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A comparative study of virulence in *Shigella* isolates from Lagos, Nigeria.

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ABSTRACT: Shigellosis and virulence in 513 patients presenting with mild and severe cases of diarrhoea in Lagos was investigated. A total of 49 *Shigella* strains were isolated with 33 grouped as mild shigellosis and 16 classed as severe shigellosis. 20 mild and 3 severe shigellosis strains were typed avirulent by congo red assay and sereny test. Plasmid analysis showed that 28 of the mild shigellosis strains lacked the 2 and 6Mdal plasmids, while 11 severe shigellosis strains harboured these plasmids. All 2 and 6Mdal plasmids' strains were virulent by conventional methods, deficient in glucose, contained rhamnose and elicited a high extracellular protease activity. Although all the strains produced extracellular protease with marked activity at 8 h of growth, the activity of this enzyme at 12 h was significantly higher ($t = 8.6$; $P < 0.05$) in virulent than avirulent strains. However, at 20 h, the difference in enzyme activity became non-significant ($t = 0.8$; $P > 0.05$). Similar pattern was observed in the controls. None of the mild shigellosis strains of *Sh. Sonnei* harboured the 120 and 140 Mdal plasmids. Strains that were avirulent by conventional methods but harboured 120 and 140 Mdal plasmids displayed protease kinetics that did not follow a clearly defined pattern. They also contained rhamnose but the amount (3-5%) was significantly lower ($X^2 = 117.9$; $P < 0.05$) than the rhamnose content (23-40%) of the virulent strains.

These results indicate that (1) the 2 and 6 Mdal plasmids are the primary accessories of virulence in bacterial pathogens of shigellosis, while the 120 and 140 Mdal plasmids play a secondary role; (2) plasmid profiling, cell wall polysaccharide sugar analysis and protease kinetic study identifies virulence in shigellosis better than the conventional methods and (3) the severity of shigellosis is a function of many other factors, which are beyond the scope of this study.

Key Words: Shigellosis; *Shigella* sp.; Virulence; Plasmid profiling

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Introduction

Shigellosis is a public health problem that displayed endemicity in communities affected by over crowding, poverty, poor hygiene, poor sanitation and inadequate health facilities (Keush *et al*, 1989; Wharton *et al*, 1990). Shigellosis accounted for 70% of the 30% infant mortality associated with diarrhoeal infection in Nigeria (Steven, 1996). In the country, the nationwide incidence of the disease was put at 24%, but incidence as high as 46% in some peri-urban communities in Lagos has been reported (Bamgbala *et al*, 1999). In an hospital study, Niemogha *et al*., (1995) reported an incidence rate of 48%. Olukoya and Oni (1990) had previously isolated 100 strains of *Shigella* species from endemic communities in Lagis and attributed multiple drug resistance and prevalence to plasmid diversity and transmissibility.

Plasmid profiling, cell wall polysaccaride analysis and protease activity determination have been used to increase the understanding of virulence and pathogenesis of shigellosis in Bangladesh and some other endemic communities (Hossain *et al*, 1993). This has enhanced surveillance and improved treatment and control of the scourge in these communities. Similar strategies have not been adopted in Lagos, thereby making knowledge of morbidity and mortality inadequate. Virulent and avirulent strains are poor differentiated and this has hindered proper management of the disease in the country. Cases of delay diagnosis have also been shown to cause prolongation and severity of disease episode, enhanced transmission, morbidity and mortality in many communities (Wells and Morris, 1981).

In the present study, virulent and avirulent strains of *Shigella* sp. in Lagos were differentiated using conventional Congo red assay and Sereny test by molecular/biochemical methods of plasmid analysis, sugar analysis and protease assays. The accuracy of these methods were also compared.

Materials and Methods

Patients and sample collection

The stool samples and rectal swabs of 513 patients with diarrhoeal were collected from referral centres in Lagos and cultured in sterile bottles containing 10ml of selenite – F broth and taken to the laboratory for subculturing and other analyses. Severe cases were based on immediate hospitalisation and intravenous rehydration therapy due to severe weight ($\geq$ 5%), water and electrolyte loss.

Identification and speciation of Shigella isolates

The stool samples and rectal swabs were subcultured into sterile sterilin plates (UK, England) containing 20ml each of McConkey agar (Oxoid, England),Xylose desoxycholate agar and salmonella – shigella agar (Difco, USA). Non lactose fermenters and hydrogen sulphide negative strains were then used to inoculate Kliger-iron-agar and motility-indole-urea (15ml/tube). Further differentiation and serotyping of Shigella strains were carried out using standard procedures (Cowan and Steel, 1972; W.H.O, 1983). Severe and mild shigellosis were classified based on the sample source.

Congo red assay and Sereny test

The congo red binding assay for the assessment of virulence in the Shigella strains isolated was carried out as described by Quadri *et al*., (1988). In brief, the standardised inoculum if each strain (1.5×10^8cfu/ml) was used to inoculate 20ml trypticase soy agar (TSA) containing 0.6% yeast extract (Gibco) and 0.003% congo red (Fisher Scientific Co. NJ. USA). The plates were incubated at 37°C for 18 h. After the incubation period, red and white colonies were attributed to virulent and avirulent strains. As gifts from N.I.M.R, Nigeria, a congo red negative mutant strain of *Shigella sonnei* (SHC – 1) obtained by reoeated subculturing on MacConkey was used as the negative control, and the positive control was a congo red positive *Sh. Sonnei* strain named (SHC – 2). Virulence was further investigated by subjecting the *Shigella* strains and controls to sereny test as described (Sereny, 1955).

Plasmid analysis

Plasmids were isolated by the method of Birnboim and Doly (1979) and separated by electrophoresis for 3 h at 30mA in 0.8% agarose. Plasmids isolated from congo red and sereny negative strains were electrophoresed for 4 h in 0.8% agarose to detect the deletion in the large plasmids (Haider *et al*, 1990).

Analysis of cell wall carbohydrates in Shigella strains.

The delipidation of the cell wall and analysis of the cell wall O-lipopolysaccharide in the *Shigella* strains were determined by the methods of Westphal *et al*., (1965) and Dubois *et al*., (1956) with a little modification. The strains were separately cultured in trypticase soy agar at 37°C for 24 h. The cells were washed and pelleted by centrifugation at 12000 x g for 10 minutes with phosphate buffered saline (PBS). LPS was extracted from the cells by phenol : water (70:30) treatment and separated pure by ultracentrifugation at 105,000 x g for 4 h. The LPS samples were then hydrolysed by 2ml of 2M trifluoroacetic acid at 120°C for 3 h. Hydrolysates were evaporated to dryness to remove the acid and re-dissolved in sterile water (0.5ml). Sugar analysis was done by TLC on silica gel using ethyl acetate : isopropanol : water : pyridine (26:14:7:2) solvent system and aniline : diphenylamine :phosphoric acid (5:5:1) as the locating reagent with heating at 100°C for 10 minutes. Spots of rhamnose, galactose, glucose and N-acetylglucosamine (1mg/ml) were used as standards. Sugar residues were identified based on R_f values. The sugar spots were eluted with 0.02M sodium acetate (pH, 6.0) and quantitated as described by Dubois *et al*., (1956).

Protease activity in Shigella strains

Twenty-four hours protease activity at 4 hourly intervals was determined in both mild and severe shigellosis strains according to Li and Yousten (1975). At the time of assays, the 18 h growing culture in TS broth was pelleted at 5000 x g for 15 minutes, the supernate filter sterilised (0.45μ M millipore) and dialysed against PBS (pH, 7.2) overnight at 4°C. Using Azoalbumin (Sigma, 0.5% in 0.1M Morpholinopropane sulphonic acid (MOPS), pH 7.0) as the substrate, a unit of protease activity was defined as the amount of enzyme, which caused an absorbance increase of 0.01 per hour under assay conditions: pH, 7.4; temperature, 37°C; incubation time 1 h.

Statistical analysis

Data were analysed as mean ± SEM and percentage composition. Differences between mean values were investigated by using student's t test and differences between percentages were analysed by the Chi square analysis with Yate's correction.(Steele and Torrie, 1987). $P < 0.05$ and $P > 0.05$ represented significant and non significant differences.

Results

Strains

Out of the 513 diarrhoeal samples collected, a total of 49 strains of *Shigella* species were isolated from mild (33) and severe (16) shigellosis cases. (Table 1).

Congo red and sereny test.

In the mild shigellosis cases, 20 strains were Congo red negative and did not display invasive character. 13 severe shigellosis strains were congo red positive and caused keratoconjuctivis in the guinea pig eye. The controls: SHC-1 and SHC-2 were congo red /sereny negative and positive respectivelt (Tables 2 and 3).

Table 1: The distribution of *Shigella* species isolated from cases of mild and severe shigellosis in Lagos, Nigeria.

Organism (No.)	Mild shigellosis	Severe shigellosis
Sh. flexneri (26)	17	9
Sh. dysenteriae (14)	8	6
Sh. boydii (5)	5	0
Sh. sonnei (4)	3	1
Total	33	16

Plasmid profiling

The 17 *Shigella flexneri* strains isolated from mild shigellosis cases harboured plasmid with sizes ranging from 2.8 to 120 Mdal. The 6 Mdal plasmid was harboured by 7 of the strains. 6 of the 9 strains isolated in severe shigellosis harboured the 2 Mdal plasmid. 6 strains of Sh. dysenteriae in mild shigellosis contained 120 and 140 Mdal plasmids, while 2 and 6 Mdal plasmids were found in 5 of the severe shigellosis strains. On the whole, 14 strains expressed plasmids of 2.3 – 140 Mdal range. Five strains of *Shigella boydii* were isolated in severe cases. None of the strains of Shigella sonnei contained 120 and 140 Mdal plasmids (Tables 2 and 3).

Sugar analysis (mild and severe shigellosis).

Sugar analysis in mild shigellosis cases revealed the presence of rhamnose, glucose, galactose, N-acetylglucosamine and other substances in the cell wall polysaccharide of *Shigella flexneri* strains. Rhamnose (5%), is found in 3 of the strains. The percentage of other sugars were glucose (33%), gal (26%), N-acetylglucosamine (21%) and other constituents (15%). Rhamnose occurred at 2, 3 and 4% in the polysaccharide preparations of five *Sh. boydii, Sh. dysenteriae and Sh. sonnei* strains. These strains also contained glucose, galactose and N-acetylglucosamine in amounts ranging from 9 to 41% (Table 2).

A higher amount of rhamnose (23-40%) was found in sixteen severe shigellosis strains that contained 2, 6, 120 and 140 Mdal plasmids. This was found to differ markedly ($X^2 = 117.9$; $P < 0.05$) from the value obtained in mild shigellosis cases (Table 3). Glucose was found in 5 of the 16 strains at low amounts (3-7%), galactose, N-acetylglucosamine and other constituents were also present at 10-30% range (Table 3).

Protease activity

All the strains grouped as mild and severe shigellosis produced extracellular protease. Marked protease activity began at 8 h of growth in all the strains. At 2 h of growth, protease activity of severe shigellosis strains was significantly higher ($t = 8.6$; $P<0.05$) than that of mild shigellosis strains. However, the difference in activity at 20 h of growth was not significant ($t = 0.8$; $P>0.05$) (Figs. 1 and 2). Similar pattern was observed in the controls (Fig. 3).

Extracellular protease activities in 120 and 140 Mdal plasmid strains of *Sh. dysenteriae, Sh. flexneri and Sh. sonnei* were shown in Fig. 4. The enzyme activity with time in these strains did not follow a similar pattern in the level of activity when compared with their respective species typed as avirulent and virulent strains in Figs. 1 and 2 (Fig. 4).

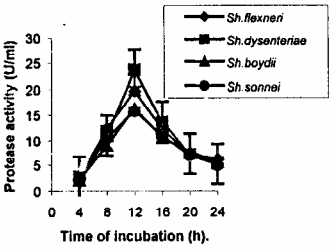

Fig 1: Extracellular protease activity of avirulent strains of Shigellae.
(Each parameter was mean ±SEM of at least 5 strains).

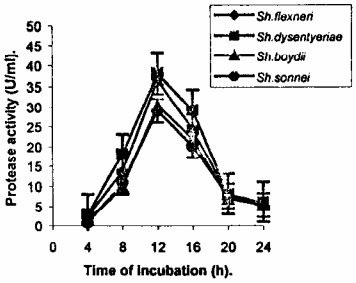

Fig 2: Extracellular protease activity of virulent strains of Shigellae.
(Each parameter wasa mean ±SEM of at least 5 strains).

Growth	t	p
12 h	8.6	< 0.05
20 h	0.8	> 0.05

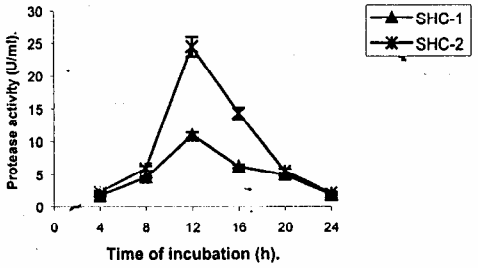

Fig 3: Extracellular protease activity of SHC-1 and SHC-2 controls. (Each parameter was mean ±SEM of 3 determinations).

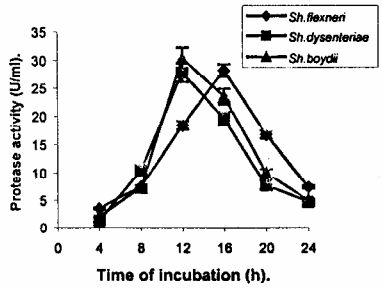

Fig 4: Extracellular protease activity of 120 and 140 Mdal plasmids strains of Shigellae. (Each parameter was mean ±SEM of at least 3 strains).

Discussion

Virulence in shigellosis is based on colonic mucosal invasion, spread of pathogens, toxin production, epithelial cell death, haemorrage and systemic complications such as bacteremia, hypoglycemia, hyponatraemia and seizure, which eventually results in morbidity and mortality (Hale *et al*, 1987). Congo red assay and sereny test have been used to determine virulence in *Shigellae* (Khaleda *et al*, 1993). Thus, they were adopted as conventional methods in this study. By these methods, 13 each of mild and severe shigellosis strains were virulent. The SHC-1 and SHC-2 controls can be said to be avirulent and virulent respectively. The genetic basis of virulence in shigellosis has been attributed to plasmids and some loci in the chromosomes of *Sh. dysenteriae, flexneri and sonnei* (Sansonnetti *et al*, 1981; Petrovyskaya *et al*, 1982). Plasmid analysis could be observed as providing more accurate typing of virulence in the studied isolates as all the 2 and 6 Mdal plasmid strains were also virulent by the conventional methods. Virulence by *Shigella sonnei* strain isolated from severe shigellosis cases may be due to the 2.2 Mdal plasmid. The plasmid might be a hybrid containing loci that mediated virulence and avirulence. Invasion association locus (ial) in Shigella and enteroinvasive *Escherichia coli* (EIEC) has been analysed by PCR to be flanked by unique sequences that allowed primers production (Frankel *et al*, 1990). The possibility of contamination of the studied strains with EIEC is also inevitable as bloody diarrhoea epidemic in Southern Cameroun due to the association of Shigella and EIEC (0157:H7) was reported recently (Patrick *et al*, 1999).

The roles of 120 and 140 Mdal plasmids in virulence as reported by Haider *et al*., (1990) is most likely in these strains as those that harboured them were virulent by conventional methods. The isolation of the 120 and 140 Mdal plasmid strains in mild and severe shigellosis cases suggest the involvement of other factors in the severity of shigellosis. Exposed latrines, camping due to political upheaval, poor sanitation and personal hygiene were among the causes of shigellosis associated with high fatality rate reported in communities in Africa (Frost *et al*, 1985), Asia (Taylor *et al*, 1991) and Central America (Gangarosa *et al*, 1970). Despite being a metropolitan city, Lagos still houses many peri-urban communities with no portable water and inadequate public health facilities and where poverty has compelled majority of the inhabitants to consume malnourished diets from unhygienic food vendors (Adebayo *et al*, 1992). Early detection of diarrhoea causing strains by plasmid profiling even in the few public centres in these communities could help in halting the tragic course of pathogenesis of these strains and the number of deadly carriers. The expected beneficial effect of this approach might result in the reduction of high morbidity and mortality rates found currently in most of the communities (Bamgbala *et al*, 1999).

Hossain *et al*., (1993) reported a variation in the sugar composition of cell wall polysaccharide of O-antigen in *Shigella dysenteriae* type 1 as the basis of virulence. In that study, the experimental Congo red and sereny test negative *Shigella dysenteriae* strains which also lacked the 6 Mdal plasmid were found to lack rhamnose, O-antigen but contained a significant amount of glucose. The high amount of rhamnose (23-40%) in the severe shigellosis strains further confirmed their virulence in line with the results of Hossain *et al*., (1993) and Dmitriev *et al*., (1976). The presence of rhamnose (2-5%) in 120 and 140 Mdal plasmid strains indicates the involvement of these plasmids in O-antigen polysaccharide synthesis in Shigallae. Although, still lower than (32-41%) in avirulent strains, the presence of glucose (2-7%) in virulent strains in severe shigellosis is at variance with the work of Hossain *et al*., (1993). This might be due to the differences in locations from which these strains were isolated.

The high amount of glucose in these strains may be due to a switch in the biosynthesis of their cell wall Lipopolysaccharide (LPS) caused by the absence of the gene products of 2 and 6 Mdal plasmids. Thus, glucose compensated for the deficient rhamnose to alter the composition and function of LPS in avirulent strains. The unidentified constituents found in both mild and severe shigellosis strains may include heptose, O-acetyl, carboxyethyl and pyruvic acid acetal. They have been found in the repeating units of some of the serotypes of *Shigella dysenteriae* (Kenne *et al*, 1982). However, their roles in virulence remains unknown.

Protease, mucinase and neuraminidase triad have been described as ‘mucinase complex’ and reported as enzymes involved in the pathogenesis of intestinal fluid production mediated by *V. cholerae* (Kabir *et al*, 1984). Mucinolytic activity of certain strains of *Shigella spp* has also been demonstrated *in vitro* (Formal *et al*, 1956). Proteases are protein hydrolytic enzymes, their extracellular production by *Shigella* strains isolated in this study may be an indication of their ability to penetrate extracellular matrix of the jejunum, ileum and colon. However, variation in the activity of extracellular protease in these strains might explain the degree of penetration of the extracellular matrix and the potential of adherence and complications. The

mean protease activity at 12 h of incubation of the virulent strains, which was significantly higher ($t = 8.6$; $P<0.05$) than that in avirulent strains implies that pathogenesis caused to virulent *Shigellae* might be shorter in duration and lethal due to enhanced extracellular matrix protease degradation. The non-significant ($t = 0.8$; $P > 0.05$) protease activity between strains at 20 h of growth might be due to biomass accumulation and cell death.

In the present study, it can be concluded that severity of shigellosis in Lagos can be reasonably diagnosed by plasmid profiling, sugar analysis of cell wall polysaccharide and extracellular protease assay. Sereny test and Congo red binding assays can be used as first line of diagnoses, although, false positive results are possible. The mode of disease management is a function of health facilities on ground and not advisable to be used as an accurate indicator of virulence and severity of shigellosis.

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