Revista Latinoamericana de Microbiología **(2000) 42:63-68**
Asociación Latinoamericana de Microbiología

Detection of *Vibrio cholerae* O:1 in Oysters by the Visual Colorimetric Immunoassay and the Culture Technique

Sergio Villalpando-Guzmán, M. Guadalupe Eusebio-Hernández*, and David Avilés-Ruíz

Departamento de Microbiología, Escuela Nacional de Ciencias Biológicas, IPN. Prolongación de Carpio y Plan de Ayala, S/N, Col. Casco de Santo Tomás, 11340 México, D.F., Mexico. Tel. (5) 729 60 00 ext. 62374, Fax (5) 7 29 62 07. E-mail: svillalpando71@hotmail.com

*Corresponding author and COFAA fellowship holder

Received 8 September 1999/Accepted 13 April 2000

ABSTRACT. The Visual Colorimetric Immunoassay (Cholera SMARTTM) [NEW HORIZONS DIAGNOSTICS] is among the quick diagnostic techniques developed during recent years for the direct detection of *Vibrio cholerae* O:1 in fecal material. In this work, this immunoassay was used together with the culture technique (Laboratorios Nacionales de Salud Pública e Instituto Nacional de Diagnóstico y Referencia Epidemiológicos de la SSA. de México) to detect *V. cholerae* O:1 in 50 oyster samples. The samples were collected in Mexico City, from markets and roadside stands, during the period from October 1994 to March 1995. Of the 50 samples analyzed, only one was found positive by both techniques. These results indicate that the Cholera SMARTTM [NHD] kit represents a quick and simple technique for the detection of *V. cholerae* O:1 in oysters. Apart from looking for the *V. cholerae* O:1, various members of the Vibrionaceae family were isolated and identified from the same oyster samples. The findings were as follows: *V. cholerae* NO O:1 (26 %), *V. parahaemolyticus* (8%), *V. alginolyticus* (46 %), other species of the *Vibrio* spp. genus (6 %) and species from the *Aeromonas* spp. genus (12 %).

RESUMEN. Dentro de las técnicas rápidas de diagnóstico desarrolladas en los últimos años para la determinación directa de *Vibrio cholerae* O:1 en materia fecal está el Inmunoensayo Colorimétrico Visual (Cholera SMART™) [NEW HORIZONS DIAGNOSTICS]. En el presente trabajo se aplicó este Inmunoensayo junto con la técnica de cultivo (Laboratorios Nacionales de Salud Pública e Instituto Nacional de Diagnóstico y Referencia Epidemiológicos de la SSA de México) para la determinación de *Vibrio cholerae* O:1 en 50 muestras de ostiones. Las muestras fueron obtenidas en mercados y puestos callejeros de la Ciudad de México, en el período comprendido de octubre de 1994 a marzo de 1995. De las 50 muestras analizadas solamente una fue positiva por ambas técnicas. En función de los resultados obtenidos, se considera que el Cholera SMART™ [NHD] funciona como una técnica rápida y sencilla para la determinación de *Vibrio cholerae* O:1 en ostiones. Además de la búsqueda de *V. cholerae* O:1, también se aislaron e identificaron en las muestras estudiadas varios miembros de la familia *Vibrionaceae*, encontrándose *V. cholerae* NO O:1 (26 %), *V. parahaemolyticus* (8%), *V. alginolyticus* (46 %), otras especies del género *Vibrio* spp. (6 %) y especies del género *Aeromonas* spp. (12 %).
Palabras clave: *Vibrio cholerae*, Inmunoensayo colorimétrico.

INTRODUCTION

Vibrio cholerae O:1 is the causal agent of cholera, which is a disease that continues to be prevalent in many developing countries.[5,6,11,19] The strains of *V. cholerae* belonging to the O:1 serogroup have been classified into three serotypes: Ogawa, Inaba and Hikojima. These serotypes contain the classical and ElTor1 biotypes[15]. In Peru, in 1991, 107,064 cases of this disease were registered, and in Mexico, during the same year, the first case within the same pandemia, was reported.[11,19] Transmission of cholera occurs by the inges tion of water or food contaminated with enterotoxigenic *V. cholerae* O:1 (17). The groups of foods commonly implicated in the appearance of cholera cases are: fish and shell fish, fruit and vegetables and other foods such as rice, potatoes, lentils, beans, egg, chicken, etc.[9,13,14,20]

Fish and other sea food may be contaminated if they have been collected from water contaminated with fecal material or from those aquatic environments in which *V. cholerae* O:1 is naturally present.[3,21] Oysters (bivalve mollusks from the genus *Crassotrea* spp.), are among the seafood that are implicated in the occurrence of cholera cases, in particular since they are frequently eaten raw.[7,22]

The incubation period of the disease may vary from 6 h to 3 days depending on various factors inherent to both the

host and the microorganism. Symptoms include, the abrupt appearance of nausea, vomiting, diarrhea and abdominal spasms, there are also metabolic alterations such as dehydration, acidosis, hypocalcimia and electrolyte imbalance.[12] Laboratory diagnosis is carried out by the isolation and identification of the causal agent or may be carried out through techniques such as, phase contrast and dark field microscopy, immunoflourescesnce, latex agglutination, coagulation, enzyme linked immunoabsorbent assay (ELISA) and the polymerase chain reaction (PCR).[2,4,9]

In 1994, Hasan and cols. reported the development and evaluation of a test based on the use of colloidal gold particles, known as the Visual Colorimetric Immunoassay [Cholera SMARTTM (Sensitive Membrane Antigen Rapid Test)], which allows direct detection of *V. cholerae* O:1 from samples of fecal material. The basis of the test is as follows: a sample suspected of containing *V. cholerae* O:1 is reacted with monoclonal antibodies against bacterial lipopolysaccharide factor A, which are marked with colloidal gold particle. If *V. cholerae* O:1 is present in the sample, *V. cholerae* O:1-monoclonal antibody complexes are formed, which are subsequently captured and concentrated by polyclonal antibodies linked to a solid phase matrix. Following the depositing of colloidal gold, and after a minimum incubation time of 5-10 minutes, the reaction appears at first sight as a developing pink stain. In the absence of *V. cholerae* O:1, the complex does not form, in which case the pink test coloring does not appear.[10]

Today there is the tendency within medical and food microbiology to develop and apply quick diagnostic techniques, that allow results to be obtained with a high degree of reliability, such that the spread of infectious diseases is prevented by cutting the diagnostic time. This is the case for the Cholera SMARTTM technique, which is analytically sensitive and specific, and therefore reliable. In other words, this technique can detect 6 x 10^6 CFU/ml of *V. cholerae* O:1 and is a test which is 100 % specific for this microorganism. The work presented here shows the first steps towards determining the application of this kit in the area of sanitary microbiology.

The objetive of this work is compare the Visual Colorimetric Immunoassay technique (Cholera SMARTTM) [NHD] with the culture technique (Laboratorios Nacionales de Salud Pública e Instituto Nacional de Diagnóstico y Referencia Epidemiológicos de la Secretaría de Salud), in order to detect *V. cholerae* O:1 and thus to validate the use of this immunoassay with oyster samples.

MATERIAL AND METHODS

50 oyster samples were collected from local markets and street stands in different parts of Mexico City. The quantity of each sample collected corresponded to one order of oysters (approximately 10 large pieces or 20 small pieces).[8] The oysters did not contain sauce, onion or any other ingredient usually used in its preparation in the form of a cocktail. The conditions of conservation in which the oysters were kept at the site of sale are as follows: a) oysters removed from their shells, kept on aluminum trays on beds of ice (29 samples collected); b) oysters removed from their shells, kept on aluminum trays or in plastic buckets, at ambient temperature (4 samples collected); and c) oysters in their shells kept at ambient temperature (17 samples collected). For this final group, the mollusks were taken out of their shell at the place of sale. Each sample was placed in disposal cups (plastic or unicel) covered with plastic lids, and each cup was placed in a polythene bag. Once the samples were obtained they were transported in an icebox for a maximum time of one hour, before the analysis was carried out.

Preliminary assays. Before applying the Cholera SMARTTM [NHD] test and the culture technique, two preliminary assays were carried out. The first of these was to determine the minimum enrichment time, at 37ºC, of a series of dilutions of pure *V. cholerae* O:1 strain, diluted in alkaline peptone water. The objective of this procedure was to determine the end point at which a positive reaction was observed when the Cholera SMARTTM [NHD] test was applied. In the second preliminary assay, a sample of oysters was inoculated with a strain of *V. cholerae* O:1 and the minimum time necessary for enrichment, in order to obtain a positive reaction with the Cholera SMARTTM [NHD] kit, was determined. In this way it was possible to determine the influence of both the components of the oyster and the time necessary for enrichment under which this "kit" functions.

Detection of *V. cholerae* O:1 in oysters by the culture technique. This technique is described in the National Public Heath Laboratories of the Mexican Health Ministry.[8] It is based on the methodologies provided by the Food and Drug Administration of the United States of America. In this investigation we only used tryptone agar with 1 % NaCl, following which we applied different tests in the order recommended by the INDRE (Instituto Nacional de Diagnóstico y Referencia Epidemiológicos).[9]

The complete sample (including both the flesh and the liquid parts of the bivalve) was placed in a sterile liquidizer and homogenized for one minute. From this suspension, 50 g were weighed out and added to 450 ml of alkaline peptone water (1:10 dilution), this was then homogenized in another liquidizer for one minute. The suspension was divided into equal volumes in two sterile flasks, and from each of these two more 10 fold dilutions were made, by transferring 10 ml to 90 ml of alkaline peptone water. In this way, two series of three dilutions were made (1:10, 1:100 and 1:1000). One series was incubated at 37 ºC and another at 42 ºC for 8 h. Then an aliquot was taken from the 1:10 and 1:1000 dilutions (from both incubation temperatures), with the use of a cotton bud, and transferred to thiosulphate citrate bile salt saccharose (TCBS) agar plates to carry out the isolation by cross streaking. All the plates

were incubated at 37 ºC for 24 h. The 1:10 dilution, incubated at 37 ºC, was kept in these conditions for 24 h.

Subsequently, three suspected colonies of *V. cholerae* were selected from each plate. Each selected colony was transferred to an agar plate with 1 % tryptone and 1 % NaCl (T1N1 agar) and isolated by cross streaking, with incubation at 37 ºC for 24 h.

Following colony isolation in T1N1 agar, Gram staining was carried out to observe the morphology microscopically. Identification was then carried out by inoculation in the following media: Kligler's iron agar, lysine iron agar, indol ornithin mobility media, Simmon's citrate agar, mythl- red Voges Proskauer broth, tryptone broth with 0, 1, 3, 6, 8 and 10 % NaCl, and arginine broth. All the media were incubated at 37 ºC for 24 h. If the biochemical tests at 24 h confirmed the presence of *V. cholerae*, the oxidase test was carried out from the lysine-iron agar and the thread test, from Kligler's iron agar. If both the oxidase test and the thread test confirmed the presence of *V. cholerae*, the serological confirmation was made with polyvalent antiserum, to identify the O:1 serogroup and then the serotype was identified using Ogawa and Inaba specific antisera. When the biochemical tests for *V. cholerae* coincided, but there was no agglutination with the polyvalent antiserum, the presence of *V. cholerae* NO O:1 was reported.

Detection of *V. cholerae* O:1 in oysters by visual colorimetric immunoassay (Cholera SMARTTM) [NEW HORIZONS DIAGNOSTICS]. The test was carried out using the enrichment broth incubated at 37 ºC for 8 and 24 h . An aliquot of enrichment broth was taken and transferred to a filtering device to half its capacity, and was homogenized for one minute by gentle shaking (it was not necessary to use the extraction regulating solution included in the commercial kit, since the sample was in a enrichment broth). Then, three drops of the liquid contained in the filtering device were extracted and added to the reaction vial in which three drops of reconstitution solution had previously been added and this was shaken gently. Later, the contents of the vial were absorbed with the sterile dacron bud which was included in the kit. The bud was place in the upper compartment of the SMART device, which was closed in the indicated area. This was incubated for 20 min at ambient temperature, and finally the test areas and negative control included in the lower compartment of the SMART device, were read. The positive control included within the same kit was run simultaneously.

Identification of other species from the *Vibrio* spp. Genus and other members of the Vibrionaceae family obtained from oyster samples. Identification of other microorganisms present in the samples studies was carried out using those colonies whose morphologies differed from those belonging to *V. cholerae* isolated on thiosulphate citrate bile salt saccharose (TCBS) agar. In addition, tests were carried out on those colonies with morphologies similar to *V. cholerae*, but which, when biochemical tests were carried out were found to be different.

The procedure for identifying the above microorganisms was similar to that used for *V. cholerae*, including gelatin agar, urea broth and phenol red broth with the addition of manitol. 2 % NaCl was added to Kligler's iron agar, lysine-iron agar, indol ornithin motility media, methyl red Voges Proskauer broth and gelatin agar. To identify *Vibrio parahaemolyticus* it was necessary to use the L-leucine assimilation test. In addition, the following media were used: bile esculin agar, blood agar and phenol red broth with the addition of arabinose to identify species from the *Aeromonas* sp. genus.[1,8,9,15]

RESULTS

In table 1 the conservation conditions under which the oysters were kept at the sampling site are shown. The presence or absence of shells at the site of sale is also shown.

The results of the preliminary assay show that the minimum enrichment time of a pure *V. cholerae* O:1 strain, at 37 ºC, is 8 h. Such that, at the end of this incubation time a positive reaction was registered when the Cholera SMARTTM [NHD] kit was applied using an inoculation of 30 CFU/ml. When a starting concentration of 10 and 3 CFU/ml of *V. cholerae* O:1 was used, the test was positive at 9 h. With respect to the other preliminary assay, in which a sample of oysters was used, a positive test was registered using a starting inoculation of 10 CFU/ml, with 9 h incubation at 37 ºC. Table 2 shows results obtained using the culture technique and the Cholera SMARTTM [NHD] test to detect *V. cholerae* O:1 in oysters. Here 49 samples are shown which were negative both with the Cholera SMARTTM [NHD] test, applied after 8 and 24 h incubation, and with the culture technique. Only one positive sample was obtained by applying the immunoassay after 8 and 24 h incubation, which was confirmed by the culture technique. The strain detected was identified as *V. cholerae* O:1 Ogawa serotype, ElTor biotype. In table 3 microorganisms belonging to the Vibrionaceae family are shown, identified from the oyster samples processed by the culture technique.

DISCUSSION

As based on the results obtained from the preliminary assays using the Cholera SMARTTM [NHD] test, the test was applied to samples enriched in alkaline peptone (APA: 1 % peptone, pH 9.0). In this way, it was possible to reveal the microorganism even in those samples in which, due to reduced numbers of microorganisms and to the analytical sensitivity of the "kit", it was not possible to obtain a positive result directly from the sample (without dilution). This has been shown in the reports of Hasan and cols. (1994), who showed that a minimum of 6 X 10^6 CFU/ml of *V.*

Table 1. Conservation conditions used for oyster samples at the sampling site.

Conditions under which oysters were kept	Conservation conditions used for oysters	Number of samples at the sampling site
With shell	At ambient temperature	17
Without shell	On ice	29
Without shell	At ambient temperature	4
Total		50

Table 2. Detection of *Vibrio cholerae* O:1 in oysters by the culture technique and the visual colorimetric immunoassay.

Number of negative samples for both techniques.	Number of positive samples detected by culture only.	Number of positive samples detected by the Cholera SMARTTM test only.	Number of positive samples detected by both techniques
49	0	0	1

Table 3. Microorganisms isolated from oysters by the culture technique.

Microorganisms	Percentage of microorganism isolation
Vibrio alginolyticus	**46**
Vibrio cholerae NO O:1	**26**
Vibrio parahaemolyticus	**8**
Aeromonas caviae	**6**
Vibrio harvegi	**4**
Aeromonas sobria	**4**
Aeromonas hydrophila	**2**
Vibrio cholerae O:1 serotipo Ogawa	**2**
Vibrio metschnikovii	**2**

cholerae O:1 are necessary to obtain an unequivocally positive result.[10] The presence of the microorganism in oyster samples was determined, using samples kept in very different micro-environmental conditions compared to samples of clinical origin (fecal material) for which this immunoassay was designed. The origin of the sample used is directly reflected in the number of CFU/ml of *V. cholerae* O:1 detected in the 2 sample types. When the Cholera SMARTTM [NHD] kit was used in the first preliminary assay, a positive result was obtained after incubating the *V. cholerae* suspension, diluted to 30 CFU/ml in APA, for 8 h. Hence, starting from this concentration of bacterial cells, the immunoassay developed a positive result after 8 h incubation, and the tests applied before this incubation time were negative. In the same way, positive results were obtained with the "kit" after 9 h incubation using a suspension of 10 and 3 CFU/ml of *V. cholerae* O:1. Furthermore, from the preliminary assay, in which an oyster sample was used, it was possible to determine if the components of the oyster itself interfered with the sensitivity of the test. It was found that after 9 h incubation, the oyster sample, diluted in APA and inoculated with an initial concentration of 10 CFU/ml, tested positive the same way as the assay in which only microorganisms in APA were used. This indicates that oyster components did not interfere with the test. Based on these results, the Cholera SMARTTM [NHD] test was used following incubation of the enrichment broth for 8 h, at 37 ºC, in the same way as the culture technique. The result was then confirmed after 24 h incubation. It is important to point out that the sample that resulted positive for *V. cholerae* O:1 was obtained form a road side stand, in which the oysters, without their shells, were kept on aluminum trays on a bed of ice. Furthermore, they were collected during the last week of March, which is important to point out given that there is seasonality involved with outbreaks of cholera cases.[12] The detection of at least one sample as positive for *V. cholerae* O:1 by both techniques, has important public health implications, and direct repercussions on the prevalence of the disease in urban areas such Mexico City. Hence, we are carrying out an evaluation of potential risks associated with the consumption of oysters which represent a transmitter of cholera within Mexico City and its environs. In addition we are investigating the various sources of contamination that allow the entrance of *V. cholerae* O:1 into oysters, for example, the contamination of origin and contamination via oyster handlers from the point of harvest to the site of sale to the con-

sumer.

With respect to the culture technique and dilutions, all the oyster flesh was homogenized along with the valve liquid. This allowed us to obtain adequate samples for analysis. Furthermore, this preparation did not have a negative effect on the viability of *V. cholerae* O:1, as may occur with the extrinsic and intrinsic parameters that are present in the food before analysis.[3,14]

Observing the data shown in table 1, the importance of obtaining samples kept on ice at the place of sale, can be appreciated. Since, it is within this group that the sample, in which *V. cholerae* O:1 was demonstrated, was found. Furthermore, it is important to mention that at the moment of acquiring this positive sample, the oysters were removed from their shells. In table 1, the conservation temperature can be seen (ambient or on ice) as well as the influence of the mollusc shell on the viability of *V. cholerae* O:1.

With respect to the immunoassay, no results were obtained that were only positive using this test. That is, false positive results were not registered, which indicates that the technique did not develop cross reactions when confronted with the variety of microorganisms that may be found in this type of sample.[16] Furthermore, positive samples were not detected by the culture technique alone, such that, false negatives were not registered. This indicates to us that the Cholera SMARTTM technique functioned well, since there was no reaction in those samples which were found to be negative for microorganisms by culture. Hence, all the reagents and the lyophilized conjugate showed specific activity.[10,18]

The low incidence of *V. cholerae* O:1 in the oyster samples analyzed in this work may be related to the time of year in which the study was carried out. The study took place during autumn and winter, and during only two weeks of spring, and although cholera cases occur throughout the whole year, they increase in summer.[3,12] Furthermore, the general handling of the oysters should also been considered, from when they were taken from their natural habitat until the places where they were consumed, in which a very important role is played by microbial competition.

The results in table 3, give an idea of the variety of microorganisms that may be present in this type of mollusk, which is strongly related to their natural characteristics (as biological concentrator organisms). Most importantly it can be seen that within these microorganisms are included potential pathogens for human beings, such as *V. cholerae* NO O:1, *V. parahaemolyticus*, *V. alginolyticus* and *Aeromonas hydrophila*.[16]

One of the most interesting aspects of the development of this work, was to determine the function of this immunoassay when confronted with a type of sample which is different from that for which the kit was originally designed. The experience obtained in this study showed that this technique can be applied to oysters, since handling of the samples was not complicated. As has already been shown, we decided to enrich the oyster samples in order to increase the probability of obtaining a positive result, since there is a direct relationship between the analytical sensitivity of the immunoassay and the concentration of the microorganisms in the oyster sample. That is, the immunoassay does not detect concentrations of the microorganism lower than 6X106 CFU/ml, and the concentration of *V. cholerae* O:1 in oysters does not necessarily reach this concentration, however there are reports of infective doses of 1X106 CFU/g.[10,12] In spite of requiring an enrichment time of 8 h to apply the immunoassay to the oyster sample, there is still an important reduction in the time required to obtain results, compared to the minimum of 78-80 h required to obtain results from culture. This type of product, therefore represents an important quick technique for the detection of *V. cholerae* O:1. Based on the advantages that this immunoassay offers, we propose that only those samples that result positive by this technique should be processed by culture, above all in those regions of the country in which epidemic outbreaks occur. This would allow the rapid application of preventative action in order to avoid the dissemination of the disease through foods such as mollusks.

As a consequence of the results obtained, it was not possible to apply any statistical model to describe the relationship between both techniques. However, it is very interesting that the total of oyster samples analyzed that resulted negative for *V. cholerae* O:1 by the Cholera SMARTTM test, were also found to be negative by the culture technique. In addition, the sample that resulted positive by the immunoassay was also positive by the culture technique. All this data has allowed us to learn more about this technique. However, it is necessary to keep a line of investigation that provides more information on its application to samples taken from their original environment (water, aquatic sediments, Phytoplankton and biofilms, etc.) as well as from food samples. Among, the other applications of this type of immunoassay, is the possibility of detecting viable forms of *V. cholerae* O:1 that are not possible to cultivate, taking as reference the isolation data of the microorganism in water, fish and seafood.[3]

In summary, it is important to point out the range of applications that may be obtained from this technique within the field of sanitary and environmental microbiology in Mexico, which will eventually provide fast and reliable detection of *V. cholerae* O:1. In this way, quality control will become more efficient and as a consequence preventive or corrective measures will be applied more rapidly to combat the problem of cholera epidemics.

In conclusion application of the Visual Colorimetric Immunoassay (Cholera SMARTTM)[NHD] to oyster samples supports its routine use for the detection of *V. cholerae* O:1 in oysters, with the aim of substituting culture techniques in emergency situations caused by cholera epidemic.

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