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Molecular diagnostics for *Vibrio cholera* based on *rec*A gene isolated from human in Diwaniyah city

Syoof Khowman Alramahy
Biology Department, Collage of Science, University of Al-Qadisiyah, Iraq

Abstract
Vibrio cholerae is gram negative bacteria, motile, has a polar flagelum, curved bacillus, causes cholera in humans. *V. cholerae* attacks the intestinal cells through the production of toxins causing the loss of large amounts of salts and water causes severe diarrhea that can lead for death.
Twenty isolates of *V. cholerae* were obtained from Diwaniyah Teaching Hospital of diarrhea infections cultivate on differential cultures media, then genetic material DNA extracted and tested by using PCR technique based on *rec*A gene primers, which were designed by using the Gene bank Site (NCBI) to obtain the *rec*A gene sequence (Gene bank code (EF990328.1), using the program design primers (primers plus) and then read the results through the ultraviolet radiation after the samples were carried by Electrical Relay.
The results showed that all *V. cholerae* isolates which were diagnosed by bacterial cultivates on culture media contain *rec*A genes by using PCR technique is a fast, highly specialized and is of a great importance in confirming the specific diagnosis of clinical and bacteriological examination.

INTRODUCTION

Cholera is a serious and common disease worldwide, especially in developing countries (1). It is an epidemic disease, endemic and spread in nature caused by *Vibrio cholerae* (2).
These bacteria can be classified into more than 200 serotypes, however, only serotype 1 and O139 are responsible for epidemiological cholera (3). Cholera outbreaks is due to type O1 and *V. cholerae*, which occur regularly. Serotypes other than O1 and O139 are frequently isolated from seafood and aquatic sources, but do not result in any major outbreaks of the disease (4). Epidemiological cholera is acute, painful and often fatal. It had a serious impact on the world during the nineteenth century (5). It can lead to mortality in untreated patients due to dehydration within a few hours (6). The optimum conditions for the growth of *V. cholerae* are average saline (slightly alkaline) water with high nutrients and of tropical moderate temperature (7). In case of human infection, the pathogen is introduced to the host upon consuming contaminated water or food (8), or by direct contact with an infected person (9). Cholera pathology is a complex process involving a number of factors that assist the pathogen in colonizing intestinal epithelial cells and producing cholera intestinal toxins (CT) (10). These toxins lead to an unbalanced ion transport in the intestinal membrane and to a significant loss of salt and water in the form of diarrhea (9).
Bacterial species are categories of breeds that share morphological pattern and genotype (11). Many problems caused by cholera have been found due to the large variation in the biochemical characteristics, as well as its close genetic similarities with many other species (12). The preservative genes characterized by slow diversity and limited variation in the alkaline sequence are suitable for determining the genetic relationship among species (13). The *recA* gene has been suggested to be a potential marker for detecting genetic relationships among taxonomic classes such as families, classes, and divisions due to the bacterial preservative nature (14). *V. cholerae recA* has the advantage of resistance recovery against oxidation, UV ray factors and increasing re-binding frequencies in the mutant *E. coli* bacteria (15). Therefore, the present study aims at diagnosing isolates of *V. cholera* by *recA* gene isolated from Diwaniyah hospitals. It is the first study in Iraq that uses this gene in the diagnosis of *V. cholerae* bacteria.

MATERIALS AND METHODS

Twenty *Vibrio cholerae* isolates were obtained from the laboratory of bacterial diagnosis at Diwaniyah Teaching Hospital, from diarrheal infection cases and diagnosed using bacterial culture in differential culture media and using Vitak test to confirm the types of the bacterial isolates.

Bacteria Isolates Culture

Vibrio cholerae isolates were cultured using BHI broth and incubated at 37 °C for 18hr. Then, bacterial growth was used in bacterial DNA extraction.

Bacterial DNA Extraction

Bacterial DNA was extracted from *Vibrio cholerae* isolates using PrestoTM Mini gDNA Bacteria Kit, which was supplied by Genaid Company, USA. One ml of bacterial culture was taken for each isolate and put in sterilized 5.1 mL tubes. The tubes were put in the centrifuge at 10,000 RPM for one minute. The surfaced fluid was then removed and bacterial cells were used in the DNA extraction process according to the manufacturer's extraction instructions. Then, the DNA was examined for concentration and purity using the NANODROP spectroscopy device. Measuring was done with two wavelengths (260/280nm) in nanogram/microliter concentration unit. DNA samples were then kept at -20 °C in the refrigerator for the PCR sequence test.

PCR Chain Reaction Test

The PCR test was conducted to determine *recA* gene in the DNA in the *Vibrio cholerae* isolates (16) using specialized primers specially designed for this study using the NCBI-Genebank site to obtain the Nitrogen alkaloid sequence of *recA* gene (Genbank Code EF990328.1); and using the primer3 plus design program as the primers were supplied by Bioneer Korea Company (See the table below).

Table 1: Lengths of Nitrogen Alkaloid Primers in the PCR Test Results

Primers	Sequence (5'-3')		PCR Results
recA gene	Front	TCGACCGGTTCTCTGTCTCT	516bp
	Rear	ACCGCCAGTGGTAGTTTCTG	

Table 2: PCR Mix Preparation

Volume	PCR master mix
5µL	DNA template
1.5µL	*rec*A gene front primer (10pmol)
1.5µL	*rec*A gene rear primer (10pmol)
12µL	PCR water
20µL	**Total**

Table 3: PCR Thermocycler conditions

PCR Step	Repeat cycle	Temperature	Time
Initial denaturation	1	95˚C	5min
Denaturation	30	95˚C	30sec.
Annealing		58˚C	30sec
Extension		72˚C	40sec
Final extension	1	72˚C	5min
Hold	-	4˚C	Forever

PCR Master Mix

PCR mix was prepared using the AccuPower® PCR Permix kit processed by Bioneer Korean Company and according to the manufacturer's instructions as in Table 2.The components of the PCR mix were then put in 0.2 ml tubes of the PCP test kit (Accupower® PCR Premix) which contains other components of the PCR mix. All tubes were then put in the mixing centrifuge (Exispin) at 3000RPM for three minutes and then put in PCR Thermocycler.The PCR test was conducted by use of PCR Thermocycler as in Table 3.

PCR Test Results Analysis

Agarose gel electrophoresis was performed using 1% agarose gel and then the read of the result of PCR product analysis was taken by dissolving 1g of agarose gel in 100 mL of 1X TBE buffer solution and put in the microwave for 5 minutes. And then the gel was left to cool at 50˚C and then 3mL of Ethidium bromide dye was added and mixed well with the gel. The agarose gel was then poured into the mold tray, which contained the comb to locate the PCR samples. Then the gel was left to harden at room temperature for 15 minutes and then the comb was removed carefully from the gel. Afterwards, 100bp DNA ladder was used and put in the first hole. After the completion of the loading process, the agarose was immersed in the 1X concentration protective solution and the cap was sealed. The device was then operated using 100V, 80Am for one hour. Finally, the gel containing the PCR product was tested by UV ray source to determine the results with the unit of measurement.

RESULTS AND DISCUSSION

The study included the collection of 20 isolates of *V. cholerae* from diarrhea cases in Diwaniyah Teaching Hospital. The isolates were gender-categorized (13 isolates, 65% males and 35% females) (Figure 1). As for age groups, ages between 11 and 20 showed the highest rate of infection (40%) and the lowest was 13-40 age group (5%) as shown in Figure 2.

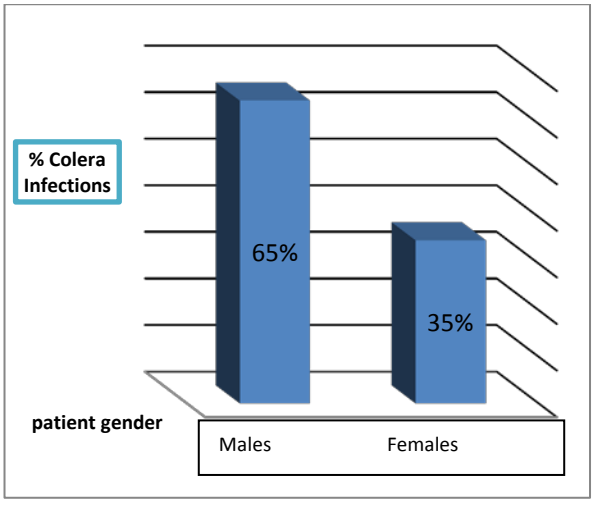

Figure 1: Gender-categorized Cholera Cases (%)

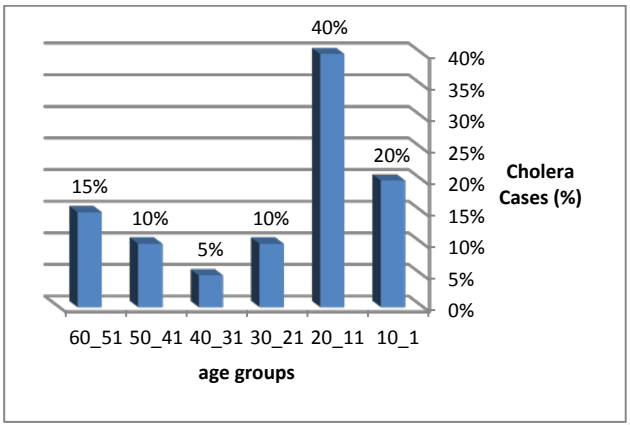

Figure 2: Cholera Infection Cases (%) According to Age Groups

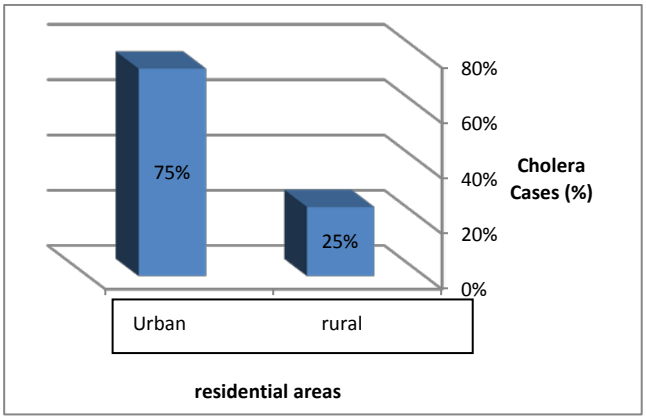

Figure 3: Cholera Infection Cases (%) According to the Residential Areas

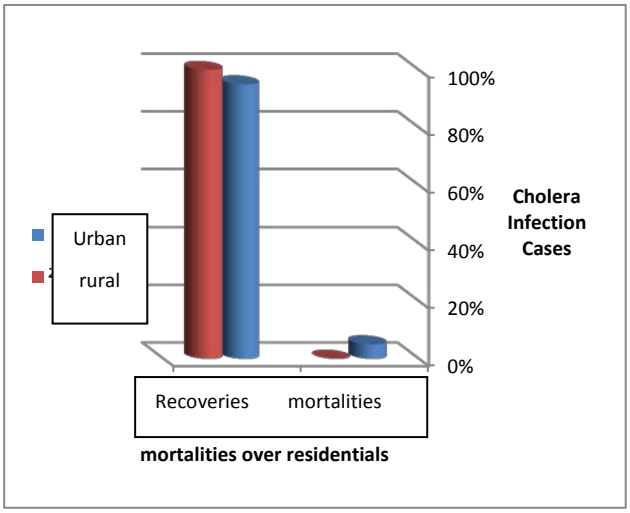

Figure 4: Mortalities Over Residential Areas

Figure 5: Electrical Relay for Agarose Gel with PCR Test Results of *recA* for Diagnosing 20 *V. cholerae* isolates Using PCR Technique on 100V, 80Am for One Hour

The bacterial isolates were geographically distributed into 5 isolates from urban population areas (25%) and 15 isolates from rural population areas (75%) (Figure 3).As for mortalities due to cholera infection, only one case found in a rural population (5%) (Figure 4).

Molecular results of the present study showed that all *V. cholerae* isolates contained *recA* gene with PCR technique because it is fast, accurate and sensitive in distinguishing bacterial species. The presence of a recA gene in all isolates confirms that these isolates are identified as *V. cholerae* isolates as shown in Figure 5

The *recA* gene is a multifunctional protein that contributes to identical re-binding, DNA repair and decomposition of DNA (18). The genetic diversity of this gene reflects the differences in the genome so it can be used as a predictor of the genomic relationships (19). The *recA* gene is highly susceptible to environmental stresses, resulting in point mutations, but they do not affect the general functions of the gene and its structure (20).

In addition, the sequence of alkaline in the *recA* gene is more distinct than the sequence of alkaline in 16SrDNA (21). This study recommends the use of *recA* gene to determine genetic relationships and differentiations among different species by analyzing the sequence of alkaline for this gene because it has diagnostic and molecular properties and characteristics.

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