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Original article

Biochemical and full genome sequence analyses of clinical *Vibrio cholerae* isolates in Mexico reveals the presence of novel *V. cholerae* strains

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Abstract

The first week of September 2013, the National Epidemiological Surveillance System identified two cases of cholera in Mexico City. The cultures of both samples were confirmed as *Vibrio cholerae* serogroup O1, serotype Ogawa, biotype El Tor. Initial analyses by PFGE and by PCR-amplification of the virulence genes, suggested that both strains were similar, but different from those previously reported in Mexico. The following week, four more cases were identified in a community in the state of Hidalgo, located 121 km northeast of Mexico City. Thereafter a cholera outbreak started in the region of La Huasteca. Genomic analyses of the four strains obtained in this study confirmed the presence of Pathogenicity Islands VPI-1 and -2, VSP-1 and -2, and of the integrative element SXT. The genomic structure of the 4 isolates was similar to that of *V. cholerae* strain 2010 EL-1786, identified during the epidemic in Haiti in 2010.

Keywords: Cholera; *Vibrio cholerae*; Phylogenomics; Disease control; PFGE

1. Introduction

Cholera is an acute intestinal disease caused by the Gram-negative *Vibrio cholerae* bacterium. It is characterized by severe diarrhea and dehydration that can lead to death within 48 h if not treated properly [1]. Cholera is endemic in more than 50 countries and is estimated that each year between 3 and 5 million cases occur with 120,000 deaths each year [2].

The magnitude of an epidemic caused by *V. cholerae* has been shown recently in Haiti, where over 700,000 cases were reported with 8700 deaths since the beginning of the epidemic in October 2010. It is estimated that in the most remote areas, up to 5% of the population could have died in the first months of the outbreak [3].

In Mexico, between 1991 and 2001 (during the 7th pandemic) 45,062 cases of cholera were confirmed affecting 97% the country with a fatality rate of 1.1% [4]. Since then, sporadic cases of infection by *V. cholerae* serogroup O1 have occurred. There was only one case in 2010, one in 2011 and two in 2012, all in the northwest state of Sinaloa. The first two cases were caused by *V. cholerae* O1 serotype Inaba, and

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the other two by *V. cholerae* O1 serotype Ogawa. These strains were characterized and found to be identical to those strains previously identified in Mexico in the 1991–2001 period [4].

V. cholerae is classified in more than 200 serogroups based on the O antigen present in the polysaccharides of the cell wall, however, so far only serogroups O1 and O139 cause cholera outbreaks. *V. cholerae* O1 is classified into two biotypes: Classical and El Tor, so named because it was first isolated in El Tor, Egypt in 1905 [1]. There are mainly two serotypes: Ogawa and Inaba. The complexity of the rapid identification of the causative agent of cholera has become more difficult by events of lateral gene transfer as it happened in 1992 in Southeast Asia where the causative agent of the epidemic was for the first time identified as *V. cholerae* O139, a strain derived from *V. cholerae* O1 El Tor that suffered a replacement of a pathogenicity island by lateral gene transfer resulting in the substitution of the O1 by the O139 antigen, but everything else identical to *V. cholerae* O1 El Tor [2]. It is noteworthy that of the seven reported pandemics, the first six were caused by *V. cholerae* O1 classical and 7th pandemic was caused by *V. cholerae* O1 El Tor. The identification of the *V. cholerae* strain causing an outbreak is further complicated by the presence of DNA sequences coding for antibiotic resistances; such is the case of the SXT element conferring streptomycin and cotrimoxazole resistance; this element has been identified in almost all strains of *V. cholerae* El Tor in the last ten years. Recent studies have reported that the SXT element has been modified and acquired resistance to different antibiotics such as tetracycline, erythromycin and ciprofloxacin, this has been described in *V. cholerae* O1 strains in Southeast Asia [5]. The major determinant of virulence is the cholera toxin encoded by the *ctxAB* genes in bacteriophage ϕCTX found in the genome of toxigenic *V. cholerae*. This toxin, along with other factors encoded in clusters called pathogenicity islands, are responsible for the serious effects of infection caused by *V. cholerae* [2].

Here we report on the study of 175 cases of cholera reported in the region of La Huasteca, (a center-east part of Mexico). Molecular Genomic analyses demonstrated that in all cases, the infectious agent was a *V. cholerae* toxigenic, serogroup O1, biotype El Tor, serotype Ogawa strain different to those previously characterized from 1991 to 2001 in Mexico and similar to those circulating in the Caribbean but clearly different as suggested by our phylogenomics results. For the first time we combined a strategy that involved biochemical and genomic molecular methods for unequivocally identify in a short period of time the causal agent of a potential case of cholera, that in this outbreak was a strain not previously found in Mexico. This report demonstrates that molecular epidemiology is an essential tool that provides valuable information to establish actions to speed disease control to avoid the spread of the agent. These advances are critical if we consider that the last pandemic in Mexico began in 1991 and was under control until 2001.

2. Material and methods

2.1. Sampling, media and culture conditions

Samples of patients suspected of being infected with *V. cholerae* were obtained by rectal swab or stool. The protocols described in the Manual of Laboratory Methods for the Diagnosis of Epidemic Dysentery and Cholera of the Center for Disease Control and Prevention, (2004, Atlanta GA, USA) were followed, briefly, samples of fresh faeces were collected with cotton swabs and introduced into a tube with Cary Blair transport medium (0.11% Disodium hydrogen phosphate, 0.15% Sodium thioglycollate, 0.5% Sodium chloride, 0.0009% Calcium chloride, 0.56% agar, pH 8.4). The swab was placed in a test tube containing Alcaline Peptone medium (AP, Peptone 1%, NaCl 1%, pH 8.5) and incubated at 37 °C for 6–8 h. Cells were then collected using a sterile loop and streaked on Thiosulfate Citrate Bile salts Sucrose agar (TCBS, Yeast Extract 0.5%, Peptone 1%, Sodium thiosulfate 1%, Ox gall 0.5%, sodium collate 0.3%, Sucrose 2%, NaCl 1%, Ferric citrate 0.1%, Bromothymol blue 0.004%, Agar 1.5%) and incubated at 37 °C for 18–24 h.

2.2. Biochemical characterization

Sucrose positive colonies (yellow colonies, about 2 mm flat generally sticky) and/or sucrose negative (green colonies, about 2 mm, usually sticky) were selected. Selected strains were grown on arginine broth tubes (AB, Base Moeller decarboxilase and L-arginine) and Peptone broth (2% peptone, 0.5% NaCl), or streaked on Motility Indole Ornithine (MIO) agar, triple sugar iron (TSI) agar or lysine iron agar (LIA) with the same colony of bacteria grown from the above observation. Nutrient broth was supplemented with 0%, 1%, 3%, 6%, 8% and 10% NaCl. The biochemical identification of *V. cholerae* was detected as previously described [6].

2.3. Serotyping

It was performed using colonies grown on a blood agar plate. Four drops of formaldehyde saline solution were placed at the upper end of the slide, at the lower end were placed a drop of saline (negative control), a drop of polyvalent serum *V. cholerae* O1, a drop of serum monovalent O1 *V. cholerae* Ogawa and one drop of serum monovalent *V. cholerae* O1 Inaba.

2.4. Cholera toxin production

To determine the production capacity of the cholera toxin, an ELISA assay using culture supernatants of the strains described above was performed as previously reported [7].

2.5. Biotypes

To determine their biotype (El Tor or Classic), strains were grown on Blood agar added with polymyxin B, the inhibition