Dhaka Univ. J. Biol. Sci. **19**(2): 189-194, 2010 (July)

NOVEL FUNCTIONS OF NON-O1 NON-O139 *VIBRIO CHOLERAE* LIPOPOLYSACCHARIDE FOR ITS HAEMAGGLUTINATING ACTIVITY

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Key words: Non-O1 non-O139 *V. cholerae,* LPS, Haemagglutination

Abstract

Haemagglutinating activity of the surface lipopolysaccharide (LPS) of non-O1 non-O139 *Vibrio cholerae* was assayed against rabbit and chicken erythrocytes. The serially diluted non-O1 non-O139 *V. cholerae* LPS showed haemagglutination of rabbit erythrocytes up to a dilution of 1 : 512, which is probably due to the hydrophilic polysaccharide moiety of the LPS. To test whether the LPS was able to bind to glycoproteins and other simple sugars, the LPS was incubated with mucin, glucose and sucrose and was assayed for the residual haemagglutinating activity. It was found that the mucin could inactivate the LPS, which indicated that the LPS of the non-O1 non-O139 *V. cholerae* strains may bind to glycoprotein and its mechanism of adherence may be similar to that of other bacterial LPS. However, the glucose and sucrose could not inhibit the LPS as has been demonstrated by the positive residual haemagglutinating activity of the LPS.

Introduction

Vibrio cholerae O1 organisms are known to be the causative agents of the fifth, sixth and seventh pandemics of cholera. These organisms are biochemically indistinguishable from their non-O1 *V. cholerae* counterparts except for their property to be agglutinated by O1 antiserum.(1) Non-O1 *V. cholerae* strains which represent a heterogeneous group comprising of more than 155 serogroups, are the natural inhabitants of aquatic environment and are known to be associated with prawns, shellfishes, etc.(2) Some of these strains are also reported to be responsible for sporadic cases and/or limited outbreaks of gastro-enteritis and extra-intestinal infections.(3) However, in 1992-1993, an epidemic of cholera was reported from India and Bangladesh which was caused by a non-O1 strain of *V. cholerae*, later to be designated as O139 Bengal.(4)

The other sero-group, non-O1 non-O139 *V. cholerae* comprise a heterogeneous group of organisms whose clinical association with humans is inadequately understood. However, it is now clear beyond doubt that some strains of non-O1 non-O139 *V. cholerae* have the capacity to cause a cholera-like syndrome and the capacity to flare into a localized outbreak.(5) Recently we reported that the environmental non-O1 non-O139 *V. cholerae* strains produce toxins, other than cholera toxin.(6) Although the study clarified the role of different toxins, including heat-labile enterotoxin, haemolysin and/or

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cytotoxin, in the pathogenesis of the non-O1 non-O139 *V. cholerae*, however, the study did not characterize the role of lipopolysaccharides (LPS) in the virulence process of the bacteria.

High haemagglutinating activity was demonstrated by the LPS of the *V. cholerae* O139 strains.[7] The bacterial haemagglutinin causing agglutination of erythrocytes has been related to the bacterial adherence to the intestinal mucosa, because the erythrocyte membrane has been believed to possess homolog(s) of the mucosal substance(s) involved in adherence.[7,8] In the present study, we provide data on the role of LPS in the haemagglutinating activity, which may be helpful in characterizing these virulence factors for a better understanding of the pathogenic mechanisms of the environmental non-O1 non-O139 *V. cholerae* strains.

Materials and Methods

Surface water was taken from small water reservoirs, ponds and rivers around Dhaka city. Water samples were enriched in alkaline peptone water at 37°C for overnight and were then inoculated on thiosulphate citrate bile salt sucrose (TCBS) agar plate. After overnight incubation for 18 - 24 hr, suspected yellow colonies were subcultured on gelatin agar (GA) and were identified by a series of biochemical tests. Serotyping was performed by slide agglutination with specific antiserum and only three strains were confirmed as non-O1 non-O139 *V. cholerae*, which were preserved in T_1N_1 (1% trypticase, 1% NaCl) soft agar at room temperature. The *V. cholerae* 569B, which is a toxigenic O1 vibrio strain, was collected from the stock culture of the Department of Microbiology, University of Dhaka and was used as a positive control.

LPS from both non-O1 non-O139 *V. cholerae* and *V. cholerae* 569B were extracted following the method of Westphal and Jann.[9] Bacteria were grown overnight in Brain Heart Infusion (BHI) broth and the cells were collected by centrifugation at 6,000 rpm for 10 min for LPS extraction. Approximately 5 gm of the bacteria were suspended in 10 ml of distilled water and were stirred with 10 ml of 90% phenol vigorously for 10 min in a water bath at 68ºC. They were cooled to 10ºC and centrifuged at 4,000 rpm for 30 min. After centrifugation, the aqueous layer containing LPS was removed and dialyzed for 48 hr against distilled water with several changes. The LPS was then lyophilized and the dry LPS was dissolved in 20 mM tris-HCl containing 0.9% NaCl (pH 7.5) to achieve a final concentration of 1 mg/ml.

The haemagglutinating activity of the non-O1 non-O139 *V. cholerae* LPS (1 mg/ml) was assayed by the method of Jones *et al.* [10] A serial dilution of 50 µl of the LPS samples were made in 20 mM tris-HCl buffer containing 0.9% NaCl (pH 7.5) and was mixed with equal volume of 1.5% rabbit erythrocytes (made in the same buffer) in the wells of a 96 - well polystyrene V-bottom microtiter plate. The plate was incubated at room temperature for 45 min. The haemagglutinating activity unit was defined as the

reciprocal of the highest dilution of the LPS sample causing visible agglutination of the erythrocytes. LPS of *V. cholerae* 569B, assayed in the same manner, was used as the positive control. The same process was also performed with chicken erythrocytes, to check the haemagglutinating activity of the LPS samples. Wells containing erythrocytes mixed with only buffer were used as negative controls.

The LPS preparations were hydrolyzed with 1% acetic acid at 100ºC for 90 min. The lipid A fraction was spun down by centrifuging the solution at 3,000 rpm for 10 min. The supernatant containing the polysaccharide moiety was checked for haemagglutinating activity following the method described above.

Glycoprotein (mucin) and sugars (glucose and sucrose) (1 mg/ml) were serially diluted and incubated with the LPS (1 mg/ml) of non-O1 non-O139 *V. cholerae* at 37ºC for 30 min. Residual haemagglutinating activity of the LPS preparation against rabbit erythrocytes was determined following the method described above. Glycoprotein and sugars incubated with the LPS of *V. cholerae* 569B and checked for haemagglutinating activity, were served as positive controls.

Results and Discussion

In the progression of enteric infections, bacterial adherence to the surface of host intestinal mucosa has been established as an essential step. This step has been documented to be accomplished by the factors possessing the ability to agglutinate erythrocytes. The factors causing haemagglutination are well-known as haemagglutinins, which recognize the specific binding sites, the so called receptors. Since erythrocytes from different animal species possess different types of receptors, the haemagglutinin-erythrocyte interaction provides the nature of the receptor(s) involved in the mucosal receptions of pathogenic bacteria. For several intestinal pathogens, including *V. cholerae*, a correlation between bacterial haemagglutinating property and adhesiveness has been found. [11-13] In this study, the haemagglutinating activity of the surface LPS of the isolated non-O1 non-O139 strain of *V. cholerae* was assayed against rabbit and chicken erythrocytes (Fig. 1). The serially diluted non-O1 non-O139 *V. cholerae* LPS showed haemagglutination of rabbit erythrocytes up to a dilution of 1 : 512. The results indicated that the non-O1 non-O139 *V. cholerae* strains had strong adhesive property and hence were pathogenic. Again as the non-O1 non-O139 *V. cholerae* LPS caused haemagglutination of rabbit erythrocytes, not chicken, therefore, it can be concluded that the non-O1 non-O139 *V. cholerae* LPS needs specific receptors for binding on the mucosal surfaces.

LPS is composed of polysaccharide moiety and the lipid A, which gives the hydrophobic nature and remains embedded in the outer leaflet of the outer membrane. To clarify whether the haemagglutinating activity of the LPS was due to the polysaccharide moiety, the LPS was subjected to acid hydrolysis using 1% acetic acid and was assayed for haemagglutinating activity. The result showed that haemagglutinating

activity was associated with the hydrophilic polysaccharide moiety of the LPS. Considering the cell surface architecture, direct interaction of the outer membrane protein with the host mucosa seems to be impossible. In contrast, the extended polysaccharide moiety of LPS may be the first to come in contact with the mucosal membrane and from this study, it seems likely that the polysaccharide moiety with strong haemagglutinating activity may function as the ubiquitous adhesin in these enteropathogenic vibrios.

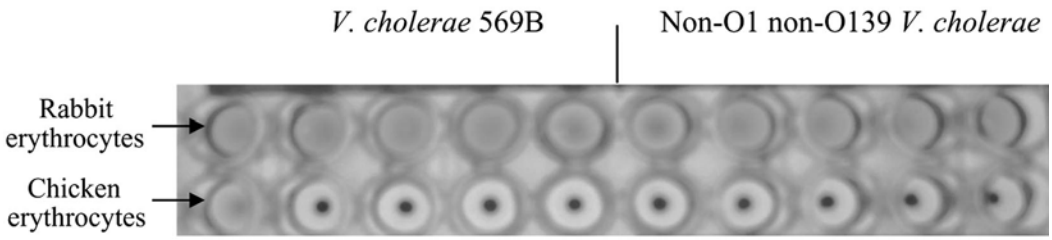

Fig. 1. Positive haemagglutination activity was demonstrated by non-O1 non-O139 *V. cholerae* and *V. cholerae* 569B for rabbit erythrocytes. The chicken erythrocytes, however, showed negative results.

The LPS binding protein in the human body is a glycoprotein with the ability to potentiate the bactericidal activity of human bactericidal or permeability-increasing protein through specific binding to LPS.[14] Although it is not known whether the homologs of the LPS binding protein is contained in the mucosa of human small intestine, the haemagglutinating activity and the affinity to glycoproteins may indicate that Gram-negative bacterial enteropathogens may employ LPS in the glycoprotein mediated anchorage to the host intestinal mucosa.[15] To test whether the LPS was able to bind to glycoproteins, the LPS sample was incubated with a glycoprotein, mucin and was assayed for the residual haemagglutinating activity. It was found that the mucin could inactivate the LPS, which indicated that the LPS of the non-O1 non-O139 *V. cholerae* strains may bind to glycoprotein and its mechanism of adherence may be similar to that of other bacterial LPS.[7,15] The LPS was also incubated with glucose and sucrose in a similar fashion. However, they could not inhibit the LPS as has been demonstrated by the positive residual haemagglutinating activity of the LPS. The inability of any sugar to abolish the adherence of *V. cholerae* O1 to the mucous coat of the human small intestine was documented by others.[16] This suggests involvement of the non-sugar mucosal component(s) in the adherence of this human pathogen. Since the major part of the small intestine is covered with a thick mucous layer containing glycoproteins,[17] sufficient elimination of the haemagglutinating ability of any vibrio LPS with the polypeptide portion of the glycoprotein may indicate the possibility of LPS-mediated intestinal adherence in the enteropathogenic vibrios, including the non-O1 non-O139 *V. cholerae*.

However, further studies can be performed to identify the receptors with which the non-O1 non-O139 *V. cholerae* LPS interacts.

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(Manuscript received on 25 August, 2009; revised on 30 March, 2010)