of the abuse of this drug. Amoxicillin is one of the drugs used in the empirical treatment of diarrhoeal related diseases, while augmentin is widely prescribed for immunocompromised and surgical patients [20]. Our isolates also displayed moderate resistance to ampicillin – sulbactam and this contradicts the result of Ling *et al*, [21]. We also observed an increased resistance trend to nalidixic acid, cefuroxime and cefotaxime. In 1990, all the *Shigella* isolates were sensitive to these drugs [6]. About a decade later, isolates resistant to nalidixic acid and cefuroxime emerged [5]. The present study reveals species dependent type of resistance to these drugs with strains of *S. sonnei* and *S. boydii* still showing susceptibility. Resistance of Shigellae to oxyimino and classical cephalosporins

has been linked to the expression of metallo- â -lactamase enzymes [22] and modification of SHV – 1 and TEM – 2 enzymes by several point mutations [23]. The increased resistance of our isolates to nalidixic acid bothers much on children health care system in the country. In the last two years, the drug has been switched from "back up" position to first line position in the treatment of pediatric shigellosis because of high frequency of treatment failures observed with previous first line antibiotics which included ampicillin, trimethoprim-sulphamethoxazole and amoxicillin. Fluoroquinolones still command a 100 percent resistance value but are not incorporated into children therapy owing to their putative toxic effects. The *Shigella* isolates studied were also found to be sensitive to gentamicin, tobramycin, piperacillin, piperacillin-tazobactam and aztreonam. Out of these drugs, it is only gentamicin that has been widely reported to be ineffective in the treatment of shigellosis [24].

This study also demonstrates variation in the utilization of â–lactam substrates by â -lactamase producing *Shigella* isolates from Lagos. The enzymes from *S. dysenteriae, flexneri* and *sonnei* hydrolyzed ampicillin 3 – 6 times greater than penicillin G hydrolysis. Hydrolysis of ampicillin by *S. boydii* â-lactamases and oxacillin by enzymes from *S. dysenteriae, flexneri* and *sonnei* were comparable with that of benzyl penicillin G, while cephalothin was utilized poorly as a substrate in all the isolates. Our findings are similar to the work of Siu *et al*, [16]. However, these workers attributed the differential substrate utilization to the preference of *Shigella flexneri* strains from Hong Kong and Shanghai for OXA – 1 – like – â -lactamase enzyme. Therefore, this suggests that the hydrolytic efficiency of *Shigellae* â - lactamases has a geographical and genetic influence.

In the present study, the genetic control and pattern of expression of the â -lactamases were not investigated. Nevertheless, the poor utilization of cephalothin by our isolates' enzymes indicates difficulty in the cleavage of this compound and further reflects the susceptibility of these isolates to cephalosporins. This seems to be at variance with the observed cefuroxime and cefotaxime resistance and the fact that extended â-lactamase (ESBLs) producing *enterobacteriaceae* showed resistance to third generation cephalosporins, were susceptible to cephamycins and inhibited by clavulanate [25]. However, third generation cephalosporin-aminoglycoside were still found efficacious in the treatment of Shigellosis and other diarrheas [26]. Our results on the inhibitory effects of clavulanate in terms of IC_{50} are in line with the report of Shiu *et al*, [17]. The results further show the species dependent response to drugs among the *Shigella* isolates tested. *S. dysenteriae* and *S. flexneri* seem to be more refractory than *S. sonnei* and *S. boydii* to Clavulanate inhibition.

It can be concluded that resistance to multiple drugs including the â-lactam antibiotics is common among â -lactamase producing *Shigella* isolates in Lagos and utilization of substrates and clavulanate inhibition of these enzymes is species dependent.Further investigations into the classes, genetic control and pattern of expression of these enzymes need to be made.

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