Patterns and Properties of Haemagglutinins Expressed by *Shigella* Serogroups in Lagos, Nigeria

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**ABSTRACT**

Forty-five strains of *Shigella* were screened for haemagglutinin production and broad-spectrum haemagglutination reaction. Mannose-sensitive haemagglutinin (MSHA) was found in 22 strains [*Shigella flexneri* (7), *S. dysenteriae* (7), *S. sonnei* (3), and *S. boydii* (5)]. Eighteen strains harboured mannose-resistant haemagglutinin (MRHA), and 8 strains were observed to be non-haemagglutinating to guinea pig erythrocyte. With the exception of human erythrocytes (O, A, B, and AB), the observed MSHA and MRHA also agglutinated the erythrocytes of rabbit, sheep, rat, chicken, and horse, suggesting a broad-spectrum haemagglutinating property. Haemagglutinins of *S. flexneri* and *S. dysenteriae* elicited a relatively stronger haemagglutinating activity with agglutinability to chicken and rabbit erythrocytes enhanced by trypsinization. Haemagglutination reaction with guinea pig erythrocyte was generally inhibited by sialic acid, while simple sugars, such as D-glucose, D-galactose, N-acetylgalactosamine, N-acetylglucosamine, and D-rhamnose, elicited no inhibitory effect. The results of the study revealed broad-spectrum haemagglutinin expression by circulating *Shigella* strains in Nigeria.

**Keywords:** Haemagglutinins; *Shigella*; Nigeria

**INTRODUCTION**

The high pathogenicity of enteric bacteria, including *Shigella*, to cause life-threatening intestinal and extra-intestinal infections has been linked to elaborate expression of virulence factors, such as toxins and haemagglutinins (1-3). Haemagglutinins, which are ubiquitous proteinaceous adhesins with cell-agglutinating and adherence properties, have also been implicated to play a crucial role in the initiation and development of clinical symptoms and complications in shigellosis (4,5). Stathopoulos et al. demonstrated the transferability of these haemagglutinating factors among Enterobacteriaceae in a manner that familiarize them with serine proteases of the Enterobacteriaceae family (SPATEs) found in *Shigella* and *Escherichia coli* (6).

As in many developing countries, *Shigella* strains in circulation in Nigeria have been found to show multiple resistance to antibiotics (7) and express proteases (8), endotoxins (9), and enterotoxins (Iwalokun BA et al. Personal communication, 2003) as virulence factors. However, information gathered so far on the virulence behaviour of this organism is not substantial since there is still a paucity of information on whether they produce haemagglutinins or not. This could be a constraint in the future design of effective vaccines against shigellosis.

Presently, use of *Shigella* vaccines as an alternative or adjunct to chemotherapy of this dreadful disease is a leading priority in many endemic communities (10), and the fact that haemagglutinins possess immunoprotective epitopes (11,12) has made their inclusion in multivalent
vaccine construct unquestionable. It was on this basis that the immunoprotective efficacy of a live oral Shigella flexneri vaccine in guinea pig was revealed (13).

Therefore, as a prelude to probable design of anti-Shigella vaccines for the Nigerian environment, 45 local Shigella strains were screened for haemagglutinin expression. The pattern of expression of these adhesions and their broadness of activity and effects of sugars other than mannose and trypsin on the haemagglutination reaction were also investigated.

MATERIALS AND METHODS

Shigella strains

Stock tryptase soy broth cultures of Shigella isolated from stool samples of patients with diarrhoea, attending six medical centres in Lagos, Nigeria, were studied for patterns of haemagglutinin expression. Stools were collected during February-November 2001 on Cary-Blair medium (Difco, USA) and Selenite F broth (Oxoid, UK). Suspected isolates were eventually identified on Salmonella-Shigella agar (Oxoid, UK) and speciated biochemically according to Cowan (14). The isolates were stocked under 16% glycerol at -70 °C. Prior to experimentation, the isolates were re-tested for viability in Mueller-Hinton broth (Oxoid, UK), Salmonella-Shigella agar, and then in trypticase soy broth (A600nm =0.5-0.8 at mid-log phase). The selected strains were: S. flexneri (n=18), S. dysenteriae (n=13), S. sonnei (n=8), and S. boydii (n=6).

Haemagglutination assay

For optimal production of haemagglutinins, each Shigella strain was statically grown in brain-heart infusion (BHI) broth (Oxoid, UK) at 37 °C for 48 hours. Cells were then harvested by centrifugation at 12,000 x g for one minute at 25 °C and adjusted to an inoculum size of 1x10⁶ cfu/mL with phosphate-buffered saline (PBS, pH 7.2). To test for haemagglutination and mannose susceptibility, citrated guinea pig erythrocytes, washed twice with PBS and re-suspended to a final concentration of 3% (v/v) with or without 50 mM mannose, were used (15,16). Two-fold serial dilutions of bacterial suspension (1:2-1:64 per row) were first dispensed in a 96-well microtitre plate, followed by the addition of equal volumes of the erythrocyte suspension. The plates were finally incubated at room temperature for 30 minutes. A mannose-sensitive haemagglutinin (MSHA) was inferred if the red blood cells agglutinated only in the absence of mannose. Haemagglutination in the presence of mannose was indicative of the expression of mannose-resistant haemagglutinins (MRHA). Generally, a positive haemagglutination reaction was defined as the settling of erythrocytes as a carpet of cells, while the settling of erythrocyte as a button-like colony indicated a negative reaction. Negative controls containing bacterial suspension (well 1) and erythrocyte suspension (well 8) were set up in parallel with the tests on a row-by-row basis. To further test for the spectrum of haemagglutination reaction, erythrocytes of horse, chicken, rat, rabbit, sheep, and human (blood group A) were used. They were prepared and assayed as previously described (16). Haemagglutination titre (HT) was defined as the reciprocal of the highest dilution of bacterial suspension at which haemagglutination occurred. HT ≤8 and HT >8 connoted weak and strong haemagglutinating activity respectively.

Haemagglutination-inhibition assay

The inhibitory effect of sugars on the agglutinability of mannose-sensitive haemagglutination (MSHA) and MRHA Shigella strains to guinea pig erythrocytes was carried out as described by Unhlenbruck et al. (17). 100 mM each of N-acetylgalactosamine, N-acetyl-glucosamine, D-galactose, D-lactose, L-fucose, D-rhamnose, and sialic acid was used in haemagglutination assay. Haemagglutination inhibition titre (HTi) was defined as the reciprocal of the lowest dilution of bacterial suspension at which no haemagglutination occurred. All the sugars were used as filtered sterilized (0.45 µm Millipore) solutions.

Effect of trypsin on haemagglutination

100 µL of trypsin solution (25 mg/mL) was pre-incubated with 10% erythrocyte suspensions of all the animals tested and human erythrocyte (blood group A, B, AB, and O) at 37 °C for 15 minutes. The trypsinedized cells, washed twice with PBS, were then adjusted to 3% (w/v) concentration for haemagglutination assay as previously described.

RESULTS

Agglutination of guinea pig erythrocyte by 45 strains of Shigella was investigated. MSHA was found in 22 of the 45 Shigella strains tested [S. flexneri (7), S. dysenteriae (7), S. sonnei (3), and S. boydii (5)]. Eight strains of S. flexneri, 5 strains of S. dysenteriae, and 1 strain each of S. sonnei and S. boydii produced MRHA.
In total, 8 strains were non-agglutinating to guinea pig erythrocyte (Fig.). S. sonnei and S. boydii was observed in the erythrocytes of horse, rat, and sheep (Table 1).

Prior to haemagglutination testing, the trypsinization of the erythrocytes tested revealed increases in haemagglutination titres and the number of MSHA and MRHA strains of S. flexneri and S. dysenteriae in chicken erythrocytes. A decrease in haemagglutination titre of

<table>
<thead>
<tr>
<th>Organism</th>
<th>Erythrocytes</th>
<th>Human</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Horse</td>
<td>Rat</td>
</tr>
<tr>
<td>MSHA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. flexneri</td>
<td>2 (12)</td>
<td>4 (24)</td>
</tr>
<tr>
<td>S. dysenteriae</td>
<td>4 (14)</td>
<td>4 (20)</td>
</tr>
<tr>
<td>S. boydii</td>
<td>3 (6.7)</td>
<td>4 (8)</td>
</tr>
<tr>
<td>S. sonnei</td>
<td>2 (12)</td>
<td>3 (6.7)</td>
</tr>
<tr>
<td>MRHA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. flexneri</td>
<td>4 (18)</td>
<td>5 (32)</td>
</tr>
<tr>
<td>S. dysenteriae</td>
<td>4 (18)</td>
<td>4 (24)</td>
</tr>
<tr>
<td>S. boydii</td>
<td>0 (0)</td>
<td>1 (16)</td>
</tr>
<tr>
<td>S. sonnei</td>
<td>1 (8)</td>
<td>2 (12)</td>
</tr>
</tbody>
</table>

* Untrypsinized erythrocytes
Open figures represent the number of strains displaying haemagglutinating activity
Figures in parentheses are mean haemagglutination titre values
MRHA=Mannose-resistant haemagglutinin
MSHA=Mannose-sensitive haemagglutinin
Haemagglutinins expressed by Shigella serogroups in Lagos, Nigeria

sheep erythrocyte was also observed in these strains. Haemagglutinating activity of *S. boydii* and *S. sonnei* was not affected by trypsinization, while human erythrocytes (O, A, B, and AB) remained non-haemagglutinated (Table 2).

Table 2. Effects of trypsinization on haemagglutinating activity of MSHA and MRHA Shigella strains

<table>
<thead>
<tr>
<th>Organism</th>
<th>Erythrocytes</th>
<th>Human</th>
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<tbody>
<tr>
<td></td>
<td>Horse</td>
<td>Rat</td>
</tr>
<tr>
<td>MSHA</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. flexneri</em> (n=7)</td>
<td>2 (12)</td>
<td>4 (24)</td>
</tr>
<tr>
<td><em>S. dysenteriae</em> (n=7)</td>
<td>4 (14)</td>
<td>4 (24)</td>
</tr>
<tr>
<td><em>S. boydii</em> (n=5)</td>
<td>3 (6.7)</td>
<td>4 (8)</td>
</tr>
<tr>
<td><em>S. sonnei</em> (n=3)</td>
<td>2 (12)</td>
<td>3 (10.7)</td>
</tr>
<tr>
<td>MRHA</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. flexneri</em> (n=8)</td>
<td>4 (22)</td>
<td>5 (32)</td>
</tr>
<tr>
<td><em>S. dysenteriae</em> (n=5)</td>
<td>4 (14)</td>
<td>4 (18)</td>
</tr>
<tr>
<td><em>S. boydii</em> (n=1)</td>
<td>0 (0)</td>
<td>1 (16)</td>
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<tr>
<td><em>S. sonnei</em> (n=2)</td>
<td>1 (8)</td>
<td>2 (8)</td>
</tr>
</tbody>
</table>

* Trypsinized erythrocytes

Open figures represent the number of strains displaying haemagglutinating activity

Figures in parentheses are mean haemagglutination titre values

MSHA=Mannose-sensitive haemagglutinin

MRHA=Mannose-resistant haemagglutinin

The data presented in Table 3 revealed non-haemagglutination inhibitory effects of N-acetylgalactosamine, N-acetylglucosamine, D-glucose, D-galactose, D-lactose, and D-rhamnose on the agglutinability of MSHA and MRHA strains to guinea pig erythrocyte. L-fucose inhibited haemagglutination reaction in four of the 22 MSHA strains (HTo=8-32) and two of the 16 MRHA strains (HTo=8-16) (Table 3), while sialic acid inhibited haemagglutinating activity of all the strains tested.

**DISCUSSION**

Haemagglutinins in enteric bacteria have been demonstrated as tools for virulence, immunogenicity, and bacterial tropism in host cells (18-20). To enable host-bacteria interaction and cellular aggregation in an infection, specific sequences and conformation of sugar residues of carbohydrate frontiers of haemagglutinins are employed by enteropathogens, including *Shigella*. It is, therefore, not surprising that the *Shigella* strains investigated in this study harboured haemagglutinins. Although there is no clarification on the fimbrial origin of these adhesions, the presence of MSHA and MRHA in our strains corroborates the findings of the study that revealed and characterized cell-associated haemagglutinin in *S. dysenteriae* (21), the work of Pascariu et al. (22) on the titration of complete and incomplete agglutinins and haemagglutinins in shigellosis, and the findings of Buchanan *et al.* (3) that revealed the presence of mannose-inhibitable agglutination factor of guinea pig erythrocyte throughout the family Enterobacte-
consistent with the findings of Adegbola and Old (23) in which non-haemagglutination observed among Enterobacter species was found to be of taxonomic relevance. The work of Lomberg et al. (24) also demonstrated non-haemagglutination in uropathogenic E. coli. Haemagglutinating activity of enteric bacteria, including Shigella, has also been shown to be dependent on cultural conditions (25,26). Therefore, the impact of storage and assay composition and conditions on the haemagglutinating behaviour of the strains tested in this study cannot be ruled out. The different geographic sources of Shigella isolates and strain variation may also have an impact on their haemagglutinating properties. The latter may explain the different patterns of haemagglutinating activity observed among strains of Vibrio cholerae (26) and even in S. dysenteriae type 1 (27).

Nevertheless, the ability of our MSHA and MRHA strains to display agglutinability to erythrocytes of rabbit, rat, chicken, horse, and sheep indicated broad-spectrum haemagglutinating activity of Shigella haemagglutinins. A similar property has been documented among haemagglutinins of Serratia species (20), E. coli (28), and V. parahaemolyticus (29). However, the observed haemagglutinins failed to agglutinate human erythrocytes even after trypsinization. This observation contradicts the haemagglutination and adhesion properties of O-polysaccharide moiety of S. dysenteriae type 1 lipopolysaccharide as demonstrated by Qadri et al. (27). This discrepancy could emanate from variation in the structure and composition of binding carbohydrates in these macromolecules.

On the basis of complementarity, specific sugar residues in the carbohydrate moiety of haemagglutinins are glycosidically linked to simple sugars in host cell receptors to effect adherence, colonization, and even non-opsonic phagocytic killing (30,5). In this study, the inability of simple sugars to inhibit haemagglutination is suggestive of requirement for complex carbohydrate binding. In the study by Guhathakurta et al., the agglutinability of haemagglutinins to erythrocytes was only found inhibitable by glycoproteins, such as mucin, by sialofetuin and asialofetuin (21). These observations may, in part, explain why sialic acid could inhibit the haemagglutination reactions of Shigella haemagglutinins tested.

That L-fucose inhibits the haemagglutination reaction of six of the Shigella haemagglutinins tested may underscore the unpredictability of agglutinability of bacterial haemagglutinins in vitro until they are tested. Haemagglutinins are emerging as a product of gene rearrangement and polysaccharide polymorphism, and point mutation has been found to alter the binding specificity of sugars to the receptor. This emerging trend has been documented in several studies (31,32).

The present study has demonstrated the presence of MSHA and MRHA with broad-spectrum haemagglutination reaction in Shigella strains in Nigeria. The adhesins, while displaying heterogeneity in haemagglutination property, also appear to conform to the requirement for complex carbohydrates for binding and cellular aggregation.

REFERENCES


