AN APPROACH FOR BETTER VISUALIZATION OF CAPSULAR MATERIAL OF *Vibrio cholerae* O139 STRAINS

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ABSTRACT
Cholera is a diarrhoeal disease caused by *Vibrio cholerae* O1 or O139, synonym Bengal. The main differences between *V. cholerae* O139 and O1 are that the former possesses a capsular polysaccharide and a different lipopolysaccharide. Transmission electron microscopy (TEM) observations of ultrathin sections of cells stained with polycationic ferritin have been used to reveal the presence of capsule on *V. cholerae* O139. Considering that *V. cholerae* O139 strain CRC266 is intended to be used as the initial progenitor for constructing an oral vaccine candidate against cholera, the main purposes of this work were: (1) evaluate the use of ruthenium red staining to visualize the capsular material of *V. cholerae* O139 strains, (2) compare the results with those of polycationic ferritin staining and (3) analyze relevant phenotypic characteristics, including the expression of capsular antigen in *V. cholerae* O139 CRC266 strain. Control strains (O1 and O139 serogroups) were exposed to polycationic ferritin and ruthenium red, and CRC266 strain was stained only with ruthenium red. TEM of ultrathin sections of ruthenium red-stained bacterial cells revealed the presence of a dense capsular material surrounding cells of *V. cholerae* O139 strains. However, no such material was seen in O1 strain (negative control). The results obtained with ruthenium red staining correlate with those of polycationic ferritin. TEM demonstrated the usefulness of ruthenium red staining to study the presence of capsule in *V. cholerae* O139 strains. Characterization of CRC266 showed that it is a rod-shaped flagellated bacterium which produces both TCP and MSHA pili, as well as a thin capsule.

Keywords: *Vibrio cholerae* O139; capsule staining; ruthenium red, transmission electron microscopy.

PROPUESTA PARA UNA MEJOR VISUALIZACIÓN DE LA CÁPSULA DE CEPAS DE *VIBRIO CHOLERAE* 0139

RESUMEN
El cólera es una enfermedad diarreica causada por *Vibrio cholerae* O1 y O139, sinónimo Bengala. Estos serogrupos difieren en que el último posee una cápsula polisacarídica y en que sus lipopolisacáridos de superficie son diferentes. La microscopía electrónica de transmisión de cortes ultrafinos teñidos con ferritina policatiónica se ha empleado para revelar la presencia de cápsula en cepas de *V. cholerae* O139. Considerando que la cepa de *V. cholerae* O139 CRC266 será empleada como punto de partida para obtener una vacuna viva atenuada contra el cólera, los principales objetivos de este trabajo fueron: (1) evaluar el uso de la tinción con rojo de rutenio para visualizar el material capsular de cepas de *V. cholerae* O139, (2) comparar los resultados con los de la tinción de ferritina policatiónica y (3) analizar características fenotípicas relevantes, incluyendo la expresión del antígeno capsular, en la cepa de *V. cholerae* O139 CRC266. Las cepas controles (serogrupos O1 y O139), se tiñeron con ferritina policatiónica y rojo de rutenio, mientras que la CRC266 se tiñó solo con rojo de rutenio. Las imágenes de los cortes ultrafinos de las células bacterianas teñidas con rojo de rutenio revelaron la presencia de un material electrodenso rodeando las células de *V. cholerae* O139; sin embargo tal material no se observó en las muestras de la cepa de serogrupo O1 (control negativo). Los resultados obtenidos con el rojo de rutenio correlacionan con los de ferritina policatiónica. Los estudios de microscopía demostraron la factibilidad del empleo del rojo de rutenio para estudiar la presencia de cápsula en cepas de *V. cholerae* O139. La caracterización de la cepa CRC266 mostró que esta es una bacteria flagelada en forma de coma que produce los pelos TCP y MSHA, así como una cápsula fina.

Palabras clave: *Vibrio cholerae* O139; tinción de cápsula; rojo de rutenio, Microscopía Electrónica.

INTRODUCTION

Cholera is a worldwide disease with an estimated incidence of more than 500,000 cases per year, most of which occur in Asia and Africa, with a case-fatality rate of more than 30% among vulnerable groups living in high-risk areas [1]. Until 1992, only *Vibrio cholerae* strains of the O1 serogroup had been associated with epidemics of cholera. However, in that year, a new serogroup termed O139, synonym Bengal, was recognized as responsible for outbreaks in India and Bangladesh [2].

The main symptoms of cholera disease are associated to cholera toxin (CT). The genes encoding CT are actually encoded within a single-stranded filamentous phage, termed CTXΦ [3]. The toxin co-regulated pilus (TCP) is considered the most important colonization factor in toxigenic *V. cholerae* [4]. The TCP is a polymer composed of a single structural subunit, TcpA, which also serves as the receptor for the CTXΦ bacteriophage. On the other hand, the mannose sensitive hemagglutinin (MSHA) is another pilus which functions as the receptor of VGJΦ, a phage able to transduce, in a very efficient way, the complete genome of CTXΦ [5, 6]. This fimbria has also a role in biofilm formation on biotic and abiotic surfaces, contributing thus to the bacterial survival in the environment.

In those areas where cholera is endemic the adult population is significantly protected against cholera caused by *V. cholerae* of serogroup O1. This observation is due of naturally acquired immunity following repeated natural exposure. In contrast to the age-related incidence of O1 disease, the majority of O139-related cholera victims are adults, suggesting that the natural immunity to *V. cholerae* O1 afforded no protection against *V. cholerae* O139 [7].

V. cholerae O139 strains have been found to be biochemically and genetically similar to *V. cholerae* O1 of the El Tor biotype. However, they differ in the composition and lengths of the O-side chains of the cell wall lipopolysaccharide (LPS) and the presence of a capsular polysaccharide (CPS) which is only found in O139 strains [8]. This capsule comprises very long polymers of the same O-subunit found in the LPS, but it is not linked to the core structure. The O antigen of the LPS is considered the most important protective antigen in *V. cholerae* O1 and O139 [9]. Furthermore, encapsulated O139 vibrios have been found to colonize better than non capsulated mutants in the infant-mouse model, implying that both O139 LPS and O-antigen capsule are virulence determinants. However, the mechanisms behind the better colonization ability of encapsulated strains are still unknown [10].

Electron microscopy studies using polycationic ferritin staining have been used to reveal the presence of capsule on *V. cholerae* O139 [11]. Those studies have shown that the O139 isolates obtained in the year of 2002 have undergone changes in ribotype, antibiotic susceptibility patterns and capsule content when compared to those isolated from 1993 to 1995 [11].

Vaccines for the O139 serogroup have been developed, including both: (i) live oral O139 vaccines (Bengal 15, CVD112 and L911) and (ii) inactivated bivalent cholera vaccine comprising the O1/O139 serogroups [12-14]. However, previous attempts to develop live attenuated O1 cholera vaccines have been hampered by the fact that even after deletion of the genes encoding for the toxins, many vaccine candidates still retained a high rate of reactogenicity when evaluated in human volunteers. So far, an effective and protective vaccine against O139 vibrios is important and remains to be obtained.

V. cholerae O139 CRC266 strain is intended to be used as the initial progenitor for constructing an oral vaccine candidate against cholera [15].

The main purposes of this work were: (1) evaluate the use of ruthenium red staining to visualize the capsular material of *V. cholerae* O139 strains, (2) compare the results with those of polycationic ferritin staining and (3) analyze relevant phenotypic characteristics, including the expression of capsular antigen in *V. cholerae* O139 CRC266 strain.

MATERIALS AND METHODS

Strains and media

V. cholerae O1 El Tor strain C7258 and the *V. cholerae* O139 reference strain MO45 (ATCC 51394, Rockville, MD, USA) were used as negative and positive controls of capsule production, respectively. CRC266 of *V. cholerae* O139 was isolated from cholera patients in Calcutta, India in the year of 2000 and was kindly supplied by Dr. G. Balakrish Nair, ICDDR, Bangladesh. Bacterial strains were grown in Luria Bertani (LB) medium at 37^{o}C, overnight, unless otherwise indicated.

TcpA and mshA analysis

CRC266 and C7258 strains were grown on blood agar plates at 37^{o}C for 18h. Afterwards, a short-time AKI culture procedure was performed in order to reach permissive conditions for TcpA production. Briefly, a 10-ml culture in freshly prepared AKI medium (1.5% Peptone, 0.4% yeast extract [Oxoid], 0.5% NaCl, 0.3% NaHCO3) was initially grown in a test tube standing still for 3h. Next, it was transferred to a flask and was incubated with shaking for 2.5h. Bacteria from short-time AKI cultures, grown at 37^{o}C, were analyzed for TcpA by SDS-PAGE and immunoblotting as described previously [16]. The protein bands were visualized by Coomassie blue staining as well as by immunoblotting using the monoclonal antibody anti-TCP 10E10E1 [16]. Bacteria were also examined for production of MSHA pili by Western blotting using anti-MSHA MAb 2F12/F1, as described previously [17].

Analysis of cell surface polysaccharides

Cell surface polysaccharides from whole-cell lysates with proteinase K digestion were analyzed by electrophoresis on 15% sodium dodecyl sulfate polyacrylamide gels. CPS and LPS O side chain expression were assessed by Western blot analyses. Samples were electrophoretically transferred to nitrocellulose membranes and probed with anti-O139 Mab O139-1:1:9 [9].

Electron Microscopy studies

For ultrastructural studies, *V. cholerae* strains CRC266, MO45 and C7258 were grown on Luria agar plates. Bacteria were harvested, washed with phosphate saline solution and resuspended in cacodylate buffer (0.1 M, pH 7.4). Negative and positive control strains were exposed to polycationic ferritin and ruthenium red, and CRC266 strain was stained only with ruthenium red during processing for resin embedding as follows.

For polycationic ferritin staining, cells were fixed in 5% glutaraldehyde for 2 h, exposed to polycationic ferritin (1 mg/ml) in sodium cacodylate buffer (0.1 M, pH 7.4) during 30 min, postfixed in 2% osmium tetroxide for 2 h, dehydrated in ethanol and embedded in Spurr resin, as described by Johnson et al, 1992. Ultrathin sections were analyzed using a JEOL JEM 100S TEM.

For ruthenium red staining, cells were left overnight in 2.5% glutaraldehyde and ruthenium red (5 mg/ml) in sodium cacodylate buffer 0.1 M, pH 7.4, washed in ruthenium red (2.5 mg/ml) in the same buffer, post-fixed for 3h in 1% OsO_4 and ruthenium red (5 mg/ml) in the buffer. Dehydration was performed in steps of 15 min of acetone 25% and ruthenium red (5 mg/ml), acetone 50% and ruthenium red (3,75 mg/ml) and acetone 75% and ruthenium red (2 mg/ml). Finally, the pellets were dehydrated in higher concentrations of acetone without ruthenium red and embedded in Araldite resin. Ultrathin sections were analyzed using a JEOL 1210 TEM. TEM

images were captured using a Megaview G2 digital camera (Olympus; Muster, Germany).

To verify the presence of flagella *V. cholerae* CRC266 strain was grown for 3h at 37^{o}C and bacteria were negatively stained with 1% (w/v) uranyl acetate for 3 min and analyzed by a JEOL JEM 100S TEM.

RESULTS AND DISCUSSION

Although *V. cholerae* O139 emerged in Bangladesh in the year of 1992, the incidence of cholera caused by this serogroup fell and subsequently remained low for about a decade. The new serogroup remained, however, as a threat in several countries. The factors that determine the emergence, disappearance or continued existence of particular variants of *V. cholerae* O139 are not clear, but several epidemiologic studies have suggested that the O139 serogroup is likely to spread over a period of time to other cholera endemic areas in the world [18].

We have previously examined different genotypic characteristics of *V. cholerae* O139 CRC266 strain to evaluate its potentiality to be used as parental strain to construct a live attenuated vaccine candidate [15]. In the present study, we investigated the production of bacterial surface antigens and potential targets of antibacterial antibodies, including MSHA, TCP, flagellum and CPS, using different methods.

The expression of MSHA and TCP in CRC266 was demonstrated by SDS-PAGE followed by immunoblotting with specific antibodies. MshA subunits of CRC266 (Fig. 1A, lane 1) were of identical sizes, ca. 17 kDa, as El Tor MshA, represented by C7258 strain (Fig. 1A, lane 2). O139 TcpA (Fig. 1B, lane 1) showed the same reactivity with anti-TCP antibodies in SDS-PAGE and immunoblotting as that found for El Tor TcpA of C7258 (Fig. 1B, lane 2).

In negatively-stained preparations of CRC266, the typical comma-shaped bacterium with a flagellum, which extends from the pole of the bacterium, was observed by TEM (Fig. 2).

The most striking difference between O1 and O139 vibrios is the presence of a capsule in the latter strain. Visualization of such material by TEM following conventional preparation is very difficult, since it may collapse during dehydration steps. Polycationic dyes such as ruthenium red or polycationic ferritin have been widely used to stain bacterial acid exopolisaccharides, due to their ability to enhance stabilization and/or staining of acidic glycosylated structures at the ultrastructural level.

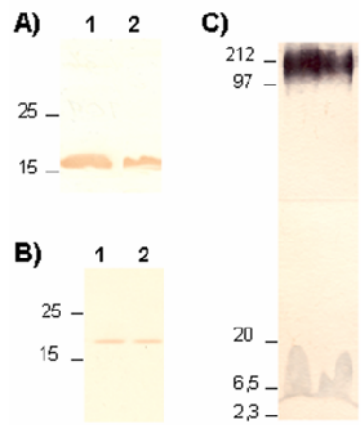


Fig. 1. Immunoblot analysis of whole cell of *Vibrio cholerae* strains, probed with A) Mab anti-MshA and B) Mab anti-TCP; lane 1, *V. cholerae* O139 CRC266 and lane 2, *V. cholerae* O1 C7258. C) *V. cholerae* O139 MO45 lysates and Mab anti-0139 LPS as probe. Relevant molecular weight markers are shown on the left in kb.

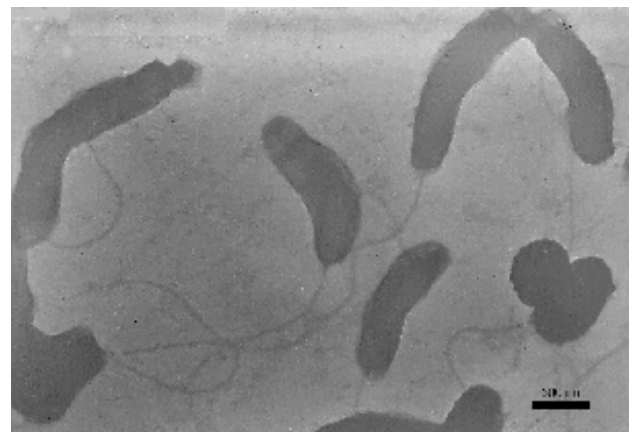
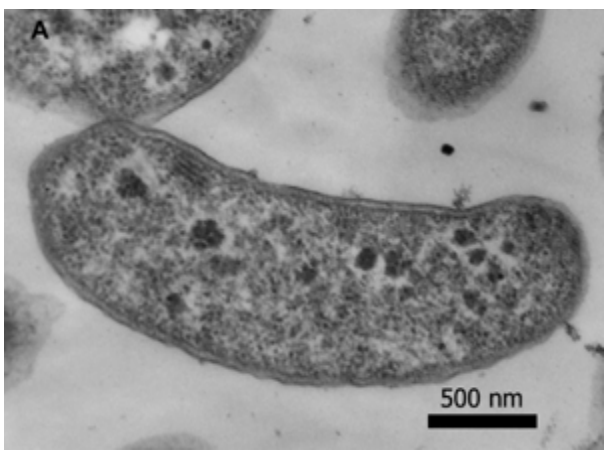


Fig. 2. Transmission electron micrograph of the flagellated, wild-type *V. cholerae* strain CRC266 negatively stained with uranyl acetate.

Ruthenium red has been used to visualize the capsular material of several microorganisms, such as *Vibrio vulnificus, Klebsiella pneumoniae* and *Pseudomona aeruginosa* [19]. Cationized ferritin has also been used to delineate the capsule of various microorganisms, as *Klebsiella sp*., *Pasteurela multocida* and others [20]. In previous studies, ultrathin sections of polycationic ferritin stained *V. cholerae* 0139 bacteria have been used to visualize the capsule [11, 21, 22], but to our knowledge, no studies have evaluated the use of ruthenium red staining to this end.

We examined *V. cholerae* O139 strain MO45 for the presence of capsule, as it is a reference strain for this serogroup. Firstly, LPS from MO45 was prepared from whole cell lysates digested with proteinase K. SDS-PAGE gels were immunoblotted with MAbs against O139 LPS. As shown in Fig. 1C, the MAbs reacted with O139 LPS and also with higher-molecular-weight material, which corresponds to the capsule, an expected result, considering that bacteria were grown on LB agar to optimize CPS production. This result is in conformity with previous observations that the CPS and LPS contain shared epitopes [9].



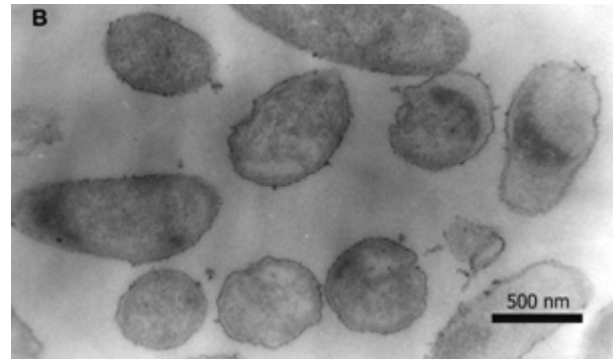
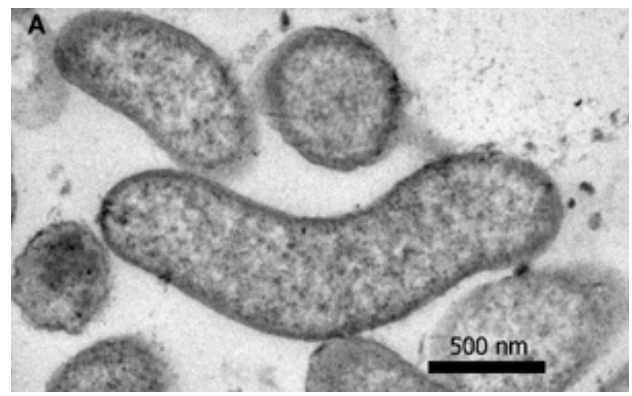


Fig. 3. Transmission electron microscopy of ferritin-labelled *V. cholerae* strains. A) C7258, non-capsulated control, B) capsulated O139 reference strain MO45. Note in figure B that ferritin particles are visible around the cell periphery of the O139 strain MO45, indicating the presence of capsule.

Later on, the presence of a capsule was morphologically confirmed by electron microscopy, using polycationic ferritin. The strain C7258 which does not present capsule was used as negative control (Fig. 3A), whereas MO45 was used as positive control (Fig. 3B). As shown in Fig. 3, ferritin particles were only visible over the cell periphery of MO45 strain, confirming the presence of a capsule; no such material was observed in the C7258 cells.

Electron microscopy observations of ultrathin sections of ruthenium red-stained bacteria revealed the presence of a dense capsular material surrounding cells of *V. cholerae* O139 strain MO45, whereas this material was not present in preparations of El Tor strain C7258 (Fig. 4).



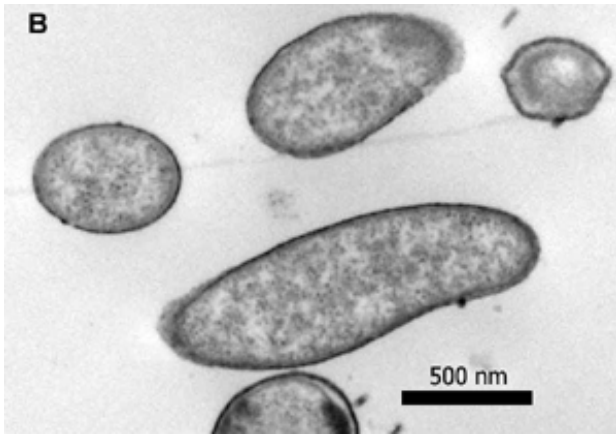


Fig. 4. TEM images of ultrathin sections of ruthenium red stained cells of *V. cholerae*. A: C7258, non-capsulated control, B: capsulated O139 reference strain MO45. Note in figure B the presence of a well-defined electron dense layer around cell periphery of the Bengal strain, indicating the presence of a capsule

The results obtained with ruthenium red showed a good correlation with those of polycationic ferritin staining, and this outcome prompt us to use the former staining method for visualization of the capsule in CRC266 strain. TEM observations demonstrated the presence of a thin capsule in this wild type strain and confirmed the usefulness of ruthenium red staining to visualize a capsule in *V. cholerae* O139 strains (Fig. 5).

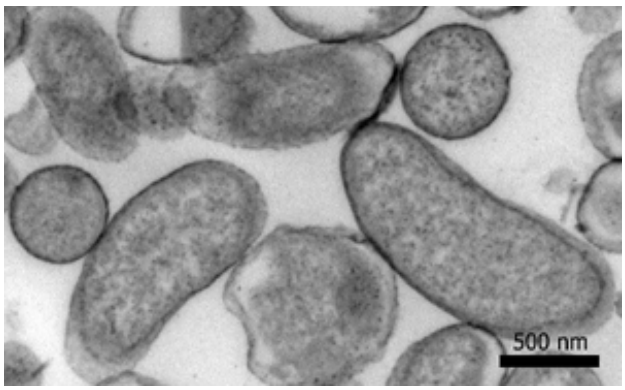


Fig. 5. TEM image of ultrathin sections of ruthenium red stained cells of *V. cholerae* O139 CRC266.

CONCLUSIONS

The results here obtained suggest that ruthenium red would be a good cationic probe, as useful as polycationic ferritin, for stabilization and visualization of capsular material of *V. cholerae* O139 strains. In addition, CRC266 is a rod-shaped flagellated bacterium which produces both TCP and MSHA pili, as well as a thin capsule. The strategy for obtaining live attenuated vaccine candidates from this strain has to consider those traits.

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