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Detection of Clinical Isolates of *Vibrio cholerae* by Dot Blot Hybridization

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ABSTRACT

Introduction: *Vibrio cholerae*, the causative agent of cholera, has attracted a great deal of attention as one of the major causes of morbidity and mortality worldwide, especially in developing countries. In most laboratories, biochemical assays are primarily performed for possible detection of these strains, which are then followed by a PCR (polymerase chain reaction) test to verify their identity. This study aimed to optimize dot blot technique in order to detect *Vibrio cholerae* bacteria for *V. cholera* as an easy-to-use and beneficial method.

Methods: A dot blot hybridization test was developed in this study to identify *V. cholerae* isolates as well as to assess the sensitivity and specificity of this test compared whit biochemical and PCR tests which are routinely performed for *V. cholerae* screening and detection in clinical specimens.

Results: Herein, the dot blot hybridization test was optimized to detect *V. cholerae*. A combination of three biochemical assays and PCR test confirmed the results of dot blot hybridization test. This test was able to identify *V. cholerae* strains with a high sensitivity and specificity of 100%. Using the newly developed method, a set of 26 collected *V. cholerae* isolates from clinical samples were accurately identified.

Conclusion: This study optimized dot blot technique as a simple and useful assay that could be employed in *V. cholerae* monitoring programs and strategies to effectively detect *V. cholerae* strains in surface water and fecal specimens.

Keywords: Detection; Polymerase chain reaction; Ctx-AB, *Vibrio cholera*; Dot blot Hybridization

1. Introduction

There are many reasons which emphasizes the need for a simple, reliable, faithful, and accurate method to detect bacteria, including the study of antibacterial agents, prophylactic measures, epidemiological studies, and further investigation of bacteria-environment relationship. Detection of bacteria based on specific culture media is one of the most basic bacterial detection methods. Bacterial genome sequencing is also used to spot different sources and types of bacteria using specific genes in a strain or family of microorganisms [1]. These methods require a significant amount of bacterial culture, which in turn is a time-consuming process due to the slow growth rate of several bacteria. In addition, special and sometimes expensive devices are needed to detect the target DNA fragment [2].

Some other methods also have their own foundamental disadvantages. For example, the complement-fixation test is a sensitive test; however, it has finite value in the early diagnosis of diseases such as hydatidosis because in this test, the serum of some people, especially those with lung cancer, becomes false-positive after receiving the rabies vaccine [3]. Similarly, the indirect hemagglutination test is also a common test used to detect some pathogenic antigens/bacteria, but it has less value in areas that are commonly infected due to cross-reactions, such as patients with cysticercosis, schistosomiasis, or nematodes. Alternatively, the latex agglutination test is a simple, sensitive, and specific test, but creates several false-positive results. Also, the indirect immunofluorescence testing is a method which requires expensive equipment [4]. In addition, the ELISA test is reasonably sensitive and specific, but in some cases, such as cysts in the brain and spleen, may increase false negatives due to less immune stimulation. Last but not least, the immuno-electrophoresis test has lengthy and complex stages [5]. After the advent of blotting techniques, the use of western blotting and later dot blotting to detect bacteria were increased. Due to very specific interactions between the antibody and the antigen, these methods do not require great amounts of bacteria or bacterial purity [6].

Additionally, no special device is usually needed to show Western blot films and the whole process is done quickly and with high accuracy [7]. In general, the blot concept refers to those techniques in which there should be an interaction between antigen and antibody. The speed and simplicity of this test makes it a very valuable diagnostic method in detecting many bacteria and even viruses. This method is even able to detect antigens up to about one ng [8].

Since the advent of blotting techniques, the use of Western blotting and later dot blotting methods to detect bacteria has increased. The main challenges related to the use of dot blot techniques include: the antibody detection by the antibody, the number of used doses by the antigen or antibody, the analysis of results, and finally the accuracy of results. Another challenge in this technique is the lack of standard antigens in some diagnostic cases [9, 10]. The dot blot technique has also been shown to be less able to normalize the signals of housekeeping genes. Many efforts have been carried out to overcome these challenges.

In a study, th radio indicators instead of fluorometric indicators was used. In this work, the required time for blotting procedures was reported to be reduced up to 70% using the innovative method [١١]. In another experiment, an attempt was made to use a modified form of this technique for methylated DNAs . In this way, oligonucleotide probes specific for methylated DNA regions were used as antibodies [12]. In a another study by Putra et al.[١٣] , two types of antigens were used in dot blot. In this type of dot blot, “one dot two development signals (ODTDS)”, the protein in question was first identified by the horseradish peroxidase (HRP) antibody, and then the antigen containing the housekeeping gene was detected by the alkaline phosphatase (AP) antigen.

In the present study, an optimized dot blot technique was used to detect *Vibrio cholerae* bacteria. Cholera has been recognized as a dangerous infectious disease for many years, infecting 3 to 5 million people and causing 100,000 to 120,000 deaths per year worldwide [١۴]. According to some researches over the past two centuries this disease has been a major cause of death and significant complications in many developing countries. It is human specific disease which has so far caused seven global catastrophes (pandemic), resulting in thousands of deaths as well as major social and economic consequences [14] . As a result, it is necessary to find appropriate

methods for rapid, inexpensive, and prompt detection of these bacteria.

2. Materials and Methods

Ethical Considerations

The research was appraised and approbated by the Research Ethics Committee of Tarbiat Modares University under (IR.MODARES.REC) approval ID before it began.

Sample collection

In this study, a total of 26 *V. cholerae* strains which were isolated from clinical specimens and obtained from the archive of Tarbiat Modares University (Tehran, Iran) were subjected to dot blot hybridization. *V. cholerae* strain ATCC 14035 from clinical samples was used in this research as a positive control. Other control isolates in this study were those strains that were identified and characterized in various clinical trials and belonged to various Gram-negative species.

Evaluation of the biochemical tests for detection of clinical isolates of *Vibrio cholerae*

Samples were identified by biochemical assays. For this purpose, initially samples were cultured on TCBS (Thiosulfate-citrate-bile salts-sucrose) agar plates and then subjected to incubation at 37 °C for 24 h. Routine bacteriological tests for all yellow bacterial colonies were later performed, including colony morphology, oxidase, sucrose and lactose fermentation, Voges-Proskauer, motility and indole tests, as previously indicated [15].

Serotyping tests for detection of clinical isolates of *Vibrio cholerae*

Fresh single colonies grown on BHI (brain heart infusion) agar were subjected to slide agglutination test to determine serogroups of isolates using polyvalent *V. cholerae* O1 and O139 antisera; serotype identification was performed using monovalent antisera against Inaba and Ogawa antigens (Mast Diagnostics, Bootle, UK). *V. cholerae* strain ATCC 14035 was employed in this research as a positive control.

Evaluation of polymerase chain reaction of CTX-AB

Identification of the CTX-AB gene in isolates was also carried out by PCR test. The primer sets used in PCR test were as follows: CTX-AB-F (5'-TATGCCAAGAGGACAGAGTGAG-3') and CTX-AB-R (5'-AACATATCCATCATCGTGCCTAAC-3'). The PCR process was conducted under the following thermal cycling program: an initial denaturation step at 94 ºC for 5 min, followed by 30 cycles of denaturation at 94 ºC for 30s , annealing at 55 ºC for 30 s, extension at 72 ºC for 1 min, and a final extension step at 72 ºC for 5 min [16].

Detection of Clinical Isolates of *Vibrio cholerae* by dot blot

DNA extraction

For dot blot technique, DNA extraction was performed on overnight cultures of bacteria grown on brain heart infusion (BHI) agar medium using phenol/chloroform/isoamyl alcohol extraction method.

Preparation of probes for *in vitro* Hybridization

For probes preparation , initially DNA was prepared and PCR process was done in order to proliferate DNA , then DNA was extracted from agarose gel and DNA was denatured with heating in boiling water followed by immediately cooling in anice water bath. DNA was labeled with DIG DNA labeling and detection kit (Rosh, Germany). Finally, was the reaction halted with 0.2 M EDTA.

Dot blot membrane preparation

For the dot blot technique, DNA extraction was performed on overnight cultures of bacteria grown on BHI agar medium using phenol/chloroform/isoamyl alcohol extraction method. The product was transferred onto positively-charged nylon membranes (Roche) and allowed to air dry. The nylon membranes were subjected to a pre-hybridization step for 30 min in a hybridization buffer consisting of 5× SSC (20× SSC is 3 M NaCl and0.3 M sodium citrate), 2% (w/v) blocking reagent (Roche), 0.1% w/v N-lauroylsarcosine, and 0.02% (w/v) SDS. The membranes were incubated in blocking buffer to block unoccupied sites on the membrane surface as wel as to hinder non-specific binding. They were then immersed in a filter paper and placed in a crosslinker machine with 1200 energy. Crosslinked membranes were later placed in hybridization buffer at hybridization temperature for 30 min. A

pre-hybridization step is generally required before the main hybridization step to reduce nonspecific hybridization sites. The following formula is used to determinate the hybridization temperature:
Tm = 49.82+ 0.41 (% G+C) – (600-L)
Topt = Tm – 20-25 ° C
L= Length of hybrid in base pairs.

Determination of the efficiency of prepared probe
The prepared probe and control DNA were diluted to 1 ng/µL using dilution buffer. About 1 µL of each diluted DNA and probe was transferred to positively-charged nylon membranes (Roche). The membranes were then located in a cross linker machine with 1200 energy. The membranes with positive charge were placed in 20 mL of malic acid buffer at 15-25 °C for 2 min.

DNA hybridization
The labeled probe with digoxigenin was heated in boiling water for 5 min and then immediately placed in an ice water bath. As a result, the labeled probe with digoxigenin was denatured. The denatured probe and hybridization buffer were mixed with the membrane. The membranes were hybridized with newly labelled and denatured gene probes at hybridization temperature for 6-18 hours.

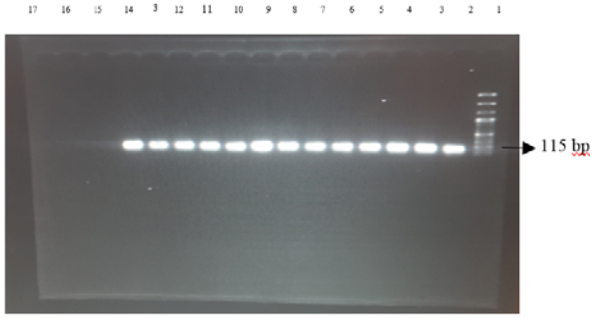
Stringency washes
The membranes were rinsed two times in buffer 1 (2× SSC and 0.1% w/v SDS) at 25 °C for about 5 min and two times in buffer 2 (0.5× SSC and 0.1% w/v SDS) at 68 °C for about 15 min. Anti-digoxigenin antibody was used to identify DNA samples undergoing hybridization over the prepared probes according to guidelines of the manufacturer.

Immunological detection
After hybridization and specific washes, the membranes were rinsed in rinsing buffer for 1 to 5 min. Following rinsing, the membranes were first incubated in 100 mL of blocking buffer for 30 min and then in 20 mL of antibody solution for about 30 min. After that, the membranes were rinsed two times in rinsing buffer for 15 min. Finally, they were balanced in detection buffer for 2 to 5 min and incubated in 10 mL of color-substrate solution in dark for 6-18 h. The effectiveness of dot blot test for its sensitivity and specificity was evaluated which 100% sensitivity in order to eliminate false negatives was experienced [17].

3. Results
Totally, 26 *V. cholerae* O1 isolates were detected by biochemical assays and serological methods, respectively. *V. cholerae* strain ATCC 14035 obtained from clinical specimens was used in this research as a positive control. Other control isolates used in this study were those strains that were identified and characterized in several clinical trials and belonged to various Gram-negative species [Table 1].

Evaluation of serological and biochemical assays and PCR test of *ctx-AB* gene
All 26 clinical isolates belonged to serogroup O1. The outcomes of biochemical assays performed for *V. choleae* isolates obtained in the present study as well as the controls are presented in Table 2. As shown in the table, among the performed biochemical tests for the isolates, both oxidase assay and sucrose fermentation on TCBS exhibited 100% sensitivity and specificity for *V. cholerae* detection. The positive *V. cholerae* isolates in biochemical assays were also positive in PCR test for the presence of the *ctx-AB* gene [Table 1 and Figure 1].

Figure 1**:** Agarose gel electrophoresis of PCR



amplified products with *ctx-AB* specific primers (115 bp). Lanes 1, DNA Size Marker (100 bp), *Vibrio cholerae* ATCC 14035; 2, clinical isolates; 2-14, negative control; 15-17.
In addition, DNAs extracted from *Escherichia coli*, *Acinetobacter* spp., *Pseudomonas* spp., and *Klebsiella* spp. were also used in this study in order to evaluate the specificity of primers used for *V. cholerae* detection but exhibited no gene proliferation in PCR assay, verifying the

specificity of primers used in this study for *V. cholerae* O1 strains detection.

Preparation of Probes for Hybridization

The used DNA concentration of in order to prepare the probe and also the incubation time of labeling reaction were calculated.

Dot blot analysis

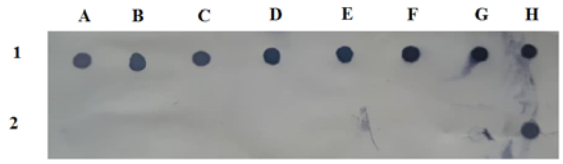
The dot blot technique is similar to other blotting methods, but does not provide data about the size of the hybridized fragment. In this technique, the extracted DNA from *V. cholerae* was spotted onto the filter without the previous electrophoresis and transfer stages. In dot blot, the probe was hybridized with the extracted DNA from *V. cholerae* strains. The probe had dot or circle shape. All examined *V. cholerae* isolates were positive in dot blot hybridization tests shown in Figure 2. The used dot blot test in this study, provided 100% sensitivity and specificity for the identification and confirmation of the *V. cholerae* isolates against the control strains that presented different biochemical test patterns from those of the *V. cholerae* strains.

The results of biochemical, PCR, and dot blot hybridization tests are shown in Table 1.

Table 1. The results of biochemical, PCR, and dot blot hybridization tests

Strain	Biochemical test							PCR for ctx-AB	Dot blot
	growth on TCBS	Lactose glucose /	Oxidase	Methyl Red	Voges-Proskauer	Indole	Motility		
Clin *V. cholerae* (n=26)	+	**K / A**	+	+	+	+	+	+	+
V. cholerae ATCC 14035	+	**K / A**	+	+	+	+	+	+	+
Pseudomonas aeruginosa ATCC 27853	-	**K / K**	+	-	-	-	+	-	-
Klebsiella pneumoniae spp.	-	**A / A**	-	-	+	-	-	-	-
Acinetobacter spp.	-	**K / K**	-	-	-	-	-	-	-
Escherichia coli spp.	-	**A / A**	-	+	-	+	+	-	-

Figure 2: Result of dot blot hybridization tests with the extracted DNA from bacteria strains by the aid of specific probe for *V. cholerae.* Line ١; A-H, Eight isolates of *V. cholerae*. Line 2; A; *Pseudomonas aeruginosa* ATCC 27853, B-C, *Klebsiella pneumoniae spp.,* D-E; *Acinetobacter spp.*, F-G; *Escherichia coli spp*., H; *V. cholerae* ATCC 14035.



Discussion

Cholera is considered as a major public health concern in developing countries. Cholera as an acute diarrhoeal illness causes about 120 000 deaths per year (WHO, 1995). Cholera disease is caused by *V. cholerae* intestinal infection through food or water contaminated with Gram-negative bacterium. When passing through the small intestine, *V. cholerae* colonizes in intestine, multiplies, and begins to produce cholera toxin. *V. cholerae* has so far been responsible for seven cholera pandemics and is considered as a main public health concern in developing countries [18].

4.

The causative agent of the seventh ongoing pandemic is *V. cholerae* serogroup O1 biotype El Tor.It is almost difficult to distinguish a patient with cholera disease from a patient infected by another pathogen causing diarrhea without a stool sample test. Various methods have been proposed for molecular detection of *V. cholerae* strains, including the analysis of intergenic sequences of 16S rRNA and 16S–23S rRNA. However, the inability of 16S rRNA sequences to explicitly differentiate diverse *Vibrio* species is well established [19].

The biochemical, serological, PCR, and dot blot hybridization tests conducted in the present research exhibited 100% sensitivity and specificity for *V. cholerae* detection and verification compared to control strains exhibiting more different patterns in biochemical assays than *V. cholerae* isolates.

All suspected bacteria with positive results in biochemical assays and PCR test for the presence of ctxA gene were subjected to dot blot hybridization test. The obtained results were completely consistent with the results of the aforementioned procedures [20].

The dot blot technique has been used to detect many bacteria and microorganisms, including *Paracoccidioides brasiliensis* [21], *Desulforhabdus amnigenus* [22], *Salmonella enterica* [23], *E. coli* [24] and *Lactobacillus* [25], *E. coli K12*, *E. coli DH5*, *Lactobacillus 5I*, and *Lactobacillus 1D* [26], as well as Group B *Streptococcal Capsular* [27].

Zhang et al. (2014) used nanoparticles containing quantum dot to detect monoclonal mouse anti-HBsAg antibodies. This nanoparticle-based technique improved the accuracy and simplicity of conventional dot blotting methods [28]. In another study, heating of nitrocellulose sheets in 10XSSC plus 0.5% SDS solution at 55 °C was investigated. According to the results, the accuracy of this detection method was up to 1000 times higher than that of the current dot blots [29]. In a different and innovative way, researchers have tried to accelerate the dot blot method by using the PVDF membrane in a 96-house microplate. several types of proteins could be examined simultaneously by this method [30]. In this study, we used an assay particular to the *V. cholerae* species that was established in our laboratory to confirm the specificity of the biochemical tests in order to detect *V.cholerae* isolates. Dot blot hybridization of the *V. cholerae* strains in pure cultures and our results was consistent with the selected biochemical tests and PCR assay in all of these isolates. It should be noted, however, that the used method was just for the detection of *V. cholerae* and not for differentiation of the pathogenic and nonpathogenic strains. Thus, according to this study, dot blot can be used as a confirmatory alternative test for the accurate identification of the species *V.cholerae*.

5. Conclusion:

Generally, the results of present study suggest that clinical specimens could be rapidly examined for the presence of *V. cholerae* strains through enriching the specimens with alkaline peptone water without NaCl, choosing yellow colonies on TCBS agar, and ultimately conducting an oxidase assay. Strains with positive results in these three experiments could be verified using dot blot hybridization test. This method could be employed in *V. cholerae* monitoring programs and strategies to efficiently detect *V. cholerae* strains in surface water as well as fecal specimens.

Authors' contributions

SBJ performed the experiments and drafted the manuscript. BB developed and supervised the work. SS and BB contributed to data interpretation. All authors reviewed the manuscript. All authors read and approved the final manuscript.

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Competing interests
The authors declare that they have no competing interests.

Availability of data and materials
The datasets used and/or analyzed during the current work are available from the corresponding authors on reasonable request.

Ethics approval and consent to participate
The study was reviewed and approved by Medical Ethics Committee of Tarbiat Modares University (Code: IR.MODARES.REC) before the study began.

Consent for publication
Not applicable

Conflict of interest
No conflict of interest was declared by the authors.

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