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Cellular Toxicity and Effects of *Shigella* Enterotoxigenic Fractions on Catalase, Retinol and α-Tocopherol Levels in Mice

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This study evaluates the patterns of enterotoxigenicity and cytotoxicity of enterotoxin fractions recovered from Shigella isolates from Lagos, Nigeria. The *in vivo* effects of the recovered enterotoxin fractions on intestinal, liver and systemic levels of total protein, retinol, α-tocopherol and catalase in mice were also determined. A total 23 *Shigella* isolates recovered from Nigerian patients presented with diarrhea at General hospitals, primary health centres and clinics in Lagos, Nigeria and speciated as *S. flexneri* (n = 9), *S. dysenteriae* (n = 6), *S. sonnei* (n = 1) and *S. boydii* (n = 4) were submitted for enterotoxigenicity and cytotoxicity evaluations. The excised tissue homogenate and blood samples were used for the determination of total protein, retinol, alpha-tocopherol and Catalase contents using HPLC and spectrophotometric methods. Of the 23 isolates tested, 12 (52.2%; $p>0.05$) from *S. flexneri* (6 of 9; $p<0.05$), *S. dysenteriae* (3 of 6), *S. sonnei* (2 of 4) and *S. boydii* (1 of 4) were found to be enterotoxigenic. Enterotoxin activity of the positive isolates ranged from 5.0-15.7 with *S. flexneri* eliciting the highest activity (15.7±1.9) followed by *S. dysenteriae* (10.0±2.7), *S. sonnei* (6.5±1.5) and *S. boydii* (5.0±3.0) ($p<0.05$). The overall mean enterotoxin activity of the 12 positive isolates was found to be 11.8±1.4 units. Reductions in intestinal protein content to 61.2-77%, catalase activity to 64.9-82.1%, retinol to 67.2-93.4% and alpha-tocopherol to 81.3-92.9% of control values were observed in mice after 24 h of exposure to culture filtrates of the Shigella isolates tested. All reductions were significant ($p<0.05$) for *S. flexneri* and *S. dysenteriae* (with the former eliciting greater effect). At extra-intestinal levels, only reduction in retinol level was significant ($p<0.05$) for these isolates. Cell culture experiments showed that *S. flexneri* and *S. dysenteriae* enterotoxin fractions inhibited growth and elicited cytopathic effect on caco-2-cells with viability reduced to 78.2-82.5% normal after 1h challenge *in vitro*. The results of this study indicate that *Shigella* enterotoxins due to *S. flexneri* and *S. dysenteriae* may play a role in intestinal antioxidant and micronutrient depletion coupled with gut epithelium disruption in shigellosis.

Key words: *Shigella*, enterotoxins, intestinal antioxidants, cell viability, cytopathic effect, mice

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INTRODUCTION

Diarrhea remains a potent killer of children and travelers world wide and is caused by multiple aetiologic agents including shigella (Keush, 1986; Gascon *et al*., 1998). The presence of *Shigella* in the gut has been found to induce abnormal ultrastructural changes in the epithelium, provokes inflammatory episodes that mediate tissue damage, incites an abnormal transport of water and electrolyte and creates a local immunodeficiency state (Donowitz *et al*., 1975; Zhang *et al*., 2001).

Enterotoxins are among the virulence factors credited for these pathogenic pathways (Keush, 1986). They have been detected in *Campylobacter jejuni*, enteroaggregative and enteroinvasive *E. coli* as well as *Yersinia enterocolitica* (Coker and Obi, 1989; Resta-Lenert and Barrett, 2003; Gascon *et al*., 1998; Pai and Mors, 1978). In shigella, two enterotoxins named enterotoxin 1 encoded by a chromosomal borne gene called set 1 and enterotoxin 2 encoded by sen 1 located on the 140 MDa plasmid have also been reported and found in all the serogroups (Vargas *et al*., 1999; Roy *et al*., 2006). The two enterotoxins have been found to mediate the watery phase of shigellosis with enterotoxin 2 together with invasion associated locus (*ial*) and invasion plasmid antigen H (*ipa*H) contributing to shigella invasion of epithelium mucosa and triggering acute phase inflammatory response in shigellosis (Vargas *et al*., 1999).

The gut provides an interface between the luminal compartment and the systemic circulation. It is endowed with a myriad of micronutrients and proteins from fetal to neonatal life obtained through maternal nutrition and gene expression (Quick and Ong, 1989; Xueping *et al*., 2002). Among these micronutrients are retinol and α-tocopherol, which function as free radical and radiation quenchers as well as attenuators of chain reactions required progressive membrane lipid peroxidation in oxidatively stressed epithelial cells (Felemovicius *et al*., 1995; Mehta *et al*., 1998). Retinol in conjunction with its metabolites such as retinoic acid is also essential for growth, reproduction, fetal development and vision (Clagett-Dame and DeLuca, 2002). The vitamin has also been found to impair intestinal absorption and movement of alpha-tocopherol and vice versa (Blakely *et al*., 1991; Basova *et al*., 2002).

Among the proteins of the gut, catalase has been found to be one of the housekeeping antioxidant enzymes known for protection against H_2O_2 toxicity through detoxification to water and oxygen (Bhor *et al*., 2004). Several studies have reported elevated level of catalase in intestinal tissues subjected to ischaemia/reperfusion injury (Deshmukh *et al*., 1997).

The influence of shigellosis on gut architecture and functions has severally been reported from animal model and human studies (Zhang *et al*., 2001). It has been associated with intestinal protein loss (i.e., protein enteropathy), urinary excretion of retinol and enterocyte villi destruction (Black and Levine, 1991; Mitra *et al*., 1988). A few clinical trials by Hossain *et al*. (1998) and Salam *et al*. (1999) have also revealed the efficacy of retinol supplementation in the management of children with shigellosis, validating a linkage between shigellosis and hypovitamin A.

Unlike shiga toxin from *S. dysenteriae* 1, enterotoxins are well recognized as secreted virulence factors in all *Shigella* serogroups. But their contribution to micronutrient depletion of the gut during the early watery phase of shigellosis remains unclear. There is also lack of data on the relationship between enterotoxins and gut antioxidant enzymes as well as the effects of these virulence factors on the homeostasis of retinol and α-tocopherol in systemic circulation and extra-intestinal tissues. Unlike shiga toxin, the toxic effects of enterotoxins on cells is also not well-known. A fuller understanding of the roles played by enterotoxins in the pathogenesis of *Shigella* infection in humans is hoped to improve management of shigellosis and provide scientific justification for a better and future *Shigella* anti-toxic vaccine constructs.

In the present study, overnight culture filtrate of 23 *Shigella* isolates recovered from Nigerian patients were tested for enterotoxigenicity in mice coupled with the determination of intestinal, systemic and hepatic levels of retinol, α-tocopherol and catalase in the exposed animals. We observed variations in enterotoxigenicity among the *Shigella* serogroups tested and loss of retinol homeostasis. We also observed growth inhibitory and cytopathic effects of the culture filtrate on caco-2-cells, suggesting the ability of enterotoxins to impair enterocyte growth and cause cell injury.

MATERIALS AND METHODS

***Shigella* strains:** A total of 23 *Shigella* isolates comprising *S. flexneri* (n = 9), *S. dysenteriae* (n = 6), *S. sonnei* (n = 4) and *S. boydii* (n = 4). The isolates were recovered from diarrheic stools of patients attending General hospitals, Primary health centres and clinics in Lagos, Nigeria between October 2004 and March, 2005 and identified by conventional biochemical methods (Cowan, 1974). They were stored at -20°C in trpticase soy broth containing 15% glycerol (TSBG). Prior to use, the isolates were tested for viability by growth in trypticase soy broth containing 0.3% yeast extract and then subcultured in trypticase soy agar medium.

Enterotoxin fraction production and enterotoxigenicity assay: The enterotoxin fractions recovered from each of the 23 *Shigella* isolates were tested for their ability to induce fluid accumulation in the ileum of 5-6 day old mice according to Dean's *et al.* (1972). For enterotoxin production, cells (300 cfu mL^{-1}) recovered from overnight culture of each isolate were inoculated into 50 mL of Brain Heart Infusion (BHI) broth and grow with shaking (300 rpm) at 37°C for 24 h. The resulting culture was centrifuged at 12,000 x g for 10 min to obtain a cell-free supernatant, decanted into another sterile capped bottle and regarded as the crude enterotoxin fraction. The enterotoxigenic fraction was filtered through a 0.45 μm Millipore filter and used immediately for the enterotoxigenicity assay.

For the enterotoxigenicity assay, twofold serial dilutions of each enterotoxin fraction was intragastrically inoculated (100 μL each) into infant mice (2 mice per dilution). Culture filtrate (100 μL) from enterotoxigenic *E. coli* Ng012 and Phosphate Buffered Saline (PBS) solution were also assayed in parallel to serve as positive and negative controls, respectively. The inoculated mice were then kept at room temperature for 24 h before they were sacrificed by cervical dislocation after a chloroform anesthesia. The animals were laparotomized, the small intestine excised with a pair of forceps and scissors and finally weighed. The ratio of gut weight to body weight >0.08 was considered positive for enterotoxin production and Enterotoxin Activity (EA) was expressed as the as the reciprocal of the highest dilution that gave gut/body weight ration >0.08 (15). Blood samples were also collected into EDTA bottles and centrifuged at 2000 rpm for 5 min to obtain plasma collected into fresh plain bottles.

Cell culture and inhibition assay: Caco-2-cells (ATCC-HTB37) derived from human colonic adenocarcinoma were cultured in Dubecco's Modified Eagle medium (D-MEM) containing 26 mM $NaHCO_3$, 10% Fetal Bovine Serum (FBS), glutamine, 0.1 mM non-essential amino acids supplemented with 50 U mL^{-1} penicillin and 50 μg mL^{-1} streptomycin, pH 7.4 at 37°C with atmospheric CO_2 at 5% (Kovbasnjuk *et al.*, 2001). The cells were grown to confluency in a 60 mm petri dish after 15 days of incubation. Five milliliter of cell suspension in PBS (10^5 cells mL^{-1}) were then subcultured in 20 mL D-MEM containing enterotoxin fraction (1 mL) from each of the tested strains. This was followed by incubation as described previously. Enterotoxin free petri dishes with or without caco-2-cells were used as control. Cytopathic effect was defined as the morphological changes in growth pattern and dislodgement of caco-2-cells. Growth inhibition was indicated by the absence of confluent monolayer of caco-2-cells after 15 days of incubation.

Cytotoxicity assay: The cytotoxic effect of the enterotoxin fraction on caco-2-cells using the MTT [formazan 3-(4,5-dimethlythiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide] reagent as described by Mosman (1983). Wells containing caco-2-cells and MTT but lacked enterotoxin fraction served as the control. The plate was incubated for 1 h at 37°C in the dark. Cytoviability of the control cells was considered to be 100%. While the viability of the treated cells was expressed as a percentage of control cells. Each enterotoxin fraction was run in triplicate assays. Absorbance of the solubilized blue formazan color formed was measured at 595 nm.

Homogenate preparation and retinol and alpha-tocopherol quantitation: The excised intestine (after weighing) and liver were immediately washed in PBS (pH 7.2) to remove blood and homogenize in 4 volumes of the same buffer using a Teflon glass homogenizer.

The concentrations of retinol and alpha-tocopherol were determined from each homogenate sample according to Craft *et al.* (2000) and Peng *et al.* (1993) with some modifications. An aliquot (40 μL) of each homogenate or plasma sample was added to a test tube containing 200 μL of ethanol. Distilled water (180 μL) was then added to this mixture followed by vortexing for 30 sec. After vortexing, the mixture was extracted on ice twice with hexane stabilized by the addition of butylated hydroxytoluene (BHT) at 0.05% final concentration to prevent lipid peroxidation and optimize the recovery of retinol and alpha-tocopherol. The hexane fractions were collected into separate tubes after centrifugation of the emulsion at 1500 rpm for 10 min, air dried and finally reconstituted with 100 μL of isopropanol. The concentrations of retinol and α-tocopherol in plasma and tissue homogenates were determined by reverse phase HPLC with 80 μL of isopropanol extract injected into C_{18} column (Shimazu, Columbia) using methanol:acetonitrile:isopropanol (3:1:1 v/v) as the mobile phase. Retinol was detected at 325 nm and α-tocopherol at 295 nm. The accuracy and precision was determined using retinyl palmitate and δ-tocopherol as internal standards. The coefficient of variability was <3% and recovery rates were 96 and 99%, respectively Concentrations of retinol and α-tocopherol were expressed as microgram per gram tissue and micromole per litre of whole blood, respectively.

Catalase and protein assays: Catalase (E.C.1.11.1.6) activity was determined by monitoring the decomposition of H_2O_2 at 240 nm and 25°C as described

by Bergmeyer *et al*. (1983). The tissue homogenate was diluted 10 times with 20 mM potassium phosphate buffer (pH 7.4) prior to enzyme assay. A catalase activity unit is defined as the decomposition of 1 mmole of H_2O_2 per second under the assay conditions and specific activity was defined as units per mg protein.

The levels of protein in the tissue homogenates were determined by the Biuret method (Gornall *et al*., 1949) with bovine serum albumin (0.2-1 mg) as standard. The protein content was expressed as mg protein per mg tissue.

RESULTS

The patterns of enterotoxigenicity of the 23 *Shigella* isolates based on ileal fluid accumulation in culture filtrate exposed-mice are presented in Table 1. Of the 23 isolates tested, 12 (52.2%; $p>0.05$) from *S. flexneri* (6 of 9; $p<0.05$), *S. dysenteriae* (3 of 6), *S. sonnei* (2 of 4) and *S. boydii* (1 of 4) 1 were found to be enterotoxigenic. Enterotoxin activity of the positive isolates ranged from 5.0-15.7 with *S. flexneri* eliciting the highest activity (15.7±1.9) followed by *S. dysenteriae* (10.0±2.7), *S. sonnei* (6.5±1.5) and *S. boydii* (5.0±3.0) ($p<0.05$). Overall enterotoxin activity of the 12 positive isolates was 11.8±1.4 and further found to be significantly ($p<0.05$) higher than those of *S. sonnei* and *S. boydii*, respectively.

Compared to the control, reductions in intestinal protein content (0.85-1.07 vs. 1.39±0.01-0.06 mg mg^{-1} wet weight) to 61.2-77%, catalase activity (2.4-3.2 vs. 3.7±0.05-0.15 U mg^{-1} protein) to 64.9-82.1%, retinol (0.82-1.14 vs. 1.22±0.02-0.03 μmole mg^{-1} protein) to 67.2-93.4% and alpha-tocopherol (0.91-1.04 vs. 1.12±0.01-0.03 μmole mg^{-1} protein) to 81.3-92.9% were observed in mice after 24 h of exposure to culture filtrates of the *Shigella* isolates tested. The reductions caused by *S. flexneri* and *S. dysenteriae* (with the former eliciting greater effect) were found to be significant ($p<0.05$) in the four parameters measured. Significant ($p<0.05$) reductions in intestinal catalase and protein were also observed for *S. sonnei* and *S. boydii*. Systemic and extra-intestinal analyses of these parameters further revealed a significant ($p<0.05$) reduction in plasma retinol level (0.98-1.04 vs. 1.21±0.01-0.04 umole L^{-1}) *S. flexneri* and *S. dysenteriae*-exposed mice but non-significant ($p>0.05$) in mice exposed to the enterotoxigenic fractions of *S. sonnei* and *S. boydii* (Table 2).

Because of the pronounced alterations of the parameters measured at intestinal level, disparity between mice negative and positive for enterotoxigenic phenotypes of the Shigella isolates was investigated. Greater and significant reduction in intestinal retinol level (0.84-0.9 vs. 1.02-1.1±0.02-0.1 μg mg^{-1}) for *S. flexneri* and *S. dysenteriae*, α-tocopherol (0.86±0.03 vs. 1.06±0.01 μg mg^{-1}) for *S. dysenteriae*, catalase (2.12-2.88 vs. 2.82-3.32±0.02-0.09 U mg^{-1} protein) for all *Shigella* serogroups and total protein (0.85-0.87 vs. 1.02-1.08±0.01-0.03 mg mg^{-1} tissue) for *S. dysenteriae* and *S. flexneri*-enterotoxin positive mice compared to their negative counterparts (Table 3). Further analysis between

Table 1: Pattern of enterotoxingenicity of the *Shigella* isolates in mice

	Enterotoxingenicity assay		
Shigella isolates	Positive, n (%)	Negative, n (%)	Enterotoxin activity (mean±SEM)^
S. flexneri	6 (66.7)*	3 (33.3)	15.7±1.9[a]
S. dysenteriae	3 (50)	3 (50)	10.0±2.7[b]
S. sonnei	2 (50)	2 (50)	6.5±1.5[c]
S. boydii	1 (25)	3 (75)	5.0±3.0[d]
Total	12 (52.2)	11 (47.8)	11.8±1.4[b]

^: Enterotoxin activity is defined as the reciprocal of maximum dilution of culture filtrate, which produces gut weight/body weight ratio >0.08. Superscripted letters of different types indicate significant ($p<0.05$) difference between mean values (ANOVA). *: $p<0.05$ (positive vs. negative), Chi-square analysis

Table 2: Intestinal, plasma and hepatic levels of retinol, α-tocopherol and Catalase of mice exposed to culture filtrates of the *Shigella* isolates

	Intestine				Plasma				Liver			
®Animals	RET	α-TC	CAT	PRT	RET	α-TC	CAT	PRT	RET	α-TC	CAT	PRT
Unexposed mice (n = 10)	1.22±0.03	1.12±0.02	3.7±0.05	1.39±0.02	1.21±0.02	1.10±0.04	5.3±0.2	5.9±0.06	1.09±0.01	1.03±0.01	4.5±0.4	1.41±0.03
S. dysenteriae-mice (n =18)	1.00±0.03[a] (82)	0.96±0.03[a] (85.7)	2.6±0.07[ac] (70.3)	1.03±0.06[a] (74.1)	1.04±0.04[a] (86)	1.09±0.02 (99.1)	5.2±0.1 (98.1)	5.7±0.07 (96.6)	1.03±0.01 (94.5)	1.02±0.02 (99)	4.7±0.4 (104.4)	1.35±0.02 (95.7)
S. flexneri-mice (n = 12)	0.82±0.02[ab] (67.2)	0.91±0.01[a] (81.3)	2.4±0.09[ac] (64.9)	0.85±0.02[ab] (61.2)	0.98±0.01[a] (81)	1.03±0.02 (93.6)	5.1±0.1 (96.2)	5.6±0.09 (94.9)	1.01±0.01 (92.7)	1.00±0.01 (97.1)	4.3±0.1 (95.6)	1.34±0.01 (95)
S. sonnei-mice (n = 8)	1.10±0.03 (90.2)	1.02±0.02 (91.1)	2.9±0.15[a] (78.4)	1.01±0.01[a] (72.7)	1.10±0.02 (90.9)	1.04±0.02 (94.5)	5.2±0.1 (98.1)	5.6±0.06 (94.9)	1.03±0.04 (94.5)	1.03±0.01 (100)	4.4±0.1 (97.8)	1.40±0.02 (99.3)
S. boydii-mice (n = 8)	1.14±0.02 (93.4)	1.04±0.02 (92.9)	3.2±0.1[a] (82.1)	1.07±0.02[a] (77)	1.14±0.02 (94.2)	1.03±0.03 (93.6)	5.2±0.4 (98.1)	5.8±0.07 (98.3)	1.06±0.01 (97.2)	1.05±0.01 (101.9)	4.5±0.1 (100)	1.42±0.02 (100.7)
All exposed mice (n = 46)	1.00±0.02[a] (82)	0.98±0.01[a] (87.5)	2.7±0.07[a] (73)	1.00±0.02[a] (71.9)	1.05±0.01[a] (86.8)	1.02±0.01 (92.7)	5.2±0.1 (91.1)	5.7±0.05 (96.6)	1.02±0.01 (93.6)	1.00±0.01 (97.1)	4.4±0.1 (97.8)	1.36±0.01 (96.4)

n = No. of animals. ®: Two mice were tested per recovered *Shigella* enterotoxin fraction. Results are expressed as means±SEM of n determinations. Differences between mean values were analyzed by ANOVA. [a]$p<0.05$ compared to unexposed mice (control). [b]: $p<0.05$ (*S. flexneri*-mice vs. other Shigella-exposed mice), [c]: $p<0.05$ (*S. flexneri* or *S. dysenteriae* vs. *S. sonnei* or *S. boydii* exposed mice). Numbers in parentheses represent percentages (%) of control values. PRT = Total protein expressed as mg mg^{-1} wet tissue or g dL^{-1}; Ret = retinol, α-TC = α-tocopherol, expressed as umole L^{-1} in plasma and microgram per mg protein in tissues. CAT = Catalase expressed as units mg^{-1} protein. 1 catalytic activity unit = 1 mmole of H_2O_2 decomposed sec^{-1} mg^{-1} protein

Table 3: Intestinal levels of retinol, α-tocopherol, catalase and protein in enterotoxigenic positive and negative mice

@Animals (n1/n2)	Retinol		α-Tocopherol		Catalase		Protein	
	Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative
S. dysenteriae-mice (6/6)	0.90±0.02[a]	1.11±0.03	0.86±0.03[a]	1.06±0.01	2.16±0.02[a]	3.03±0.04	0.87±0.03[a]	1.08±0.03
S. flexneri-mice (12/6)	0.84±0.02[a]	1.02±0.02	0.92±0.02	0.96±0.02	2.12±0.02[a]	2.82±0.06	0.85±0.02[a]	1.02±0.01
S. sonnei-mice (4/4)	1.10±0.07	1.10±0.01	1.00±0.04	1.07±0.01	2.53±0.16[a]	3.22±0.03	0.99±0.01	1.04±0.01
S. boydii-mice (2/6)	1.13±0.02	1.16±0.02	1.04±0.01	1.05±0.01	2.88±0.03[a]	3.32±0.09	0.96±0.02	1.10±0.01
All exposed-mice (24/22)	0.93±0.03	1.09±0.01	0.93±0.02	1.03±0.01	2.26±0.06[a]	3.09±0.05	0.89±0.02[a]	1.06±0.01

n1 = No. of enterotoxin positive (Pos) mice; n2 = number of enterotoxin negative (Neg) mice. @Two mice were tested per recovered *Shigella* enterotoxin fraction.Results are expressed as means±SEM of n determinations. Differences between mean values were analyzed by ANOVA. [a]: $p<0.05$ compared to enterotoxin negative-mice. PRT = Total protein expressed as mg mg^{-1} wet tissue; Retinol and α-Tocopherol levels were expressed microgram mg^{-1} protein in tissues. Catalase activity was expressed as units per mg protein. 1 catalytic activity unit = 1 mmole of H_2O_2 decomposed sec^{-1} mg^{-1} protein. CF = Culture filtrate

Table 4: Growth inhibition and cytopathic effect of *Shigella* enterotoxin fractions on caco-2-cells

Strain	Cytopathic effect (days) 1-3	4-6	7-9	10-12	13-15	Growth inhibition
Shigella flexneri 005	2±	4±	4±	4±	4±	a
Shigella flexneri 013	±	3±	4±	4±	4±	a
Shigella flexneri 017	2±	4±	4±	4±	4±	a
Shigella flexneri 025	2±	4±	4±	4±	4±	a
S. dysenteriae 003	3±	4±	4±	4±	4±	a
S. dysenteriae 038	3±	4±	4±	4±	4±	a
S. dysenteriae 039	2±	3±	4±	4±	4±	a
S. boydii 011	0	0	0	0	0	b
S. sonnei 033	0	0	0	0	0	b
Control	0	0	0	0	0	b

0 = No morphological changes in cells growth pattern; 1±to 4±= 25, 50, 75 and 100% of caco-2 cells eliciting cytopathic effect. a = Inhibition of growth of the caco-2 cells; b = No growth inhibition of the caco-2-cells. Control = Caco-2 cells exposed to sterile water

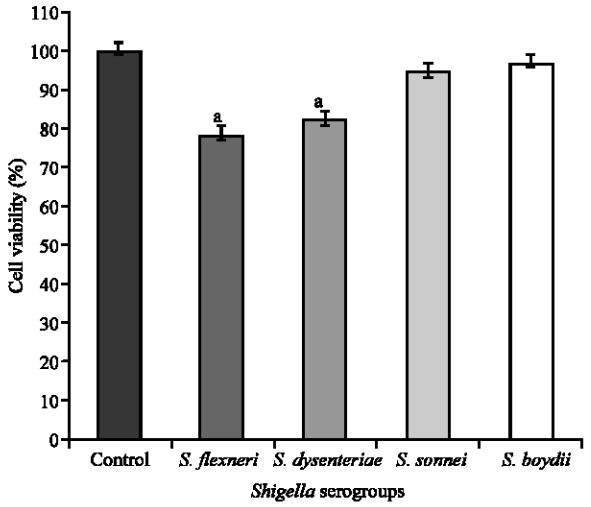

Fig. 1: Cytotoxic effect of Shigella enterotoxigenic fractions on caco-2-cells. Each bar represents mean±SEM percent viability of caco-2-cells exposed to Shigella enterotoxigenic fractions for 1h at 37°C. a $p<0.05$ significant reduction (test vs. control) (ANOVA)

enterotoxin-positive (n = 24) and enterotoxin-negative mice for all the isolates (n = 22) revealed significant disparity for catalase (2.26±0.06 vs. 3.39±0.05 U mg^{-1} protein) and total protein (0.88±0.02 vs. 1.06±0.01 mg mg^{-1} tissue) only (Table 3).

Furthermore, the growth inhibitory and cytopathic effects of 9 of the 12 enterotoxin positive *Shigella* isolates were investigated. Results indicate that the enterotoxin fractions of *Shigella flexneri* and *S. dysenteriae* strains elicited cytopathic effect on caco-2-cells from day 1 and characterized by 25-75% cell dislodgement (Table 4). These fractions also reduced cell viability to 78.2-82.5% of the control normal after 1 h challenge *in vitro* (Fig. 1).

These observations on caco-2 cells were not found with the enterotoxin fractions of *S. boydii* and *S. sonnei* (Fig. 1 and Table 4).

DISCUSSION

Enterotoxin secretions into the culture filtrates of enteropathogens during a 24 h growth in non-selective media such as brain heart infusion and trypticase soy broth have been used to assess the enterotoxigenicity of *Yersinia enterocolitica*, *E. coli* and *Campylobacter jejuni* in suckling mice (Pai and Mors, 1978; Singh and McFeters, 1986; Coker and Obi, 1989). Using this approach, we have found 60.8% of our *Shigella* isolates to be enterotoxigenic in mice similar to the finding of Donowitz *et al.* (1975) in rabbit ileum. At the molecular level, variations in the expression of enterotoxin genes in shigella have previously been reported by several researcher. Vargas *et al.* (1999) found 31 (60.78%) of the 51 *Shigella* isolates tested to be enterotoxigenic, while Roy *et al.* (2006) recently reported 49.1% of the *Shigella* isolates tested as enterotoxin 2 producers. Noriega *et al.* (1995) had also reported less (~ 3.3%) enterotoxin 2 production in 150 *Shigella* isolates tested.

More importantly the present study has revealed that *Shigella* isolates in Nigeria are also enterotoxigenic similar to the report of Coker and Obi (1989) for *Campylobacter jejuni*.

Regarding the enterotoxigenicity of our *Shigella* strains, we have found *S. flexneri* to elicit highest

enterotoxigenic activity followed by *S. dysenteriae*, *S. sonnei* and *S. boydii*. In clinical manifestation terms, our finding suggests that shigellosis due to *S. flexneri* is mostly likely to exhibit the watery phase compared to other forms of shigellosis. Molecular studies have been in support of this possibility as they have consistently ascribed enterotoxin 1 or enterotoxin 1 and 2 production to *S. flexneri* alone, while other *Shigella* serogroups are mostly noted for enterotoxin 2 production (Noriega *et al*., 1995; Vargas *et al*., 1999; Roy *et al*., 2006).

Meanwhile, *S. dysenteriae* 1 is well recognized for its toxigenicity based on shiga toxin production (Cantey, 1985). But this virulence factor is neither secreted nor involved in the watery phase of shigellosis (Cantey, 1985).

Furthermore, intestinal reduction in the levels of retinol, α-tocopherol and Catalase in enterotoxigenic positive greater than negative mice and the control was also observed in this study. Therefore, present finding provides a strong support for the involvement of enterotoxins in shigellosis-associated protein enteropathy and urinary retinol excretion previous reported in afflicted patients (Black and Levine, 1991; Mitra *et al*., 1988). Present study also reveals the possible urinary secretion of α-tocopherol in shigellosis with enterotoxin involvement.

The movement and metabolism of retinol in the gut is governed by key proteins such as cellular retinol binding protein type 2 (CRBPII) for binding hydrophobic retinol to be esterified for storage by another protein lecithin:retinol acyltransferase (LRAT) and retinol to be oxidized sequential to retinal and retinoic acid by retinol dehydrogenase (RDH) and retinol oxidase (RO) (Zhang *et al*., 2002a, b). These proteins account for greater than 1% of total intestinal proteins (Xueping *et al*., 2002). Therefore, the possible loss of these proteins in shigellosis would undoubtedly compromise retinol absorption and storage in the gut mucosa. A low density lipoprotein receptor in the mucosa has also been found to be involved in movement and metabolism of α-tocopherol (Cohn and Kuhn, 1989). The loss of this protein due to shigellosis associated-enteropathy may also account for the loss of α-tocopherol in the gut.

Interestingly, the observed reduction in the intestinal levels of these parameters in exposed mice that did not manifest enterotoxigenicity provides an indication that toxigenic factors may also participate in mediating micronutrient depletion observed in this study. Virulence factors such as autotransporter toxins have also been implicated in the pathogenesis of *Shigella* infection. Their secretion into the culture filtrate is also a possibility but their roles in intestinal micronutrient and antioxidant depletion has not been defined (Roy *et al*., 2006).

We also observed reduction in plasma retinol level in our enterotoxigenic positive mice compared to the control. However, plasma levels of α-tocopherol and hepatic levels of both micronutrients were not significantly altered. Present finding indicates that retinol highly sensitive enterotoxin action with loss of its homeostasis in mice. In children with shigellosis, lower serum retinol level has been reported by Mitra *et al*. (1998). Present findings appear to support this clinical manifestation in the early phase of shigellosis due to enterotoxigenic strains.

Studies in rodents have shown that the hepatocyte is marginal in retinol storage and subsequently non-essential in maintaining retinol homeostasis during retinol scarcity (Dann, 1934). In this study, the mice used are also in their neonatal period life with liver retinol and protein eliciting no significant difference between enterotoxin exposed and control animals. Therefore, the observed reduced plasma retinol may be due to loss of mobilization of retinol from the gut of the tested mice as a result of enterotoxin assault.

The observed non-significant alterations in the plasma and hepatic levels of α-tocopherol on the other hand indicates that (1) systemic homeostasis of this micronutrient is maintained at least for a short period despite their intestinal tissue reduction after 24 h of enterotoxin assault in mice and (2) its homeostatic pathways and thus compensatory mechanisms are different from those of retinol. However, in the present study we did not investigate the possible buffering effects of other extra-hepatic tissues to maintain the systemic homeostasis of α-tocopherol but the fact that no significant hepatic alteration of α-tocopherol was observed between the tested and control mice rules out the liver as a source of compensation for the intestinal α-tocopherol depletion. Meanwhile, in rats fed diets that are marginal in α-tocopherol during gestation, systemic and tissue homeostasis of this vitamin has been observed in resulting pups for 21 days (Pazak and Scholtz, 1996).

Intestinal reduction in catalase activity was also observed in the enterotoxigenic mice, indicating that enterotoxins may contribute to decreased intestinal antioxidant capacity observed in shigellosis (Grisham and Granger, 1988; Perdomo *et al*., 1994). Present finding also aligns with the research of Nieto *et al*. (2000). The workers reported decreased intestinal antioxidant defense system in weanling rats with chronic diarrhea.

Catalase, a housekeeping enzyme accounts for up to 1% of total intestinal proteins of rodents and protein enteropathy due to shigellosis (Black and Levine, 1991) my account for its loss. In addition, shigellosis has been demonstrated in several animal models and humans to

evoke mobilization of pro-oxidant cytokines mitochondrial destruction and increase macrophage and neutrophil respiratory burst activity in intestinal mucosa in the early phase of infection (Perdomo *et al*., 1994; Korteski *et al*., 2005; Islam *et al*., 1997). The resulting preponderance of free radicals in the intestine may further contribute to catalase consumption.

However, this observation is contrary to the severally reported elevated catalase response to intestinal injury inflicted by ischaemia/reperfuson episodes (Deshmukh *et al*., 1997). The disparity in catalase response between the two distinct intestinal injuries could be attributed to intestinal protein loss found in this study and previous studies but which has not been reported in ischaemia/reperfusion injury (Deshmukh *et al*., 1997).

Culture filtrate of *Shigella* isolates were also observed especially those of *S. flexneri* and *S. dysenteriae* to elicit cytopathy and cell lysis in caco-2-cells *in vitro*. This suggests that enterotoxins are cellular growth inhibitors and cytotoxic in action. However, contribution to cytotoxicity by other non-enterotoxin virulence factors cannot be ruled out since their absence in the culture filtrate was not validated in this study. Similar to the cytotoxic actions of *Shigella* enterotoxin 2 (Fernadez-Prada *et al*., 1997; Jensen *et al*., 1998; Hilbi *et al*., 1998), *Shigella* cell wall lipopolysacharide (LPS) and invasion plasmid antigen B (IpaB), a type three secretion protein have also been shown to elicit cellular cytotoxicity in macrophages and dendritic cells *in vitro* through activation of caspase 1, which subsequently triggers apoptosis of these cells (Edgeworth *et al*., 2002).

The observed effects of the enterotoxigenic fractions especially those of *S. flexneri* and *S. dysenteriae* on caco-2 cells also suggest the possibility of mucosal deaths occurring earlier than expected during shigellosis since enterotoxins predominate the acute phase of shigellosis (Vargas *et al*., 1999).

The present study has consistently revealed the greater virulence of *S. flexneri* and *S. dysenteriae* over the other serogroups based on the magnitude of depletion of parameters analyzed and caco-2-cell toxicity outcome. Our previous study on the ability of these isolates to cause keratoconjuctivitis in guinea pig and absorb congo red dye as well as studies conducted elsewhere have also aligned with this observation (Iwalokun *et al*., 2002, 2003). It also justifies the fact that serotypes of *S. flexneri* and *S. dysenteriae* that are highly virulent outnumbered those of *S. sonnei* and *S. boydii* in most endemic populations in developing countries (Haider *et al*., 1989). However, because serotyping of our isolates was not done, explanation for why *S. flexneri* displayed greater virulence than *S. dysenteriae* could only be based on ileal fluid accumulation outcome in the infant mice and greater number of *S. flexneri* strains tested in this study. The highest number of *S. flexneri* strains tested in this study attests to the epidemiologic status of shigellosis in Nigeria with *S. flexneri* exhibiting serogroup dominance followed by *S. dysenteriae*, *S. sonnei* and *S. boydii* (Iwalokun *et al*., 2001). There is currently lack of data on serotype distribution of *Shigella* isolates in Nigeria and this is thus a major limitation of the present study.

It can be concluded that *Shigella* enterotoxins acutely elicit cellular toxicity, intestinal depletion proteins, retinol?, alpha-tocopherol and catalase as well as systemic loss of retinol homeostasis without compromising the hepatic levels of these parameters in mice.

However, further studies will conducted to unravel the genetic determinants of enterotoxins in a larger number of *Shigella* strains and determine the pathogenic contributions at intestinal level of other toxins that may be present in *Shigella* culture filtrate.

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