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Original Article

Antigenic Cross-Reactivity Between *Escherichia albertii* DM104 and Different *Shigella* spp.

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Environmental *Escherichia albertii* strain DM104 has been found to induce protective immunity against *Shigella dysenteriae* in guinea pig model and intranasal immunization showed promising results in terms of antibody response and protective efficacy. For selecting a proper immunodiagnostics marker against shigellosis, the current study investigated the antigenic cross-reactivity between DM104 and four different *Shigella* spp (*S. dysenteriae* type 4, *S. flexneri* type 2a, *S. boydii* type 15 and *S. sonnei*). At least six antigenic protein bands (85, 72, 34, 30, 23 and 20 kDa) of the surface components from all these species reacted strongly with both homologous and heterologous antisera, suggesting common distribution of antigenic determinants/epitopes in these bacterial species. This experiment, thus gave a clear idea of the level of antigenic determinants/epitopes sharing and variations between the DM104 and four *Shigella* spp. Results from this study suggest that the 34, 23 and 20 kDa antigenic proteins may be incorporated as immunodiagnostic marker for the detection of different *Shigella* spp.

Keywords: *Escherichia albertii*, *Shigella*, shigellosis

Introduction

Shigellosis, an acute gastrointestinal infection, caused by *Shigella* has long been a major global public health risk, particularly in developing countries like Bangladesh. Shigellae are considered to be highly infectious due to their low infectious dose (10–100 organisms)[1]. They are the cause of extensive paediatric morbidity and mortality. Approximately 1.1 million deaths result from the 164.7 million annual cases worldwide, with about 70% of episodes and 60% of deaths involving children younger than 5 years[2]. The incidence declines after the age of 5 years, and a serotype specific protective immunity develops after exposure[3].The asymptomatic carrier of *Shigella* spp. is believed to play a significant role in sustaining the organism and in the spread of the disease within the community. A survey conducted in Nigeria and Bangladesh showed that the carrier rate of this organism in healthy children was about 2–3%[4].

In recent years, our group has isolated a number of *Shigella*-like bacteria from freshwater environments in Bangladesh that serologically cross-reacted with different *Shigella* spp.[5, 6]. One of these strains, DM104 was phylogenetically identified as *E. albertii* and showed a similar lipopolysaccharide (LPS) gel banding profile to that of *S. dysenteriae* type 4[7]. Recently, it was also demonstrated that the DM104 isolate was non-invasive, did not produce any entero- or cytotoxins, and showed negative results in the mouse lethal activity assay[8]. The non-pathogenic DM104 strain gave, however, a high protective efficacy as an ocularly administered vaccine in the guinea pig eye model against *S. dysenteriae* type 4 challenge. It also induced a high titer of serum IgG against *S. dysenteriae* type 4 whole cell lysate (WCL) and LPS. We also assessed three different immunization routes, such as intranasal, oral, and intrarectal routes, and revealed differences in immune responses by measuring both the serum IgG and mucosal IgA antibody titers[9]. Protective efficacy of different routes of immunization was also determined by challenging immunized guinea pigs against live *S. dysenteriae*. It was found that intranasal immunization showed promising results in terms of antibody response and protective efficacy. In current study, we investigated the antigenic cross-reactivity between vaccine strain DM104 and other *Shigella* spp for selecting a proper immunodiagnostics marker against shigellosis.

Materials and Methods

Bacterial strains

E. albertii strain DM104[7], *S. dysenteriae* type 4, *S. flexneri*type 2a, *S. boydii*type 15 and *S. sonnei* were all obtained from the stock cultures of the Department of Microbiology, University of Dhaka, Dhaka, Bangladesh.

Animals

New Zealand white rabbits (2–2.5 kg body weight) were maintained in the Department of Microbiology, University of Dhaka, and all experiments using animals were undertaken following the ethical issues set by the Faculty of Biological Sciences, University of Dhaka.

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Preparation of water extracted material (WEM)

Water extracted material containing the surface proteins were prepared from DM104 strain as described by Oaks *et al*. (1986)[10]. Bacterial strains were grown in BHIB(Brain Heart Infusion Broth)for 18 h at 37ºC in a shaker-incubator. Cells were harvested by centrifugation and washed with normal saline (0.85% NaCl solution) for three times. The washed pellet was then re-suspended in 5 ml of sterile distilled water and was put on a shaker for 6 h at room temperature. Finally, the materials were centrifuged at 12,000×g for 15 min. The supernatant was then filtered through 0.45µm pore size membrane filter and stored at -20ºC until use. Similar procedure was also applied for the preparation of WEM from *S. dysenteriae* type 4, *S. flexneri* type 2a, *S. boydii* type 15 and *S. sonnei*.

Estimation of protein concentration

The amount of protein in the WEM was estimated using Bio-Rad protein assay (Bio-Rad, USA), which is based on the Bradford method[11].It involved the addition of an acidic dye solution, and subsequent measurement of optical density at 595 nm. Comparison with a standard curve provided a relative measurement of protein concentration.

Extraction of lipopolysaccharide (LPS)

Lipopolysaccharide from the DM104 isolate was extracted following the method of Westphal and Jann (1965)[12]. Bacteria were grown overnight at 37°C in 200 ml BHIB; cells were harvested at 8,000 rpm and were suspended in 5 ml of distilled water. This was stirred vigorously in 5 ml of 90% phenol for 10 min in a water bath at 68°C. The suspension was 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 h against distilled water. The crude LPS was then lyophilized and dissolved in 20 mM Tris-HCl (pH 7.5) in normal saline to obtain a final concentration of 1 mg/ml.

Preparation of formalin-killed whole cell

Live DM104 grown in BHIB was harvested for 24 h and centrifuged at 10,000× g for 10 min. The bacterial pellet was washed twice and resuspended in phosphate buffer saline (PBS). They were inactivated with 1% formalin at 4°C for 18 h and the viability was checked onMacConkey agar plates[13]. The formalin-killed DM104 was used to raise polyclonal antisera in rabbits. Similarly, formalin-killed whole cells of *S. dysenteriae* type 4, *S. flexneri* type 2a, *S. boydii* type 15 and *S. sonnei* were also prepared to raise polyclonal antisera in rabbits.

Production of rabbit-sera

Antisera against formalin-killed DM104, *S. dysenteriae*, *S. flexneri*, *S. boydii* and *S.sonnei* were raised in rabbits as described by Chen et al. (1997)[14]. Briefly, New Zealand white rabbits were separately injected intramuscularly with 0.5 ml of formalin-killed whole cell (10^9 cells/ml as compared to the Mac Farland standard) emulsified with equal volume of Freund's complete adjuvant followed by two boosters with Freund's incomplete adjuvant at 15 days intervals. The rabbits from each group were bled for sera through ear veins 15 days after second booster and the antibody titers in the blood sera were checked by ELISA. After obtaining a desired antibody titer (1:1600), the rabbits were sacrificed and polyclonal antisera were prepared. These sera were used to determine the antigenic cross-reactivity between the DM104 strain and the four different *Shigella* spp. included in this study.

Antigenic cross-reactivity between the DM104 strain and the four different Shigella spp

To determine the antigenic cross-reactivity between the DM104 strain and the four different *Shigella* spp., Western Blot (WB) analysis was done. WEM and LPS of DM104, *S. dysenteriae*, *S. flexneri*, *S.boydii* and *S. sonnei* were all used in different WB analysis against the rabbit sera raised against them.

Western Blot Analysis

WEM and LPS samples of *S. dysenteriae*, *S. flexneri*, *S. boydii* and *S. sonnei* were fractionated by SDS-PAGE using 15% polyacrylamide gels following the procedure described by Laemmli[15] using a Mini Protean III Cell system (Bio-Rad, USA). WB was performed following the method described by Towbin *et al*.(1979)[16]. In brief, after separation by SDS-PAGE, the WEM and LPS samples were transferred to nitrocellulose membranes. The membranes were blocked at room temperature for 30 min using a blocking solution (2% nonfat dried milk in a PBS Tween 20 solution), washed twice for 1 min with PBS-Tween 20, and incubated for 1 h at 37°C with polyclonal antisera raised in rabbit against DM104. After incubation with the primary antibody, membranes were washed twice and then incubated with HRP conjugated secondary antisera (Sigma, USA) in 2% skim milk at 1:2000 fold dilution for 1 h at 37°C. The strips were then washed and soaked in substrate solution (1 mg Diaminobenzoic acid) dissolved in 2 mL citrate buffer of pH 5.2 with 3 ¼L H_2O_2) for a few minutes to obtain positive signals, and then finally washed in deionized water to stop the reaction. Molecular weight of the antigenic bands was determined by calculating the relative migration (Rf) values of the antigenic bands.

Similarly, a reverse cross-reactivity was checked by loading WEM of DM104 in different wells and by blotting with rabbit sera raised against DM104, *S. dysenteriae*, *S. flexneri*, *S.boydii* and *S. sonnei* as primary antibody. Similarly, LPS was also electrophoresed in10% polyacrylamide gel and were blotted following the procedure as described for WEM.

Results

Antigenic cross-reactivity between the DM104 WEM and with four *Shigella* spp., revealed more than 20 antigenic bands, when reacted with homologous antiserum (Fig. 1, Lane-3). The WB analysis also revealed high degree of cross-reactivity between the surface components of the DM104and the four *Shigella* spp (Fig. 1, Lane-3 to 7). At least ten antigenic bands (105, 85, 72, 53, 41, 34, 30, 26, 23 and 20 kDa) of the surface components from all the five bacterial species reacted strongly with both homologous and heterologous antisera, suggesting common distribution of antigens/epitopes in these bacterial species. This cross reactivity was repeated by the reverse experiment using the DM104 WEM as antigen in all the lanes and blotted with homologous, DM104 and heterologous, *S. dysenteriae*, *S flexneri*, *S. boydii*, *S. sonnei* antisera (Fig 2). At least six prominent

antigenic bands of 85, 72,34, 30, 23 and 20 kDa were found to be common or cross-reactive when these two results were compared (Table 1).

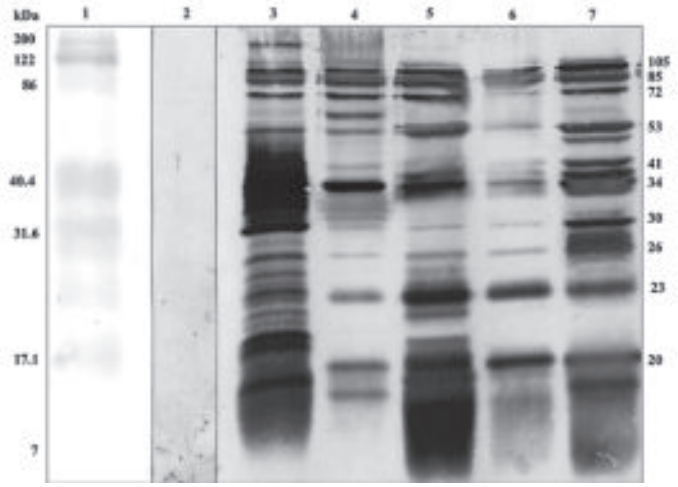

Fig. 1. *Surface proteins that share common epitopes between DM104 and Shigella spp. Cross-reacting antigenic components of WEM of DM104 and four Shigella spp. were determined against anti-DM104 polyclonal antibody raised in rabbit. Lane-1, pre-stained protein markers; Lanes-2 and 3, WEM of DM104; Lane-4, WEM of S. dysenteriae; Lane-5, WEM of S. flexneri; Lane-6, WEM of S. boydii; Lane-7, WEM of S. sonnei. In lane-2, non-immune rabbit serum was used as primary antibody. Ten prominent protein bands of 105, 85, 72, 53, 41, 34, 30, 26, 23 and 20 kDa were found to be common in WEM of DM104 (Lane-3) and four Shigellaspp. (Lanes-4, 5, 6 and 7) when checked against anti-DM104 rabbit sera.*

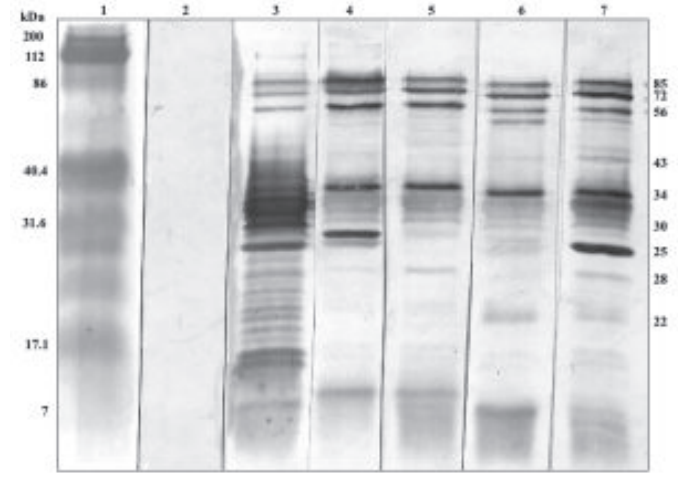

Fig. 2. *Surface proteins of DM104 recognized by different antisera against Shigella spp. Cross-reacting antigenic components of WEM of DM104 were recognized against polyclonal antisera of four different Shigella spp. raised in rabbit. Lane-1, pre-stained protein markers; Lane-2, non-immune rabbit sera; Lane-3, anti-DM104; Lane-4, anti-S. dysenteriae; Lane-5, anti-S. flexneri; Lane-6, anti-S. boydii; Lane-7, anti-S. sonnei. Nine prominent protein bands of 85, 72, 56, 43, 34, 30, 28, 23 and 20 kDa were found to be common in DM104 WEM when checked against homologous (Lane-3) and heterologous antisera (Lanes-4, 5, 6 and 7).*

Table 1. *Antigenic bands of the DM104 WEM against homologous and heterologous antibodies*

WEM of different bacteria (DM104, *S. dysenteriae*, *S. flexneri*, *S. boydii* and *S. sonnei*) against antibody of DM104	WEM of DM104 against different antibodies (DM104, *S. dysenteriae*, *S. flexneri*, *S. boydii* and *S. sonnei*)	Common bands in both the WB analysis
Protein bands (kDa)		
105		
85	85	85
72	72	72
	56	
53		
	43	
41		
34	34	34
30	30	30
26		
23	23	23
20	20	20

Similarly, LPS of DM104 and four *Shigella* spp. were run in SDS-PAGE and blotted with serum against DM104. The homologous antibody could recognize both long-chain and core LPS preparation of the DM104 strain (Fig. 3, Lane-3) and only the long chain in case of *S. dysenteriae* (Fig. 3, Lane-6)[17]. This result was repeated

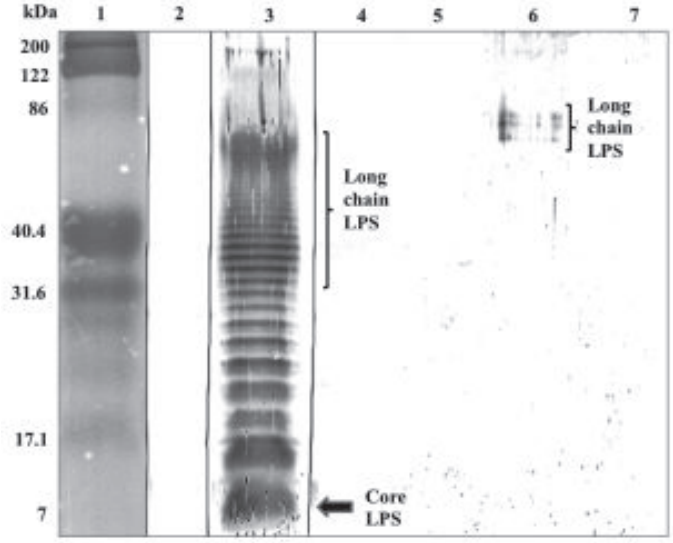

Fig. 3. *Immune reaction of anti-DM104 polyclonal antibody with LPS of different Shigella spp.Cross-reacting antigenic components of LPS of DM104 and four Shigella sp were determined against anti-DM104 polyclonal antibody raised in rabbit. Lane-1, pre-stained protein markers; Lanes-2 and 3, LPS of DM104; Lane-4, LPS of S. flexneri; Lane-5, LPS of S. boydii; Lane-6, LPS of S. dysenteriae; Lane-7, LPS of S. sonnei.In lane-2, non-immune rabbit serum was used as primary antibody. Anti-DM104 antibodies bound to both homologous long-chain (braket, Lane-3) and core LPS (arrowed, Lane-3) whereas only core LPS (braket, Lane-6) of S. dysenteriae was recognized in heterologous antibody reaction.*

by the reverse experiment using DM104 LPS in all five lanes and blotted with sera against formalin-killed DM104, *S. dysenteriae* type 4, *S. flexneri* type 2a, *S. boydii* type 15 and *S. sonnei* respectively. However, in this case both long-chain and core LPS of DM104 were recognized in all the lanes by the heterologous antisera, although the intensity of the LPS bands were higher in homologous than the heterologous reactions (Fig 4).

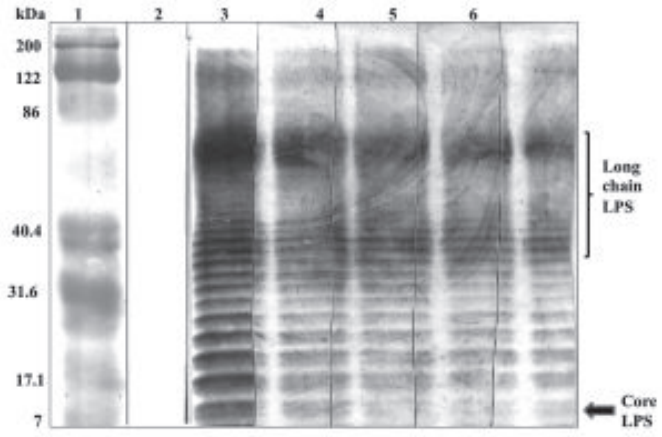

Fig. 4. *Components of DM104 LPS that demonstrated immune reaction with different Shigella spp. antisera.Cross-reacting antigenic components of LPS of DM104 were recognized against polyclonal antisera of four different Shigella spp raised in rabbit. Lane-1, pre-stained protein markers; Lane-2, non-immune rabbit sera; Lane-3, anti-DM104; Lane-4, anti-S. dysenteriae; Lane-5, anti-S. flexneri; Lane-6, anti-S. boydii; Lane-7, anti-S. sonnei. Both long-chain (braket) and core LPS (arrowed) were recognized in DM104 LPS when checked against homologous (Lane-3) and heterologous antisera (Lanes-4, 5, 6 and 7) but the intensity of the LPS bands were higher in homologous than the heterologous reactions.*

Discussion

In this study, we tried to demonstrate the serological cross-reactivity and the degree of antigenic relatedness between the *Shigella*-like *E. albertii*DM104 strain and four *Shigella* spp. At least six antigenic protein bands (85, 72, 34, 30, 23 and 20 kDa) of the surface components from all these species reacted strongly with both homologous and heterologous antisera, suggesting common distribution of antigenic determinants/epitopes in these bacterial species (Table 1). In an earlier study Mukhopadhaya and his coworkers[18] demonestrated the immunogenicity of certain outer membrane proteins (mainly 38, 34, 23 and 20 kDa) of *Shigella* spp in animal model as well as their protective immunity, which was supported by this study. At least three of the antigenic proteins (34, 23 and 20 kDa) were found to be common in these two studies. The distribution of these common epitopes between different species of *Shigella* remains to be the subject of future studies. On the other hand, antisera against DM104 also recognized long-chain and core LPS in homologous reaction but only long chain portion of heterologous *S. dysenteriae* type 4 LPS.

Serological relationships have previously been observed between different members of the *Enterobacteriaceae* family, e.g., between *Salmonella* and *Citrobacter*, *Escherichia* and *Shigella*, *Escherichia* and *Proteus* strains, etc[19]. Serological cross-reactions have also been demonstrated between three Gram-negative bacteria, e.g., *Proteus, Escherichia* and *Salmonella.* These cross-reactions are mostly based on the heat stable LPS antigens or common outer membrane protein antigens[20]. In our rabbit antigenicity experiment, the results strongly suggest that the surface components, protein or LPS of DM104 are highly immunogenic in the rabbit model, when given intramuscularly. Some of the specific antigenic components may be of great use in differential diagnosis and seromonitoring of the bacterial diseases. This experiment, thus gave a clear idea of the level of antigenic determinants/epitopes sharing and variations between the DM104 and four *Shigella* spp. and 34, 23 and 20 kDa antigenic proteins may be incorporated as immunodiagnostic marker for the detection of different *Shigella* spp.

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