BACTERIAL CHARACTERISATION

A comparative study of the properties of *Vibrio cholerae* O139, O1 and other non-O1 strains

R. K. NANDY, T. K. SENGUPTA, S. MUKHOPADHYAY and A. C. GHOSE*

Department of Microbiology, Bose Institute, Calcutta 700-054, India

Summary. *Vibrio cholerae* O139 organisms isolated from different parts of India and from Bangladesh were characterised with respect to their haemagglutination (HA) activity, plasmid content, cholera toxin (CT) production, cell surface protein and lipopolysaccharide (LPS) profiles, and antigenic properties. Of 28 *V. cholerae* O139 isolates tested, 14 (50%) were shown to agglutinate chicken erythrocytes; the HA activity was sensitive to D-mannose 0.1%. In parallel experiments, 12 (92.3%) of 13 *V. cholerae* O1 (El Tor) and 12 (75%) of 16 non-O1, non-O139 strains agglutinated chicken erythrocytes. Plasmid analysis of 32 O139 isolates showed that 12 (37.5%) carried one or more plasmids of 35.8–2.6 MDa. Plasmids were not detected in any of the *V. cholerae* O1 strains, although plasmids were demonstrable in 35% of the non-O1, non-O139 strains tested. *V. cholerae* O139 isolates showed an ability to produce CT that depended on media composition and other cultural conditions. A comparison of envelope and outer-membrane protein profiles between O1 and O139 isolates failed to show any significant differences. LPS analysis of O139 isolates revealed that these organisms were devoid of long “O” side-chain polysaccharides. Some of the non-O1, non-O139 strains also showed similar LPS profiles whereas others showed the presence of long repetitive “O” side-chain polysaccharides similar to those seen in O1 organisms. An antiserum raised against *V. cholerae* O1 strain O395 did not show any significant reactivity towards O139 and non-O1, non-O139 strains although it reacted with other O1 strains. Furthermore, the anti-O1 serum induced marked protection against challenge with an O1 strain but not with an O139 strain in passive protection experiments. All these results indicate that, despite sharing some common properties with *V. cholerae* O1, the O139 isolates possess certain characteristics that make them distinct from their O1 counterparts.

Introduction

Vibrio cholerae strains belonging to serogroup O1 have been responsible for the diarrhoeal disease cholera in its epidemic form.¹ Strains belonging to the non-O1 serogroups, on the other hand, have so far been implicated as the causative agents of sporadic cases of gastro-enteritis and extra-intestinal infection in man.^{2,3} However, cholera-like epidemics have been reported recently from India and Bangladesh that are caused by novel strains of *V. cholerae* assigned to a newly designated serogroup O139.⁴ Limited information available so far suggests that the O139 organisms produce cholera toxin (CT)^{5,6} and appear to be related to *V. cholerae* O1 strains, particularly those belonging to the El Tor biotype.^{7,8} However, this does not exclude the possibility that O139 strains

could be related to other non-O1 strains and share common characteristics. This is an issue of considerable epidemiological significance and more information is needed to resolve it. Therefore, a study was undertaken to obtain detailed information on certain important characteristics of *V. cholerae* O139 organisms isolated from recent epidemics in India and Bangladesh. These characteristics were chosen for their importance in the pathogenesis and epidemiology of cholera. Furthermore, results obtained with O139 organisms were compared with those obtained with *V. cholerae* O1 and other non-O1, non-O139 strains to determine possible relatedness and differences amongst these organisms.

Materials and methods

Bacterial strains

V. cholerae strains belonging to serogroup O139 were isolated from diarrhoeal stool samples during

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*Correspondence should be sent to Professor A. C. Ghose.

1992–93 epidemics in different parts of India.⁹ A few O139 strains isolated from epidemics in Bangladesh were kindly provided by Dr J. Albert (ICDDR, Bangladesh). *V. cholerae* O1 strains used in this study include old¹⁰ as well as some recent isolates from cases of diarrhoea at Calcutta. The reference O1 strain (O395) was kindly made available to us by Dr J. J. Mekalanos, Harvard Medical School, Boston, USA. The majority of the other clinical and environmental isolates of non-O1 *V. cholerae* strains used were described previously.¹¹ Others were isolated locally from diarrhoeal cases and environmental sources over the past few years.

Determination of haemagglutinating (HA) activity

The HA activity of *V. cholerae* strains was determined with chicken erythrocytes (CRBC) by the slide agglutination test¹² or by the microtitration plate method.¹³ For the latter, organisms were grown in tryptic soya broth with (TSB) or without glucose (SF TSB), AKI¹⁴ or Luria broth (LB), for 16 h or 4 h at 37°C or 30°C for comparisons.

Sugar-mediated inhibition of the HA activity was studied in the presence of D-mannose or L-fucose 0.1%.¹⁵ A reduction of HA titre of four-fold or greater was taken as the positive index for the sugar-mediated inhibition of HA.

Analysis of bacterial lipopolysaccharide (LPS)

LPS profiles of *V. cholerae* strains were analysed by SDS-PAGE of proteinase K-treated¹⁶ whole cell lysates followed by silver staining¹⁷ of the electrophoresed material. Purified LPS from a limited number of strains was also analysed by SDS-PAGE. For this, LPS was extracted from acetone-dried cells of *V. cholerae* by the hot (65°C) phenol-water extraction method.¹⁸ Any residual protein remaining in these LPS preparations was removed by protease digestion.¹⁹

Preparation of bacterial envelope (ENV) and outer membrane (OM)

V. cholerae cells grown in AKI medium were harvested and homogenised by an Ultrasonic disintegrator (Braunsonic 1510; Braun). Crude ENV fraction was collected by differential centrifugation of the homogenate as described previously.¹⁰ The OM fraction was isolated from the ENV fraction by selective solubilisation of inner membrane with Sarcosyl 0.5% solution.²⁰ Both the ENV and OM preparations were analysed by electrophoresis in polyacrylamide 12.5% gels followed by staining with Coomassie Brilliant Blue.

Plasmid analysis

Plasmid DNA of *V. cholerae* cells grown in LB was extracted by the alkaline lysis method.²¹ Extracted

DNA material was electrophoresed in agarose 0.8% gel and stained with ethidium bromide. An approximate estimate of the plasmid molecular size was obtained by use of the reference *Escherichia coli* strain V517²² harbouring plasmids of known mol. wts.

Assay for cholera toxin (CT) production

The ability of one O1 strain (O395) and one O139 strain (SG25) to produce CT was determined by the GM₁-ELISA method.²³ For this, organisms were grown under desired culture conditions and culture supernates were used for the assay. Results were expressed as µg of CT produced/ml of supernate/opacity unit (at 540 nm) of the bacterial cell suspension.

Raising of antiserum

An antiserum to *V. cholerae* O1 strain O395 was raised in rabbits by weekly injections of bacterial whole cells in graded doses of (1×10^7) – (1×10^8) cfu/ml.²⁴ Initially, two injections were given by the subcutaneous route followed by intravenous injections. Rabbits were bled 7 days after the last injection and sera were collected. Agglutinability of the antiserum for *V. cholerae* whole cells was determined as described previously.¹⁹

ELISA

Serological reactivity of the antiserum was tested against *V. cholerae* O1 and O139 strains by the whole cell-ELISA.²⁵ Serological reactivity of the antiserum was also tested against LPS purified from O1 and O139 organisms. For this, ELISA plates were coated with appropriate LPS preparations (2 µg/well) as described previously.²⁶

Protection experiments

The protective activity of an antiserum to strain O395 was evaluated in passive protection experiments in a suckling mouse model.²⁷ For this, separate groups of mice (six in each group) were challenged with 10 LD₅₀ doses of O1 or O139 organisms, pre-incubated with appropriate dilutions of the antiserum.²⁸ Pre-immune serum was used as control in these experiments. The level of protection was estimated by recording the number of mice surviving in each group 24 h after oral challenge.

Results

Haemagglutinating activity

HA activity of the *V. cholerae* strains was initially tested with CRBC 2.5% in the slide agglutination test. Of 28 O139 isolates tested, 14 (50%) showed HA activity. This was in contrast to the results obtained

Fig. 1. Distribution of HA titres amongst *V. cholerae* strains belonging to serogroups O139 (a, 28 strains), O1 (b, 13) and non-O1, non-O139 (c, 16): $\square$ AKI, pH 7.4, 30°C, 16 h; $\square$ LB, pH 6.5, 30°C, 16 h; $\square$ SFTSB, pH 7.4, 37°C, 4 h; $\blacksquare$ TSB, pH 7.4, 37°C, 16 h.

with 13 O1 strains (all of El Tor biotype) of which 12 (92.3%) showed HA activity. Of 16 non-O1, non-O139 strains tested, 12 (75%) agglutinated CRBC in a similar assay. The HA titres of *V. cholerae* cells grown in different media were determined in microtitration plates and the results are shown in fig. 1. No significant difference in the distribution of HA titre was apparent in O139 cells grown under different conditions. Similar results were obtained with *V. cholerae* O1 and non-O1, non-O139 strains. The results presented in fig. 1 also show that the microtitration plate assay was more sensitive than the slide agglutination test for HA as the

Table 1. CT production by *V. cholerae* O1 and O139 strains grown under different culture conditions

Culture conditions*			Amount† of CT ($\mu\text{g/ml}$ /opacity unit) produced by <i>V. cholerae</i> strains	
Medium	pH	Temperature of incubation	O395‡	SG25§
NB	7.4	37°C	0.030	0.037
AKI	7.4	37°C	0.160	0.040
AKI	7.4	30°C	0.267	0.016
LB	6.5	30°C	0.625	0.010
LB	7.4	30°C	0.477	0.013
LB	6.5	37°C	0.032	0.006
LB	7.4	37°C	0.031	0.004

NB, nutrient broth; AKI, AKI broth; LB, Luria broth.

*Organisms grown for 16 h.

†Assay by GM₁-ELISA method.²³

‡Serogroup O1.

§Serogroup O139.

latter usually became positive for a strain showing an HA titre ≥ 16 in microtitration plates.

The HA activity of all the HA-positive strains—except two non-O1, non-O139 and one O139 strain—was inhibited by D-mannose 0.1%. One non-O1, non-O139 isolate also showed both mannose- and fucose-sensitive HA activity.

Plasmid analysis

Of 32 O139 strains tested, 12 (37.5%) possessed one or more plasmids which were usually in the range 35.8–2.6 MDa. The agarose gel electrophoresis patterns of eight plasmid-containing O139 strains are shown in fig. 2. Interestingly, of 17 non-O1, non-O139 strains tested, six (35%) possessed plasmids whereas all O1 strains tested were plasmid-free (data not shown).

Fig. 2. Agarose gel electrophoresis patterns of plasmid DNA from *V. cholerae* O139 strains. Positions of reference plasmids of known mol. wt are shown. Chr, fragmented chromosomal DNA.

Fig. 3. SDS-PAGE analysis of envelope (Env; lanes 1, 3, 5, 7) and outer-membrane (OM; lanes 2, 4, 6, 8) proteins of *V. cholerae* O139 and O1 strains.

CT production

V. cholerae O139 strains expressed CT when grown in liquid medium at 37°C. One of the O139 strains (SG25) was subsequently chosen to study the effect of medium composition and other culture conditions on the expression of CT. Similar data were also generated with the reference O1 strain O395 and results are presented in the table. It can be seen that the O139 strain SG25 produced considerably smaller amounts of CT than those produced by strain O395 in similar conditions. However, both strains showed variations in their ability to produce CT in different media and culture condition (temperature of incubation, pH of the medium). Whereas CT production by O1 strain O395 was maximum in LB, pH 6.5, at 30°C, O139 strain SG25 produced CT maximally in AKI at 37°C. Furthermore, the amount of CT produced by strain O395 was lowest in nutrient broth (NB), but CT production by strain SG25 was close to its highest level in the same medium.

Cell surface protein profiles

ENV and OM preparations of two O139 and two O1 (one classical and one El Tor) strains were subjected to SDS-PAGE and the results are presented in fig. 3 for comparison. It is evident that ENV protein profiles of all these isolates were more or less similar to each other. The same was generally true for their OM protein profiles; however, positions of the major protein bands around 40–43 kDa showed minor variations.

LPS

LPS profiles of randomly chosen strains of O139 are shown along with the LPS profiles of O1 and non-O1,

non-O139 strains (fig. 4). LPS profiles of O139 strains were remarkably similar to each other but were different from those of O1 strains which possessed long “O” polysaccharide side-chains. Interestingly, a few of the non-O1, non-O139 strains (fig. 4c, lanes 3 and 7) showed LPS profiles similar to those of O139 strains, whereas others showed the presence of repetitive units of “O” polysaccharide side chains with some degree of variability.

Serological reactivity of an antiserum raised against *V. cholerae* O1 strain O395

Serological reactivity of an antiserum raised against *V. cholerae* O1 strains O395 was tested against different *V. cholerae* strains by whole cell-ELISA and the results are presented in fig. 5. The antiserum showed considerably greater reactivity with *V. cholerae* O1 strains than with O139 or non-O1, non-O139 strains. Similar results were also obtained when reactivity of the antiserum was tested against purified LPS from O1, O139 and one of the non-O1, non-O139 strains.

Protection experiments

Protective efficacy of the anti-O395 antiserum was determined separately against *V. cholerae* O1 (O395) and O139 (SG25) strains. When used at an arbitrarily chosen dilution of 1 in 800, the antiserum protected all six mice against challenge with strain O395. Further dilution of the antiserum (1 in 1600) also protected four of six mice challenged with the same strain. On the other hand, the antiserum at a dilution of 1 in 800 failed to protect any of the six mice challenged with strain SG25. Furthermore, the use of a higher concentration (1 in 400) of the antiserum protected only one of six mice challenged with this strain.

Discussion

V. cholerae O1 strains belonging to biotype El Tor can be differentiated from classical biotype strains by their HA activity towards chicken erythrocytes in the slide agglutination test.^{12,29} However, this phenotypic property does not appear to be restricted exclusively to El Tor strains, as a significant percentage of *V. cholerae* non-O1 strains also exhibit similar activity.¹¹ The O139 organisms studied here showed apparent heterogeneity with respect to HA properties, as c. 50% of these strains were shown to express HA activity (titre ≥ 16) which was more or less independent of their culture conditions. However, the HA activity of the O139 strains was inhibited by D-mannose 0.1% suggesting similarity with the O1 (El Tor) and the majority of non-O1, non-O139 strains. The mannose-sensitive haemagglutinin (MSHA) of *V. cholerae* O1 (El Tor) strains was characterised recently and shown to be a 17-kDa protein of pilus origin.³⁰ Whether or not

Fig. 4. LPS profiles of proteinase K-digested cells of *V. cholerae* O139 (a), O1 (b) and non-O1, non-O139 (c) (lanes 1-7). Lane 8 shows the LPS profiles of a diarrhoeagenic *E. coli* strain DS92 (O125) under similar experimental conditions.

Fig. 5. ELISA reactivity of an anti-O395 antiserum determined against different strains of *V. cholerae* O1 (a), O139 (b) and non-O1, non-O139 (c) whole cells. Broken lines show the reactivity of the antiserum to LPS preparation of *V. cholerae* strains indicated in the figure. (a) ●—● O395, ○—○ 154, ×—× AD48, ▲—▲ 1797, ●—● O395 LPS. (b) ●—● SG25, ○—○ B01, ■—■ API, ▲—▲ MD012, ●—● SG25 LPS. (c) ●—● VCE 328, ○—○ V59, ■—■ 10325, ▲—▲ 10259, ●—● 10325 LPS.

the MSHA activity of O139 organisms is mediated by the same or a related protein remains to be seen.

A significant percentage of *V. cholerae* O139 and other non-O1, non-O139 strains contained one or more plasmids. It may be noted here that *V. cholerae* O1 El Tor strains rarely carry plasmids,³¹ although these could be demonstrated in some O1 (classical)³² as well as non-O1, non-O139 strains.^{33,34} Some of these plasmids were shown to encode antibiotic resistance markers.^{34–36} However, plasmids associated with the majority of *V. cholerae* strains reported earlier were cryptic in nature.^{31,37} The O139 plasmids also appear to be cryptic in nature as removal of plasmids from one of the O139 strains (SG25) by successive passages *in vitro* failed to show any alteration in antibiotic resistance, CT production or colonising properties (R. K. Nandy *et al.*, unpublished data).

The O139 strains have been shown already to express CT-like toxins.^{5,6} The epidemic isolates of O139 strains studied here also possess *ctxA* genes when probed by appropriate primers (R. K. Nandy *et al.*, unpublished data). However, optimum cultural conditions for the expression of CT are different for O1³⁸ (classical strain O395) and O139 (strain SG25).

Earlier studies¹⁹ showed that the OM protein profiles of different *V. cholerae* O1 strains were remarkably similar to each other and independent of their serotypes and biotypes. Results presented here extend the earlier observation, as no significant difference could be noted in the cell surface protein profiles between O1 and O139 strains. On the other hand, marked differences were noted between the LPS profiles of O1 and O139 strains. In fact, the LPS

profiles of O139 strains resembled rough or semi-rough variants¹⁷ of *V. cholerae*. Also, the similarity in the LPS profiles between O139 and some of the non-O1, non-O139 isolates may be of considerable interest, as both these groups of organisms were shown to express polysaccharide capsules,^{39,40} that are not seen in O1 strains.

Antigenic differences between O1, O139 and non-O1, non-O139 strains are also documented in this study. Serological reactivity (fig. 5) appeared to correlate well with the protective activity of anti-O1 serum, which was shown to provide passive protection against O1 but not O139 challenge. These results are consistent with the epidemiological observation that pre-formed immunity against the O1 organisms failed to protect against O139 disease in man.^{6,41}

Results presented in an earlier study,⁹ as well as this report, show certain variations within O139 strains which cannot be correlated with their geographic origin. Whether or not these differences arose as a result of clonal divergence once a strain was introduced into a given area is difficult to prove. However, our results suggest that, despite sharing some common properties with *V. cholerae* O1, the O139 strains possess certain characteristic features (common to non-O1, non-O139 strains) that make these organisms distinct from their O1 counterparts.

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