SHORT ARTICLE

Cross-reaction between a strain of *Vibrio mimicus* and *V. cholerae* O139 Bengal

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Of 200 isolates of *Vibrio mimicus* screened, one from water (N-57) agglutinated with *V. cholerae* O139 polyclonal antiserum (absorbed with a rough strain of *V. cholerae* only) and not with O139 polyclonal diagnostic antiserum (absorbed with the rough strain and *V. cholerae* O22 and O155). The antigenic relationship between *V. cholerae* O139 and N-57 is of a,b–a,c type, where a is the common antigenic epitope and b and c are unique epitopes. Strain N-57 was assigned to a new serogroup of *V. cholerae* O194. It gave negative results in a monoclonal antibody-based rapid test and a PCR test specific for *V. cholerae* O139. It did not possess the ctx gene or produce cholera toxin. Antiserum to strain N-57 cross-protected infant mice against cholera on challenge with *V. cholerae* O139. Structural studies of the surface polysaccharides and studies of the rfb genes will shed more light on the extent of relatedness between *V. mimicus* N-57 and *V. cholerae* O139.

Introduction

Since the outbreaks of cholera in 1992–1993, *Vibrio cholerae* O139 Bengal has become established as a causative agent of endemic cholera in the Indian subcontinent. Genetic studies indicated that *V. cholerae* O139 is probably a mutant of *V. cholerae* O1 El Tor, and that it might have arisen by substitution of rfb genes that encode the lipopolysaccharide (LPS) antigen in *V. cholerae* O1 by genes that encode the LPS antigen and the capsular antigen of *V. cholerae* O139 [1]. The organism that would potentially donate rfb genes for surface polysaccharide synthesis in *V. cholerae* O139 has not been identified. *V. cholerae* O139 also cross-reacts with a number of bacteria, including *V. cholerae* serogroups O22 and O155 [2], and *Aeromonas trota* [3]. In addition, as the core antigen is common to all *V. cholerae* serogroups, *V. cholerae* O139 also shares this antigen with other *V. cholerae* serogroups [2]. Therefore, for production of specific diagnostic polyclonal antiserum to *V. cholerae* O139, the antiserum must be absorbed with a rough strain of *V. cholerae* and with *V.*

cholerae O22 and O155 [2]. A rapid test based on specific monoclonal antibodies (MAbs) to *V. cholerae* O139 has been developed that detects the bacterium directly from the samples [4]. The objectives of a search for bacteria that cross-react with *V. cholerae* O139 are three-fold: to identify the bacterium that would have donated the rfb genes for O139 antigen, to continually ensure the specificity of monoclonal and polyclonal sera to *V. cholerae* O139 for diagnosis, and to characterise cross-reacting bacteria for general microbiological interest. The continuing search for cross-reacting bacteria identified a water isolate of *V. mimicus* that agglutinated with *V. cholerae* O139 antiserum. This isolate was characterised with respect to its antigenic and virulence properties.

Materials and methods

Bacterial strains

One hundred *V. mimicus* strains isolated from diarrhoeal stools of patients treated at the Dhaka treatment facility of the International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B), during 1968–1993, and 100 strains of *V. mimicus* isolated from surface and well waters in Dhaka and Matlab, Bangladesh, during 1989–1993, were studied. *V. mimicus* was identified by standard methods [5].

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Production of antisera

Antisera were produced in adult New Zealand White rabbits against live cells of *V. cholerae* O139 (strain AI-1852), cross-reacting *A. trota* (strain 1354) [3] and cross-reacting *V. mimicus* (strain N-57). The preparation of antigen and the immunisation schedule have been described previously [3]. Antibody to core antigen was removed by absorption of antisera with a rough strain of *V. cholerae* (strain CA385) [3]. Antisera to *V. cholerae* O22 and O155 were those prepared previously at the National Institute of Health, Tokyo, Japan [2].

Slide agglutination test

Each of the 200 strains of *V. mimicus* was tested in a slide agglutination test with two antisera to *V. cholerae* O139: antiserum absorbed with the rough *V. cholerae* strain CA385, and diagnostic antiserum absorbed with strain CA385, *V. cholerae* O22 and *V. cholerae* O155 [2]. Strains that gave positive results were further tested for agglutination with antisera to *V. cholerae* O22 and O155, and *A. trota* strain 1354, and with two mouse MAbs (ICL11 and ICL12) specific for *V. cholerae* O139, as described previously [6].

Tube agglutination test

The tube agglutination test was performed as described previously. Doubling dilutions of antisera were tested from a starting dilution of 1 in 20. Agglutination was scored on a 0 to 4+ scale, and the endpoint titre was defined as the highest dilution of antiserum yielding a 2+ agglutination of cells [3].

Agglutinin-absorption test

After incubation of antigen-antibody mixture, the supernate was filter-sterilised. Antisera were absorbed either with a single culture or multiple cultures as required [3].

Serogrouping

The cross-reacting *V. mimicus* strain was subjected to O serogrouping with antisera to the 155 serogroups of *V. cholerae* previously recognised [2] and to 38 other serogroups (serogroups O156–O193) recognised subsequently (unpublished data). *V. cholerae* and *V. mimicus* have a common serogrouping scheme.

Studies with LPS antigens

LPS was extracted by the hot phenol-water extraction method, separated on SDS-polyacrylamide 13.5% gels and visualised by silver staining. For immunoblotting, the separated LPS was probed with rabbit polyclonal antiserum to the relevant bacteria, and then with alkaline phosphatase-conjugated swine anti-rabbit IgG [3].

Bengal SMART test

The Bengal SMART test, which is a rapid test for *V. cholerae* O139, is a colorimetric immunoassay that uses the colloidal gold-labelled MAb ICL12 to *V. cholerae* O139 [4]. The ability of the SMART test to detect cross-reacting *V. mimicus* was tested according to the manufacturer's instructions.

PCR assay for *V. cholerae* O139 antigen

The cross-reacting *V. mimicus* was tested in a PCR assay with primers specific for the O-antigen of *V. cholerae* O139 as described previously [7].

Enterotoxin production

Culture filtrate was tested in mouse Y1 adrenal tumour cells for cholera toxin-like enterotoxin and in the suckling mouse assay for heat-stable enterotoxin [3]. The criterion for a positive test in the Y1 cell assay was rounding of >50% of the cell monolayer, and that for the suckling mouse assay was a ratio of >0.085 for intestinal body weight to remaining body weight from five mice per test [3].

The cross-reacting *V. mimicus* strain was also tested in a PCR assay for cholera toxin gene (ctx) with two primers corresponding to the ctx operon of *V. cholerae* O1 as described previously [8].

Passive protection in infant mouse cholera model

To study the protective efficacy of polyclonal antiserum to the cross-reacting *V. mimicus*, 0.1 ml of a 1 in 10 diluted antiserum in physiological saline was mixed with a 100 LD₅₀ dose of a clinical isolate of *V. cholerae* O139 (strain AI-1852) and injected into a group of 4-day-old mice. Mortality was observed for up to 48 h [9].

Results and discussion

Of 200 *V. mimicus* strains tested, only one strain, isolated from pond water, agglutinated with *V. cholerae* O139 polyclonal antiserum (absorbed only with *V. cholerae* CA385) in a slide agglutination test. However, this strain did not agglutinate with the *V. cholerae* O139 diagnostic antiserum (absorbed with CA385, and *V. cholerae* O22 and O155). It also agglutinated with antisera to *V. cholerae* O22 and cross-reacting *A. trota* (strain 1354), but not with antiserum to *V. cholerae* O155. The strain was designated N-57.

The results of agglutination and agglutinin-absorption tests are shown in Table 1. Cross-absorption of antisera to *V. cholerae* O139 and *V. mimicus* with heterologous bacteria of *V. mimicus* and *V. cholerae* O139 caused reductions in antibody titres to the homologous bacteria. Such reductions in homologous titres occurred when antisera to *V. cholerae* O22 and *A. trota* were

Table 1. Agglutination and agglutinin-absorption tests with antisera to *V. mimicus* (N-57), *V. cholerae* O139 (AI-1852), *V. cholerae* O22, *V. cholerae* O155 and *A. trota* (1354)

Antiserum*	Absorbing organism†	Titre of antisera to O antigens from				
		<i>V. mimicus</i> (N-57)	<i>V. cholerae</i> O139 (AI-1852)	<i>V. cholerae</i> O22	<i>V. cholerae</i> O155	<i>A. trota</i> (1354)
<i>V. mimicus</i> (N-57)	None	2560	160	40	—‡	80
	<i>V. cholerae</i> O139	640	—	—	—	—
	<i>V. cholerae</i> O22	1280	40	—	—	—
	<i>A. trota</i>	640	40	20	—	—
<i>V. cholerae</i> O139 (AI-1852)	None	320	1280	640	40	640
	<i>V. mimicus</i>	—	640	640	40	640
<i>V. cholerae</i> O22	None	160	320	640	—	640
	<i>V. mimicus</i>	—	160	320	—	640
<i>V. cholerae</i> O155	None	—	80	—	640	—
	<i>V. mimicus</i>	—	80	—	640	—
<i>A. trota</i> (1354)	None	320	1280	160	—	2560
	<i>V. mimicus</i>	—	320	40	—	1280

*Antibody to core antigen in these sera was absorbed with a rough strain of *V. cholerae* (CA385).

†Absorbed with live and boiled organisms; boiled (100°C for 1 h) cultures were used.

‡No agglutination at a dilution of 1 in ≥ 20 .

absorbed with *V. mimicus*. These data suggested that the antigenic relationship between *V. mimicus* N-57 and *V. cholerae* O139, *V. cholerae* O22 and *A. trota* is that of a,b–a,c type where a is the common epitope and b and c are unique epitopes. This is because absorption of an antiserum with a heterologous strain failed to remove antibodies to the homologous strain completely. This type of antigenic relationship has been shown previously between *V. cholerae* O139 and other cross-reacting bacteria such as *V. cholerae* O22 and O155 and *A. trota* [2, 3]. Consistent with the slide agglutination test, *V. mimicus* did not agglutinate with *V. cholerae* O155 antiserum. *V. cholerae* O155 also did not agglutinate with *V. mimicus* antiserum. Different patterns of reactions were observed among *V. cholerae* O139, O22 and O155 and *A. trota* when antisera to these organisms were absorbed with *V. mimicus*.

The SDS-PAGE separation profile of LPS from *V. mimicus* N-57 and *V. cholerae* O139 AI-1852 is shown in Fig. 1. The profile of *V. mimicus* resembled that of smooth bacteria revealing a core antigen (thick band at the bottom of the gel) and an O-antigenic repeating polysaccharide (ladder-like portion in the top part of the gel). However, the LPS pattern of *V. cholerae* O139 resembled that of semi-rough bacteria as reported earlier [1] revealing a modified core structure and no high mol. wt O antigen-specific side chain. Immunoblot studies with antisera to *V. cholerae* O139 and *V. mimicus* recognised homologous and heterologous LPS antigens equally well. In Fig. 2, the immunoblots are shown with antiserum to *V. mimicus* only. Compared with the LPS pattern in Fig. 1, the core antigens in the bottom portion of the gel in Fig. 2 are not stained because the core antibody has been absorbed out. These studies further confirmed the antigenic relationship between *V. cholerae* O139 and *V. mimicus* N-57.

The LD50 of the challenge strain of *V. cholerae* O139,

Fig. 1. LPS profile of *V. cholerae* O139 strain AI-1852 (A) and *V. mimicus* N-57 (B). The LPS preparations were electrophoresed on an SDS-polyacrylamide 13.5% gel and were silver stained.

AI-1852, for infant mice was 10^3 cfu in 0.1 ml of inoculum. A 1 in 10 dilution of polyclonal antiserum to isolate N-57 completely protected mice against a challenge dose of 100 LD50 of strain AI-1852. However, all the mice died when a 1 in 10 dilution of pre-immune rabbit serum was used. This suggested

Fig. 2. Immunoblot analysis of LPS from *V. cholerae* O139 strain AI-1852 (A) and *V. mimicus* N-57 (B) separated on an SDS-polyacrylamide 13.5% gel by electrophoresis, blotted on to a nitrocellulose membrane and probed with antiserum to *V. mimicus* N-57.

that the shared antigenic epitope(s) between *V. cholerae* O139 and *V. mimicus* N-57 is protective.

Some strains of *V. mimicus* produce a cholera toxin-like enterotoxin and a heat-stable enterotoxin [10]. However, strain N-57 did not produce these toxins, and also gave a negative result for ctx in a PCR assay. This indicated that this strain may not produce watery diarrhoea.

Strain N-57 did not agglutinate with *V. cholerae* O139-specific MAbs (ICL11 and ICL12) and also gave a negative result in the Bengal SMART test, which uses ICL12. It also gave a negative result in the PCR assay specific for *V. cholerae* O139 as it failed to generate an amplicon of 416 bp [7]. These data suggest that such cross-reacting *V. mimicus* strains will not be misdiagnosed as *V. cholerae* O139. The cross-reacting *V. mimicus* strain, N-57, could not be serogrouped with antisera to the 193 O serogroups of *V. cholerae*. Therefore, a new serogroup, O194, was assigned to this strain.

Structural analysis of surface polysaccharide of *V. cholerae* O139 suggested that it contains two unique

constituents, galactose cyclophosphate and colitose [11]. Some cross-reacting bacteria share either or both of these structures [12, 13]. A previous study found that the cross-reacting between *V. mimicus* strain N-1990 and *V. cholerae* O139 is due to sharing of the galactosyl residue substituted with a cyclic phosphate by both bacteria [14]. The structural basis of cross-reaction as well as the homology of the rfb genes encoding the surface polysaccharides in both *V. cholerae* O139 and strain N-57 will need to be determined to further explore the relatedness between these two bacteria.

Worldwide efforts are being made to contain the threat of 'emerging and re-emerging pathogens'. *V. cholerae* O139 is a newly emerged pathogen, and is considered to be the causative agent of the 'eighth' pandemic of cholera. The potential of cross-reacting bacteria for use as vaccine strains against O139 cholera should also be explored.

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