Purification and Characterization of Fimbriae of *Vibrio cholerae* O1, Classical Biotype, Inaba Serotype

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Abstract: Fimbrial subunits have been shown to share common antigenicities between *Vibrio cholerae* biotype El Tor and biotype classical (Ehara *et al.*, 1987). The above fact was confirmed by western blotting and immunoelectron microscopy. Net−work and bundle formations were observed in the fimbrial preparations by immunoelectron microscopy. Purified fimbriae of *Vibrio cholerae* O1, classical biotype possessed haemagglutination activity (HA) against chicken erythrocytes. This HA was inhibited in the presence of D−mannose but not by L−fucose. Antigenic determinants of the fimbriae were exposed on the sides of the fiber. The low hydrophobicity of the fimbriae molecule revealed by the amino acid composition suggests that the fimbriae may interact with epithelial cells not by hydrophobic interaction. Development of the antibody against fimbrial subunit of *V. cholerae* O1 was confirmed in the blood sera obtained from convalescent cholera patients.

Key words: Vibrio cholerae O1, fimbriae, component vaccine

INTRODUCTION

The colonization of enteropathogenic *Vibrio cholerae* O1 to the intestinal epithelium is an essential step in the production of watery diarrhoea. Whether *V. cholerae* O1 produce any colonization factor during the interaction between vibrios and epithelial cells of

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small intestine was a controversial problem. The answer to the problem was obtained by the observation of colonized vibrios after the detachment of the vibrio cells from epithelial cells of small intestine. The detached vibrios were shown to possess several long flexible fibers (fimbriae) (Ehara *et al.*, 1986).

The purified fimbriae of an El Tor strain was shown to have the haemagglutinating activity against human type O red blood cells as well as chicken erythrocytes. This HA was sensitive both to D−mannose and L−fucose (Ehara *et al.*, 1987). Since fimbriae of classical vibrios were not characterized in the previous paper, this paper describes the purification of fimbriae of *Vibrio cholerae* O1, classical biotype, Inaba serotype and their partial characterization.

Materials and Methods

Bacteria: Non−motile mutant strain Bgd 17−1 was obtained from the strain Bgd 17 (x−23751) by treatment with nitrosoguanidine (50 μg/ml).

Bgd 17 (x−23751) of the classical biotype Inaba serotype was isolated in Bangladesh in 1982 and kindly provided by Dr. Y. Takeda.

Non−motile mutant derivatives from Bgd 17 were positive in a rabbit ileal loop test and were confirmed to adhere to the ileal epithelium under scanning electron microscopy.

Medium: Vibrios were grown on TCG agar (Ehara *et al* ., 1986) (1 % Bactotryptone, 0.2 % Yeast extract, 0.5 % NaCl, 0.3 % $NaHCO_3$, 0.02 % Thioproline, 0.1 % Monosodium L−glutamate, 1 mM EGTA, and 2 % Agar) at 30 ℃ for 48 h.

Purification of fimbriae: Vibrio cells were cultured on TCG agar in 80 Roux bottles containing 100 ml medium at 30 ℃ for 48 h.

After the addition of 10 ml of 0.1 M potassium phosphate buffer pH 6.8 (PPB) containing 0.5 mM $MgCl_2$ and 1 mM EGTA, cells were harvested by pipette and centrifuged at 10,000 x*g* for 1 h at 4 ℃. Whole cells were resuspended in PPB to 0.5 g (wet weight) / ml, then homogenized in a cooled Sorvall Omnimixer at setting 5 for 5 min. The homogenate was centrifuged at 12,000 x*g* for 30 min.

The resultant supernatants were pooled at 4 ℃ and the pellet was resuspended with fresh PPB and the homogenization repeated three times. Each supernatant was combined and kept at 4 ℃ overnight after the addition of DNase I and RNase, each at 100 μg / ml. The mixture was centrifuged at 48,000 x*g* for 1 h at 4 ℃ to remove cell debris. The supernatant was concentrated to 4 ml over an Amicon PM 10 membrane. The concentrated sample was loaded onto the top of 20−50 % sucrose linear gradient solution and centrifuged with SW 41 rotor (Beckman) at 200,000 x*g* for 16 h at 4 ℃. Fractions of 0.5 ml were taken from the top of the tube and monitored for purity by electronmicroscopy and sodium dodecyl sulphate polyacryl amide gel electrophoresis. Fimbriae−rich fractions were combined and loaded onto Sepharose CL−4B column chromatography equilibrated with 20 mM PPB with 10 % sucrose.

Each fraction was monitored for purity as mentioned earlier.

Preparation of antiserum to El Tor fimbriae: Fimbriae purified from K 23 strain were mixed with an equal volume of Freund's complete adjuvant (0.5 ml fimbrial solution of 200 μg/ml and 0.5 ml of the adjuvant) and used to parenterally immunize rabbits for the production of high titre antibody against native El Tor fimbriae. The rabbits were injected subcutaneously at two sites in the back and intramuscularly at two sites in the thigh each week for the first month and every two weeks for the second month substituting incomplete adjuvant for complete adjuvant. They were bled one week after the last injection. The IgG fraction of the antisera was prepared by DEAE−Sephadex A−50 column chromatography and was used for immunoblotting and for immunoelectron microscopy.

SDS−polyacrylamide gel electrophoresis: polyacrylamide gel electrophoresis (PAGE) was performed in 1.5 mm thick slab gels according to the system of Laemmli (1970). Fimbrial preparations were heated at 100 ℃ for 5 min in sample buffer (0.05 M Tris−HCI pH 6.8 containing 2.5 % (w/v) sodium dodecyl sulfate (SDS), 10 % (v/v) glycerol, 5 % (v/v) 2−mercaptoethanol, and a trace of bromophenol blue as tracking dye). Samples were applied to the polyacrylamide slab gel (12 %) using a 4 % stacking gel and were electrophoresed for 7 h at 20 mA constant current per slab. The protein bands were stained with Coomassie brilliant blue R 250. For molecular weight calibration, the low molecular weight market kit (Pharmacia Fine Chemicals) was used.

Western blotting: Immunoblotting was performed according to the techniques of Towbin *et al.* (1979) and Burnett (1981). Purified fimbriae were subjected to SDS−PAGE in duplicate on different gels, one of which was stained with Coomassie blue and the other electroblotted onto nitrocellulose membrane (BIO−RAD) using a BIO−RAD electroblotting apparatus (30 V for 16 h and 70 V for 1 h). Horseradish−peroxidase−conjugated goat anti−rabbit IgG was from Cappel Laboratories Inc., USA, and 4−chloro−1−naphthol (BIO−RAD Laboratories) was used in the development of the color reaction.

Haemagglutination and haemagglutination inhibition tests.

Purified fimbriae of strain Bgd 17−1 were dialysed against 20 mM PPB to remove sucrose. Techniques for quantification of haemagglutination (HA) and HA inhibition with sugars were adapted from Jones *et al.* (1976). Serial two−fold dilution of fimbrial preparations (200 μg/ml) were prepared in a U−bottomed microtitre plate in 25 μl of 20 mM PPB. Chicken red blood cells (2 % in 20 mM PPB) were added to 25 μl, the plate was tapped to mix the interactions and the erythrocytes were settled at 25 ℃ for 30 min.

The titre is defined as the reciprocal of the highest dilution in which HA was clearly visible. To test whether the HA reaction was inhibitable by specific monosaccharides, erythrocytes with 1 % (w/w) D−mannose or L−fucose were added to the wells containing serially diluted fimbriae and HA reactions were read after 30 min at 25 ℃.

Amino acid composition: Amino acid analysis was carried out on the fimbrial subunit eluted from a preparative SDS−PAGE. The extracted fimbrial subunit solution was first dialyzed against 0.2 M NaCl to remove glycine in the electrophoresis buffer before dialysis against distilled water. The sample was hydrolyzed with 6 N HCl at 110 ℃

for 24 h in an evacuated sealed tube. The hydrolysate was analyzed with a JEOL JLC−200A amino acid analyzer.

Detection of antibody against fimbrial subunit in blood sera from convalescent cholera patients: Paired blood sera were taken from cholera patients admitted to Bamrasnaradura Hospital by one of the authors (S. T.). Detection of the antibody was carried out by western blotting, using crude fimbrial fraction as antigen. Blood sera were diluted 200-fold with Tris buffered saline.

Electron microscopy: For negative staining, one drop of the sample was placed on a sheet of PARAFILM and a Formvar−coated copper grid was floated on the drop for 2 min. The excess liquid was washed three times with distilled water each time for 10 s, then stained with 1 % uranyl acetate for 30 s. The excess stain was removed with the tip of a filter paper. For immunoelectron microscopy, the copper grids coated with purified fimbriae or cell−suspension were reacted with the primary antibody (anti−El Tor fimbriae antibody) and with the secondary antibody (anti−rabbit IgG goat serum conjugated with 15 nm colloidal gold, E. Y. LABS, INC. SANMAEO, CA 94401 USA) before negative staining. The specimens were examined with a JEM 100 cx electron microscope operated at 80 kV.

RESULTS

Purification of fimbriae: Fimbriae rich fractions (fraction No.12 to 16) obtained by sucrose density gradient centrifugation were combined and loaded onto a Sepharose CL−4

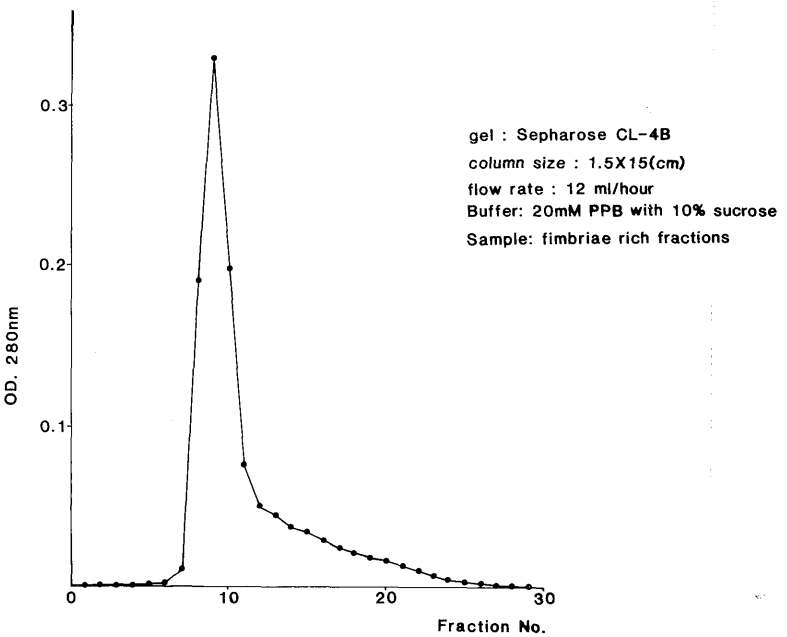


Fig. 1. Gel filtration profile.

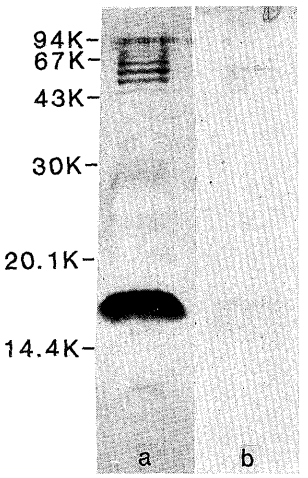
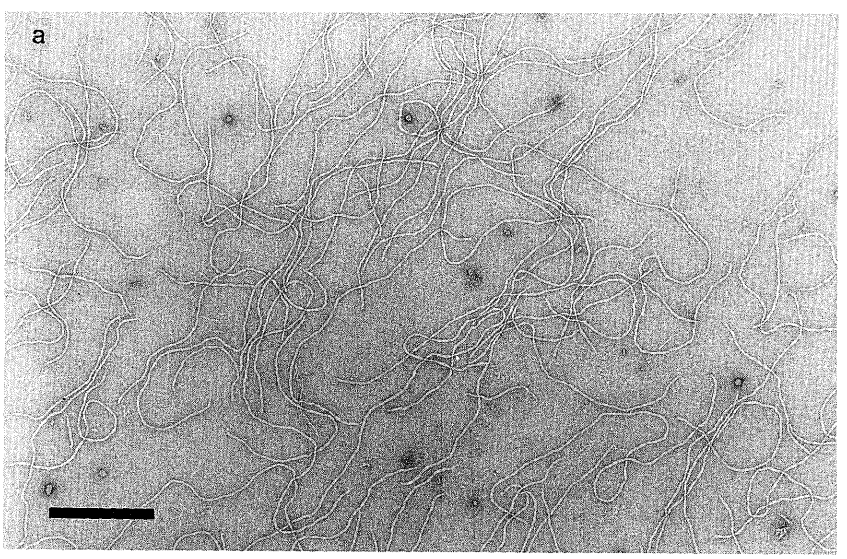
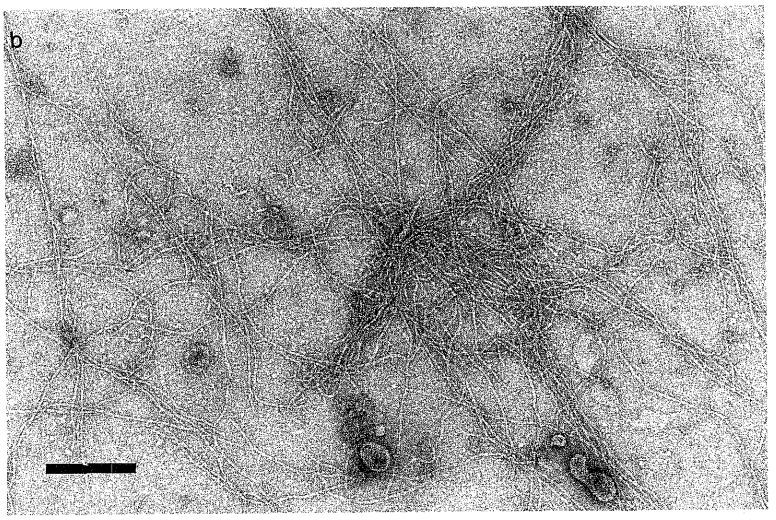


Fig. 2. a:western blot
b:SDS−PAGE.

B column. Fimbriae were eluted at the void volume fraction (Fig. 1). The fimbrial preparations obtained by the above procedure were analyzed by SDS−PAGE and Western blot. The molecular weight of the structural subunit of fimbriae (fimbrillin) was ca.16,000. As reported in the previous paper, fimbriae of a classical strain were stained faintly in the Laemmli system, although the same specimen showed numerous fibrils under electron microscopy and was densely immunostained in a western blotting (Fig. 2). Electron microscopic examination of the purified fimbriae from *V. cholerae* O1 strain Bgd 17−1 showed the presence of numerous, thin, non−channeled fimbriae of external diameter 5−7 nm, typical of the fimbriae present on intact, non−homogenized bacteria (Ehara *et al.*,

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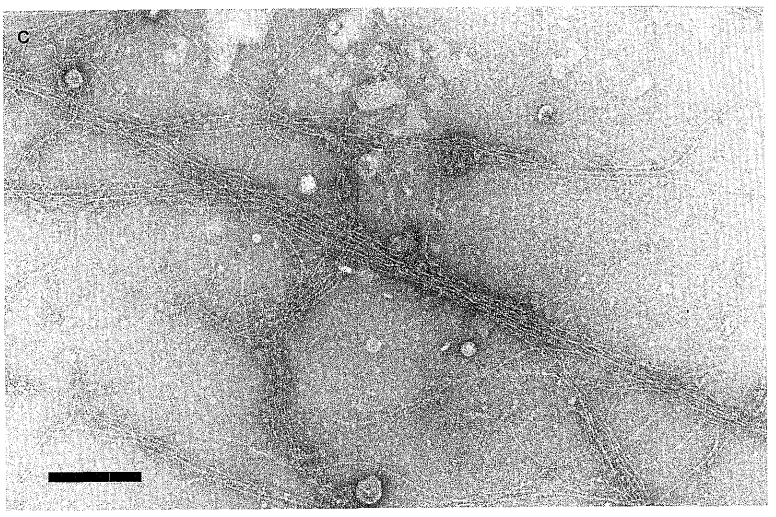
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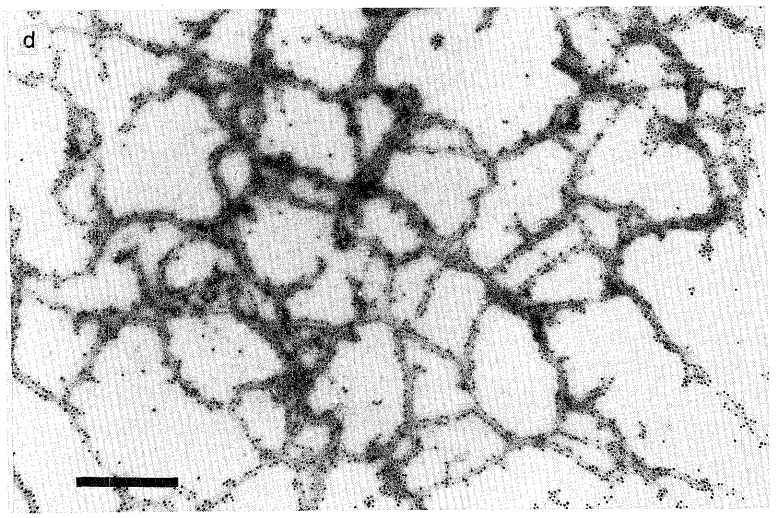
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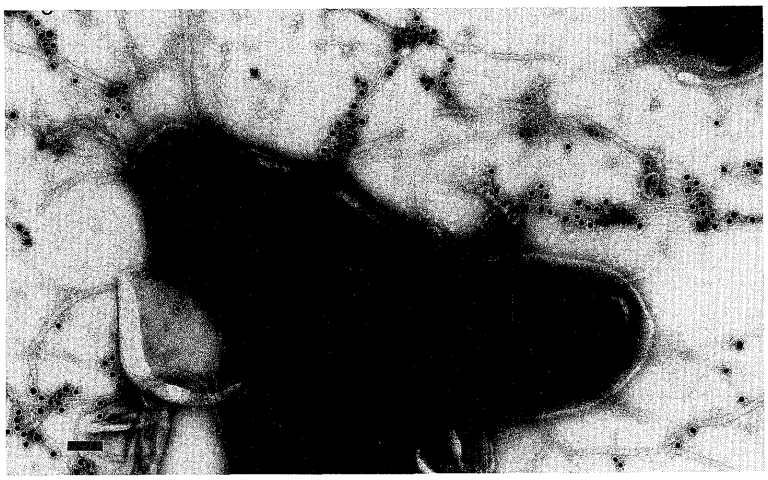

Fig. 3. Electron micrographs of purified and immunodecorated fimbriae.
(a) fimbriae purified through a gel filtration; (b), (c) fimbriae reacted with anti−El Tor fimbriae antibody, (d) fimbriae reacted with anti−El Tor fimbriae antibody for 15 min following the reaction with anti−rabbit IgG goat serum conjugated with 15 nm colloidal gold, (e) Bgd 17(parent strain) reacted with anti−El Tor fimbriae antibody in the same method as used in sample(d).
Bars indicate 250 nm in (a)−(c), 500 nm in (d) and 100 nm in (e).

1986, Faris *et al.*, 1982, Tweedy *et al.*, 1968) as shown in Fig. 3 a. When reacted with anti−El Tor fimbriae antibody, net−work and bundle formation (Fig. 3 b, c) were observed in the specimens of classical fimbriae. The electron micrographs of classical fimbriae treated with anti−El Tor fimbriae antibody following the reaction with anti−rabbit IgG goat serum conjugated with 15 nm colloidal gold were shown in Fig. 3 d and 3 e. The antibodies bound to the sides of the fimbrial net−work.

Haemagglutination activity of fimbriae: Fimbriae of classical vibrio had low HA titer to chicken erythrocytes and HA inhibition was obtained in the presence of D−mannose but not in the presence of L−fucose as shown in Table 1.

Detection of antibody against fimbrial subunit: The raised antibody against fimbrial subunit was detected in each blood specimen taken 3, 4, 5 and 7 weeks after onset (Fig. 4).

Table. 1. Haemagglutination activity (HA) to chicken erythrocytes

Classical fimbriae (120 μg/ml)	8
in the presence of	
D−mannose	2
L−fucose	8

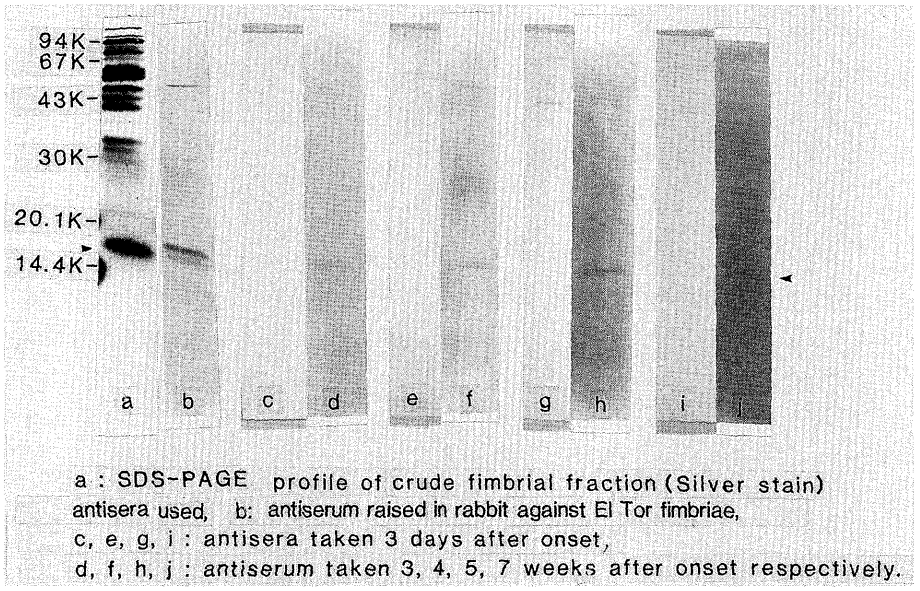

Fig. 4. Detection of the fimbrial subunit by convalescent antisera from cholera patients.

Table. 2. Amino acid compositions of the fimbriae purified from *V. cholerae*, 17−1 strain, classical biotype, Inaba serotype

Amino acid	Residues per molecule
Aspartic acid	10
Threonine	5
Serine	17
Glutamic acid	20
Proline	5
Glycine	26
Alanine	10
Valine	4
Methionine	1
Isoleucine	4
Leucine	6
Tyrosine	3
Phenylalanine	3
Histidine	5
Lysine	7
Arginine	8

The proportion of hydrophobic amino acids (proline, alanine, valine, methionine, isoleucine, leucine, and phenylalanine) was 24.6% in the fimbriae molecule of *V. cholerae* O1, classical biotype, Inaba serotype.

The calculated molecular weight was 16,189.

Amino acid composition: A quantitative amino acid analysis was performed on the fimbrial subunit purified by a preparative SDS−PAGE (Table 2). The amino acid composition was characterized by low concentrations of hydrophobic amino acids. Values of Ser and Gly residues may be overestimated due to contamination during the preparative SDS−PAGE.

Discussion

It is well known both from epidemiological studies of cholera in endemic areas (Ehara *et al.*, 1985, Glass *et al.*, 1982, Mosley, 1969, Woodward, 1971) and from volunteer studies (Cash *et al.*, 1974, Levine *et al.*, 1981) that a natural infection with *V. cholerae* O1 resulting in diarrhoea gives rise to long−lasting protective immunity against a second attack of cholera. A recent study by Lycke et al. (1986) using the RITARD model has also described a state of protective immunity which extends to strains of unrelated serotype and biotype. From the data obtained by a western blotting (Fig. 2 a) and by immunoelectron microscopy (Fig. 3 d) it is apparent that classical fimbrial subunit has a molecular weight of 16,000 and shares antigenicity independent of biotype and serotype. This study shows that the major antigenic determinants of fimbriae of classical vibrios are exposed on the sides of the fimbriae and easily detected by the antibody. It is noteworthy that the antibody against fimbriae was detected in the blood sera obtained from convalescent cholera patients. Rabbits parenterally immunized with El Tor fimbriae were protected from colonization of vibrios unrelated serotype and biotype (Ehara *et al.*, 1988). Our data mentioned as above suggest that the fimbriae of *V. cholerae* is one of colonization factors and highly antigenic, independent of biotype and serotype. Therefore, the fimbrial antigen of *V. cholerae* may be a suitable antigen to be analyzed in the blood sera of convalescent cholera patients and of healthy carriers in cholera endemic areas.

The relatively low hydrophobicity of the fimbriae molecule of classical vibrio may suggest that this protein interacts with epithelial cells by ionic interaction or with specific affinity to certain receptor and not by hydrophobic interaction.

The cell−associated haemagglutinin of classical vibrios has been reported to be sensitive to L−fucose and not to D−mannose (Hanne and Finkelstein). Our data show that purified fimbriae of classical vibrios are D−mannose−sensitive haemagglutinin (MSHA) and are not affected in the presence of L−fucose. This discrepancy may suggest the presence of different type of cell−associated haemagglutinin in classical vibrios or the presence of one type of filamentous haemagglutinin with different chemical modifications due to culture conditions. Furthermore, the low HA titer of purified fimbriae of a classical strain may suggest that native cell−associated haemagglutinins have different structure and function from purified ones.

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