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SHORT COMMUNICATION

Comparison of DOT-ELISA and Standard-ELISA for Detection of the *Vibrio cholerae* Toxin in Culture Supernatants of Bacteria Isolated from Human and Environmental Samples

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Abstract A comparison of DOT-ELISA and Standard-ELISA was made for detection of *Vibrio cholerae* toxin in culture supernatants of bacteria isolated from human and environmental samples. A total of 293 supernatants were tested in a double blind assay. A correlation of 100 % was obtained between both techniques. The cholera toxin was found in 20 Inaba and 3 Ogawa strains. Positive samples were from seafood (17 samples), potable water (1 sample) and sewage (5 samples). The DOT-ELISA was useful as the standard-ELISA to confirm the presence of cholera toxin in the environmental samples.

Keywords Cholera · Mexico · DOT-ELISA

Introduction

Vibrio cholerae is the etiological agent of epidemic cholera. The serogroups O1 and O139 have been associated with large epidemic outbreaks, the former clusters the serotypes Inaba and Ogawa. The serogroups referred as non-O1 and non-O139 have been associated with occasional outbreaks of cholera-like disease and they can be abundantly isolated from aquatic and estuarine sources [1]. Since clinical cases of severe watery diarrhoea are found to be associated with the isolation of toxin producing bacteria, the identification of cholera toxin is an important step in the diagnosis of cholera disease. Indeed, the detection of cholera toxin has been evaluated using animals models [2], cell lines, immunoassays and molecular-based assays [3–5]; most of these techniques are time-consuming and the mRNA synthesis not always correlates with protein expression. Because the only way to confirm the presence of the cholera toxin is by mean of laboratory diagnosis, the aim of this work was to compare DOT-ELISA and Standard-ELISA for detection of the *Vibrio cholerae* toxin in supernatants of bacteria isolated from human and environmental samples.

Vibrio cholerae bacteria were obtained through the national permanent epidemiological survey to identify the prevalence of cholera in Mexico. Isolation was made from human faeces, food, sewage or drinking water in local laboratories. Serogroups were characterized using biochemical tests and serologically confirmed by slide agglutination using specific polyvalent antisera to *V. cholerae* O1 and O139. Serotypes were characterized using polyvalent O1 and monovalent Inaba and Ogawa antisera [6]. Bacteria were propagated in heart infusion agar during 18 h at 37 °C and then transferred to Craig medium and incubated for 48 h at 30 °C. The culture medium was centrifuged at 503×*g* for 10 min and the supernatant was separated and stored at 4 °C until use. Supernatants were divided in two groups. Group 1 was constituted by 46 supernatants of cholera toxin producing bacteria (22 of Inaba and 24 of Ogawa) and 21 of not producer (non-O1). Group 2 was constituted by 226 supernatants of bacteria which condition of cholera toxin producing remained

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unknown at the moment of the study. Supernatants were 51 of Inaba, 44 of Ogawa, 119 of non-O1, 11 of O139 and 1 of O1 rough strain. The supernatants from VC13 O1 Inaba, VC12 O1 Ogawa, VC O139 and VC Non-O1 were used as reference strains. To obtain hiperimmune serum against the cholera toxin, three adult albino rabbits of 2.5 ± 0.1 kg weight were subcutaneously immunized during six weeks, once a week with 0.1 mg of cholera toxin (Sigma-Aldrich, St. Louis, MO, USA) with incomplete Freunds adjuvant. IgG antibodies were isolated by affinity chromatography and conjugated to biotin [7]. The cholera toxin was determined in the supernatants by a standard-ELISA. Briefly, microtiter plates (Corning-Costar, Bloomington, MN, USA) were coated with 0.002 mg/well GM1 ganglioside (Sigma-Aldrich, St. Louis, Missouri, USA) diluted in 0.015 M phosphate-buffered, pH 7.2 (PBS) for 1 h at 37 °C and then incubated overnight at 4 °C. After washing with 0.05 % tween 20 in PBS, the plate wells were blocked with 1 % bovine serum albumin (BSA) in PBS during 30 min at 37 °C. Bacteria supernatants were tested by triplicated as the routine diagnosis work using 0.1 mL/well and incubated for 1 h at 37 °C. At this point, a number of wells were supplied with supernatant from no-toxin producing bacteria and then, 0.1 mL of cholera toxin (0 to 0.1 mg/mL) in PBS was added to create a dose–response curve. The supernatants of *V. cholera* reference strains were used as positive controls. The rabbit IgG anti-cholera toxin (1 mg/mL) was added 1:2000 in BSA and incubated 1 h at 37 °C. Goat anti-rabbit IgG alkaline phosphatase conjugate (Life Technologies, Carlsbad, CA, USA) diluted 1:1000 in BSA was added and incubated 1 h at 37 °C. Finally, 0.1 mL/well of 1 mg/mL p-nitrophenyl phosphate (Sigma-Aldrich) in diethanolamine buffer, pH 9.8 was added during 30 min. The enzyme reaction was stopped with 0.05 mL/well of 3 M NaOH. Absorbance values were read at 405 nm. The cutoff was the average absorbance plus three times the standard deviation obtained with the 21 supernatants of no-toxin producing bacteria. In order to determine the optimal conditions to perform the dot-ELISA, several concentrations of anti-cholera toxin (0.001 to 0.10 mg/circle) to coat the nitrocellulose membrane were tested. In addition, the optimal time and conditions of incubation of reagents and the best dilution of immunological conjugate were also determined. The best conditions identified were as follow: six milimeters circles of nitrocellulose membrane were coated with 0.003 mg/circle of IgG anti-cholera toxin and then dried during 30 min at 37 °C and washed with 0.2 mL of 0.3 % Tween 20 in PBS (PBS-T). The circles were blocked with 0.5 mL/well of 5 % skimmed milk in PBS-T (SM-T) during 15 min at room temperature. Supernatants were added (0.5 mL/circle) and incubated 1 h at 37 °C. Each supernatant was tested by triplicate. At this point, a number of circles were added with 0.2 mL of cholera toxin (0 to 0.1 mg/mL) diluted in PBS to create a dose–response curve. A total of 0.5 mL of anti-cholera toxin biotin conjugate (1 mg/mL) diluted 1:50 in SM-T was added and incubated 30 min at room temperature. Afterwards, a conjugate of avidin and horseradish peroxidase was added 1:500 in SM-T and incubated 30 min at room temperature. The colour reaction was developed with 0.5 mg/mL 3,3′diaminobenzidine and 0.003 % hydrogen peroxide in PBS. After 10 min of incubation at room temperature, the reaction was stopped with distilled water. A brown dot in the circle was considered as a positive reaction while, the colourless were

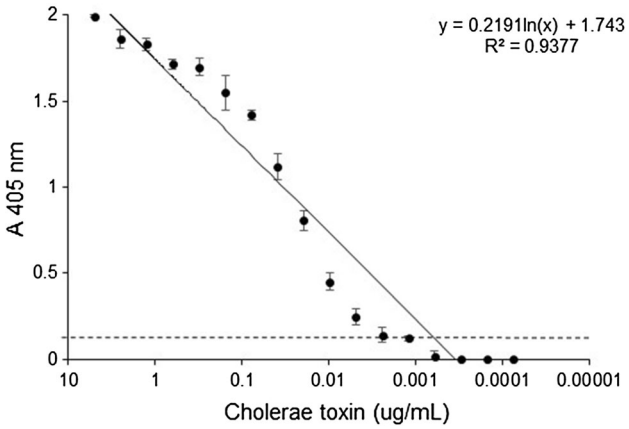

Fig. 1 Analytic sensitivity of a standard ELISA to determine the cholera toxin in supernatants of *Vibrio cholerae* bacteria. Microtiter plates were coated with GM1 ganglioside and after washing and blocked, a number of wells were supplied with supernatant of no-toxin producing bacteria and then, cholera toxin (0 to 0.1 mg/mL) was added to create a dose–response curve; each concentration was tested by triplicate. The reaction was developed with a rabbit IgG anti-cholera toxin and Goat anti-rabbit IgG alkaline phosphatase conjugate. The *horizontal dotted line* is the cut off obtained with 21 supernatants of no-toxin producing bacteria. Data of linear regression is show at *right*

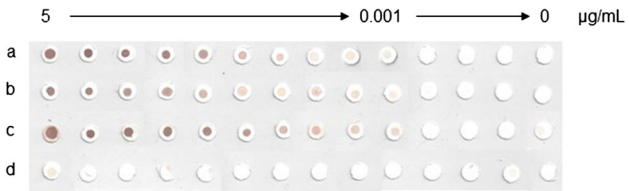

Fig. 2 Analytic sensitivity of a DOT-ELISA to determine the cholera toxin in supernatants of *Vibrio cholerae* bacteria. Circles nitrocellulose membrane were coated with IgG anti-cholera toxin. After blocked with skimmed milk, a number de circles were incubated with the cholera toxin (0 to 0.1 mg/mL) and supernatants of no-toxin producing bacteria to create a dose–response curve. The reaction was developed with an anti-cholera toxin biotin conjugate and avidin-horseradish peroxidase The colour reaction was developed with 3,3′diaminobenzidine and a *brown dot* in the circle was considered as a positive reaction while, the colourless were considered as negative. Results of three different supernatants are show in *a*,*b* and *c* lines, while a supernatant without cholera toxin is show in *d* line

Table 1 *Vibrio cholerae* bacteria serotype and source isolation analysed to determine the cholera toxin in the culture supernatants

Serogroups						Total	Source of isolation					Total
	O1			O139	Non-O1		W	S	F	H	ND	
	I	O	R									
BCS	0	0	0	0	1	1	0	1	0	0	0	1
CAM	2	9	0	0	0	11	6	3	0	2	0	11
CHP	0	1	0	0	0	1	1	0	0	0	0	1
DIF	1	0	0	0	5	6	0	0	0	6	0	6
GRO	0	0	0	0	2	2	2	0	0	0	0	2
HID	0	4	0	6	3	13	4	5	2	2	0	13
JAL	1	0	0	2	4	7	0	0	5	2	0	7
MIC	3	0	0	0	2	5	0	3	2	0	0	5
NAY	26	4	0	1	86	117	9	10	45	0	53	117
NLE	1	16	0	0	4	21	0	21	0	0	0	21
OAX	0	1	0	0	0	1	0	1	0	0	0	1
PUE	0	2	0	0	1	3	0	0	2	1	0	3
QTO	0	0	0	0	2	2	0	2	0	0	0	2
ROO	0	0	0	0	1	1	0	1	0	0	0	1
SIN	1	0	0	0	0	1	0	1	0	0	0	1
SON	5	0	0	0	3	8	0	9	0	0	0	9
TAB	0	2	1	0	1	4	3	0	0	0	0	3
TAM	1	5	0	0	2	8	0	2	2	4	0	8
TLA	0	0	0	2	0	2	0	2	0	0	0	2
VER	10	0	0	0	0	10	0	5	3	2	0	10
YUC	0	0	0	0	2	2	2	0	0	0	0	2
Total	51	44	1	11	119	226	27	66	61	19	53	226

Analysed serotypes were Inaba (I), Ogawa (O) and O1 rough (R). The source of isolation was potable water (W), sewage (S), seafood (F) or human faeces (H). ND = data not available. The names of the Federal States were simplified according to the ISO 3166-2, International Standard for country codes and codes for their subdivisions

Table 2 Number and source of isolation of *Vibrio cholerae* O1 serotypes toxin producing

	Serotypes		Total
	Inaba	Ogawa	
Potable water	1	0	1
Sewage	2	3	5
Seafood	17	0	17
Human faeces	0	0	0
Total	20	3	23

considered as negative. The kappa coefficient was used to compare the immunoassays [8].

The analytic sensitivity of both the standard-ELISA (Fig. 1) and the DOT-ELISA (Fig. 2) was of 0.001 μg/mL. The concentration of cholera toxin was similar in the Inaba and Ogawa serotypes. The 22 Inaba and the 24 Owaga serotypes were positive while, the 21 Non-O1 were negative (kappa index = 1). To compare the immunoassays, 226 supernatants of *Vibrio cholerae* with unknown condition for toxin producing were analysed. Bacteria were obtained from 21/32 Federal States of Mexico; 51.8 % were from Nayarit, 9.3 % from Nuevo León and the last 38.9 % were distributed between the other 19 States. The samples were 22.6 % of Inaba, 19.5 % of Ogawa, 4.9 % of O139, 53 % of Non-O1 and 1 sample was O1-rough (Table 1). Samples were obtained of potable water (11.9 %), sewage (29.2 %), seafood (27 %) or human faeces (8.4 %); data on 23.5 % of samples were unavailable. Cholera toxin was determined in 23 supernatants (Table 2). A general prevalence of 10.2 % was observed although it is worthy to say that all positive samples were from the State of Nayarit; thus, the local prevalence could be recorded as 19.7 %. The Inaba serotype and the seafood were the factors where the cholera toxin was predominant (8.8 and 7.5 %, respectively). No cholera toxin producing bacteria were identified in human faeces.

In a cholera outbreak as well as other epidemic disease, the time of analysis of samples is a primordial factor to establish the appropriate control measures in the shortest possible time. Indeed, there is an urgent requirement of

biomarkers for rapid identification and, a number of strategies with this propose has been tested, including the genome analysis in silico [9]. Nevertheless, the use of the immunoassays is not discarded due to its versatility. Here, we made a comparison of DOT-ELISA and Standard-ELISA for detection of *Vibrio cholerae* toxin in culture supernatants of bacteria isolated from human and environmental samples. The implications of the DOT-ELISA here reported would be associated as a screening test during outbreaks or epidemic studies, since the execution time is of 3 h in contrast to the 24 h of working with the ELISA; even, the cost of the DOT-ELISA is 10 times cheaper than the ELISA and, the results can be interpreted at naked eye, which minimizes the use of an ELISA reader. A number of rapid diagnostic tests for *Vibrio cholera* in human faecal samples have been described [10] and, since routine microbiological and biochemical analyses need 3 working days for cholera diagnosis, some DOT-ELISAs have been described, proving useful in determine outer membrane proteins and O1 and O139 antigens in faeces [11], rectal swabs [12] and bacteria of environmental samples [13]. Analytical sensibility has been reported between 2 and 10 ng/mL with an execution timing of 2–3 h [14]. However, to our knowledge, this is the first report of a DOT-ELISA, where the cholera toxin is captured between two polyclonal antibodies where the reaction was amplified by the avidin–biotin system; thus, the DOT-ELISA here described had an analytical sensitivity of 1 ng/mL. Data here reported suggest that the Inaba serotype and the seafood were the factors where the toxin producing bacteria were predominant. Actually in Mexico, the analysis of environmental samples suggests a permanent circulation of toxin producing bacteria [15–17]; thus, the DOT-ELISA here described could be a very useful rapid diagnostic tests designed to enhance a rapid response in epidemiological conditions.

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