A comparative study among clinical and environmental isolates of *Vibrio cholerae* at their antibiotic resistance and plasmid profile

دراسة مقارنة لمقاومة المضادات الحيوية والمحتوى البلازميدي لعزلات مرضية وبيئية لبكتريا *Vibrio cholerae*

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Abstract

In this study, the comparison of the antibiogram and molecular level among different serotype of clinical and environmental isolates of *V. cholerae* was done. Antibiogram of five clinical and three environmental isolates showed high susceptibility to all tested antibiotics, but non-O1 serotype had Ampicilin resistant. Plasmid profile screening revealed that *V. cholerae* O1 isolates and non-O1 Dial131 had no small size plasmid while non-O1 ab, 1J, 1R harbored three different small size plasmids with different appearance profile, two mega plasmids detected in the O1 isolate and only one mega plasmid resolute in non-O1 clinical and environmental isolates.

المستخلص

اظهر التحري عن المقاومة للمضادات الحيوية لخمس عزلات مرضية وثلاثة بيئية حساسية عالية لجميع المضادات المفحوصة عدا عزلات النمط المصلي غير المتلازن مع O1 حيث اظهر مقاومة لمضاد الامبسلين . اثبت التحري عن العوامل الوراثية البلازميدات ان عزلات النمط المصليO1 والعزلة البيئيةDial131 غير المتلازنة مع النمط المصلي O1 خالية من البلازميدات صغيرة الحجم بينما احتوت العزلة المرضية غير المتلازنة non-O1 ab والعزلات البيئية الاخرى على ثلاث بلازميدات مختلفة الحجم والانماط . امكن الكشف عن بلازميدين كبيري الحجم في عزلات النمط O1 وبلازمد مفرد كبير الحجم في عزلات النمط non-O1 المرضية والبيئية .

Introduction

Vibrio cholerae is an environmental species that has been linked with seven pandemics of cholera since 1817. More than 200 serogroups of *V. cholerae* have been identified to date; however, epidemics cholera has been associated with a limited number of closely related strains of O1 and O139 seotypes [1]. Also epidemic cholera has been well documented in many countries as being spread by contaminated water whereas sporadic cases of cholera have been associated with the aquatic environment [2]. While, [3] mentioned that the survival of *V. cholerae* in the environment was thought to be short less than one week in sea water; contaminated water required continual recontamination with feces from carriers of the organism. Therefore, the need for the emergence of newer molecular subtyping approaches which can identify specific outbreak strains and can often determine the geographic origins of strains [2].

Treatment of cholera consists essentially of replacing fluid and electrolytes. The solutions contain a usable energy source such as glucose, antibiotic like tetracycline and deoxycycline and ciproflaxin, to which *vibrios* are generally sensitive [1]. However, in 1979, an outbreak of cholera due to multiple -drug resistant *V. cholerae* O1 occurred in Mtlab, Bangladesh. The outbreak showed that 16.7%

were resistant to five antibiotics including tetracycline in which a conjugative antibiotic resistant plasmid was identified in these isolates [4]. While, [5] mentioned that the susceptibility of *V. cholerae* O1 strains to certain antibiotics changes depending on the isolation time and geographical location.

On the other hand, plasmid profile analysis was one of the first subtyping tools and has been an effective epidemic marker system for many diarrheal pathogens. It has been of limited value for *V. cholerae* O1; since most susceptible strains to antimicrobial agents, appear to be plasmidless [2].

The present study was undertaken with two goals: (1) to screen the antibiotic susceptibility and plasmid profile analyses from clinical isolates of O1 and non-O1 and the environmental isolates of *V. cholerae*. (2), to compare, at a molecular level the differences among clinical and environmental isolates of *V. cholerae.*

Materials and methods

Bacterial isolates

Eight isolates of *V. cholerae*; five were collected from clinical specimens and three were represented to environmental isolates were used in this study [6, 7]. Their designations, serogroupes and types of toxins produce were indicated in Table (1).

Table(1): Serotypes and toxins production of clinical and environmental isolates of *V.cholerae*

Isolate name		Clinical isolates		
	Serotype	TCP	CT or CT-like	ST
***V. cholerae* S**	**O1 Ogawa**	+	+	-
***V. cholerae* 18**	**O1 Inaba**	+	+	-
***V. cholerae* 22**	**O1 Inaba**	+	+	-
***V. cholerae* 13**	**O1 Ogawa**	+	+	-
***V. cholerae* ab**	**Non-O1**	+	+	+
		Environmental isolates		
***V. cholerae* 1J**	**Non-O1**	+	+	-
***V. cholerae* 1R**	**Non-O1**	-	-	-
***V.cholerae* dial131**	**Non-O1**	-	-	-

TCP= toxin coregulated pili; CT= cholera toxin; ST=heat stable toxin; += positive; -=negative

Antibiotic susceptibility test

Each isolate was tested for susceptibility to twelve antibiotics by the disc incorporation method (8) using Muueller- Hinton agar. The following antibiotics were used (microgram per disk): Ampicillin, 10; Amoxicillin, 25; Cephazolin, 30; Ciproflaxin, 5; Chloramphenicol,30; Cloxacillin, 1000; Gentamycin, 10; Nalidixic acid, 30; Nitrofurantin,300; Rifampicin,5; Streptomycin, 10; Tetracycline, 30.

Plasmid profile

DNA was extracted from clinical and environmental isolates and plasmid profile was screened from each isolate firstly addapting salting out method described by [9] with few modification in which using small volume of bacterial cultures and omitting the use of proteinases as described below:

Bacterial growth from overnight cultures were centrifuged, decanted the supernatant and bacterial pellet was resuspended with 1 ml of SET buffer (75mM NaCl, 25mM EDTA-Na_2, 25mM Tris base pH 8). Solution of 10% Sodium dodecyl sulfate solution (SDS) was added to give 1% as a final concentration of SDS. Bacterial lysates were watched for complete lysis. To each tube volume of 4M sodium chloride solution was added to give 1.2M of sodium chloride as a final concentration. Bacte

mixed with chloroform; isopropanol was added to upper layer and kept at room temperature for 30 min. DNA pellets were resuspended using TE buffer (10 mM Tris base, 1mM EDTA-Na_2 pH8). Complete DNA resuspended was achieved by incubation tubes at 55°C water bath.

Another method was adopted for plasmid profile screening based on [10] with modification briefly, bacterial growth from overnight cultures were centrifuged, decanted the supernatant and bacterial pellet was resuspended with 1 ml of lysis buffer which consist of 50mM Tris base, 50mM EDTA-Na_2, 8% sucrose and 0.03% Triton X100 without using proteinase and Rnase, after complete lysis tubes were removed to boiling water bath for 45 sec, bacterial debris was removed with toothpick and 5%cetydl-trimethyl ammonium bromide (CTAB solution) was added at 1/10 volume. Tubes were left at room temperature for 5 min, centrifuged, supernatant was removed and solution of 1.2 M sodium chloride solution was added to dissolve the pellet. Finally isoprobanol was added and left for 30 minute at room temperature, centrifuged and pellets resuspended by 100 µl of TE buffer.

Agarose gel electrophoresis

Each sample of DNA was prepared by mixing 20 µl of DNA sample with 5 µl of loading buffer (Bromophenol blue 0.25 and 15% Ficol).

Horizontal gel electrophoresis of 1% agarose was used for detection of DNA which visualized with the aid of Ethedium bromide and UV transilluminator. Tris borate buffer TBE 5X(Tris base 54 mM, boric acid 27mM and Edta-Na2) was used to prepare TBE 1X buffer which added to the electrophoresis tank, tray with agarose was immersed in electrophoresis tank and buffer was about few milliliters above the surface of agarose. Each well was loaded with 25 µl sample and standard molecular weight DNA lader (λ DNA EcoRI+Hind III marker) was loaded in a well. Tank was closed and electrophoreses run at 5 volt/cm of the gel. Agarose was removed from the tank and visualized with the aid of UV transilluminator and photographed [11].

Results and Discussions

The results showed that all clinical and environmental *V. cholerae* either sensitive to all the antibiotics used except the clinical non-O1 ab and the three environmental isolates that shows resistant to ampicillin as shown in Table (2). These results were closely resembling to those obtained by [12] who tested different serogroups of *V. cholerae* to a variety of antimicrobial agents. But on the contrary to the results of [13] who found that clinical strains of *V. cholerae* were resistant to a greater number of antibiotics.

Clinical O1 *V. cholerae* which belongs to Ogawa and Inaba serotype, nonO1 and the three environmental isolates showed high sensitivity to tetracycline which is regard as the drug of choice. The outcome is in agreement with the result of [14] who found that Ogawa isolates in Iraq sensitive to Tetracycline but 29% of Inaba isolates reflected Tetracycline resistant, and disagreement with the finding of [15] which indicated 100% Tetracycline resistant among Iranian isolates of *V. cholerae.* Also, the results are in disagreement with [16] who found that 75% of Clinical non-O1 had Tetracycline resistant.

Table (2): Antibiotic susceptibility of *V. cholerae* isolates

Types of antibiotic	Susceptibility results							
	Clinical isolates					Environmental isolates		
	S	18	22	13	Ab	1R	1J	Dial131
Ampicillin	S	S	S	S	R	R	R	R
Cephazolin	S	S	S	S	S	S	S	S
Chloramphenicol	S	S	S	S	S	S	S	S
Ciproflaxil	S	S	S	S	S	S	S	S
Cloxacillin	S	S	S	S	S	S	S	S
Gentamycin	S	S	S	S	S	S	S	S
Nalidixic acid	S	S	S	S	S	S	S	S
Nitrofurantin	S	S	S	S	S	S	S	S
Rifampicin	S	S	S	S	S	S	S	S
Streptomycin	S	S	S	S	S	S	S	S
Tetracyclin	S	S	S	S	S	S	S	S

S: Sensitive

R: Resistant

The results indicated that the antibiogram of the three environmental isolates were very similar or closely related to clinical non-O1 *V. cholera*. For this purpose non- O1 non-O139 strains should be monitored carefully not only to detect new serotypes with a possible epidemic potential but also to determined the development, mechanisms of antibiotic resistance, and the demonstration that the modification of a lineage that is already epidemic or closely related to such a clone may be the cause of existing of a new strains. The susceptibility of isolates to a wide range of antibiotic tested in this study which used extensively in diarrhea treatment may be due to the non epidemic occurrence of disease in Iraq at the last years in addition to the fact that cholera disease was treated primarily with Tetracycline antibiotic and water replacement therefore, there was no chance for antibiotic resistant emergence.

Plasmid profile

The constraction of plasmid profile was be one of the characterization techniques which is based on the genetic content of strains and gave the relationship between them. The results of both extraction methods showed that only clinical non-O1ab and environmental isolates 1R and 1J harbored three different small size plasmid bands migrated before chromosomal fraction on agarose gel while, clinical O1 *V. cholerae* contained no small size plasmid as shown in figure (1). The non-O1 *V. cholerae* was found to harbor more than one plasmid and 83% of clinical O1 *V. cholerae* isolated in Iraq harbored no small size plasmid [14].

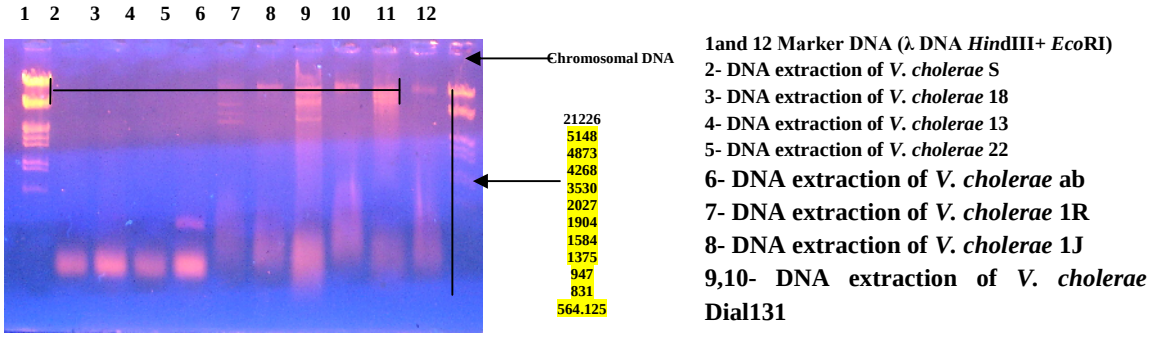
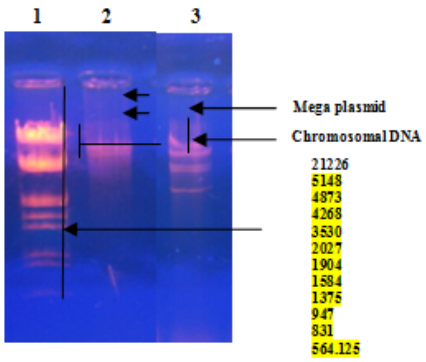


Fig (1): Gel electrophoresis of DNA extraction of *V. cholerae*
(1% agarose; 5 volt/cm; 1.5 hor electrophoresis)

This small size plasmids which were gained by isolates (ab,1R,and 1J) may or may not express any phenotypic characters a suggestion that was confirmed by earlier work of [12] which clarified that the existing of low- molecular weight plasmid that do not express any phenotypic characters. Our findings revealed a relationship might exists among the Ampicillin resistant of non-O1 ab, 1J, and 1R and plasmid content. In spite of this relationship, the results found that plasmidless environmental Dial 131 isolate showed also Ampicillin resistant. The Ampicillin resistant may be due to the presence of R plasmid that encodes for antibiotic resistant determinants, or to another kind of plasmid which could transfer between bacteria as found [17] that *V. cholerae* contain conjugative plasmid pSj15 responsible for Ampicillin resistant transfer.

The results of agarose gel electrophoresis clarified a considerable diversity among *V. cholerae* clinical non-O1 ab, and environmental 1J, 1R, Dial 131 attributed to the dissimilarity in overall genotype suggesting that the original of clinical non-O1 was not the environment. Alternatively, even the environment was the source; the clinical isolate had apparently acquired extra elements in their genome to differ significantly in overall genotype from their environmental counterpart. According to [18] and [13] demonstration in a species that has pathogenic and non- pathogenic microorganisms, the pathogenic counterparts frequently possess one or more large genetic insert known as "pathogenic island" which contains genes exclusively associated with pathogenic phenotype; likewise non-O1, non-O139, serogroups capable of initiating a cholera-like disease may have had some unknown supplementary element present in their genome, which are likely to contribute to the capacity for virulence as compared with the innocuous environmental strains.

Gel electrophoresis also showed that both of the extraction methods succeeded in the extraction of mega plasmid from all clinical O1 and non-O1 and environmental isolates. In addition, *V. cholerae* S,18,22,13, which contained another mega plasmid, did not exist in the extraction of non-O1 isolates Figure (2), the study results were in accordance with the report of [15] when found a naturally conjugative plasmid of 100 Kb in 61% of the isolates of *V. cholerae* O1, from different provinces in Iran. The second mega plasmid may be related with a kind of plasmid known as toxin -linked cryptic plasmid (pTLC) which accordingly all toxigenic strains of O1 and O139 carried the nucleotide sequence of this plasmid, but non toxigenic strains did not [19].



1. Ladder of DNA marker;
2. DNA sample of *V. cholerae* S;
3. DNA sample of *V. cholerae* 1J

Fig (2): Megaplasmid isolation from *V. cholerae* S and *V. cholerae* 1J (1% agarose; 5 volt/cm; 3 hor electrophoresis)

The study investigation reflects that the yield of chromosomal fraction with modified salting out was greater than the yield of DNA obtained by modified CTAB minipreparation and this might be due to the specification of the Salting out method in the isolation of large fragment with high yield of DNA [9] However, the preparation of DNA by modified CTAB method gave very clear DNA extraction Figure (3) agreement with work of [20] who used the detergent CTAB in DNA preparation. The purity of DNA extraction with CTAB may be due to property of the cationic detergent that while proteins and neutral polysaccharide remain in solution in its presence at low- salt concentration, less than 0.6 molar, nucleic acid and neucleic polysaccharide which had negative charge would precipitate.

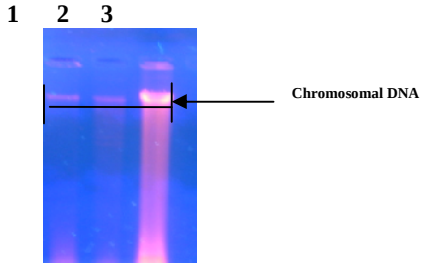


Fig (3): Gel electrophoresis of DNA extraction of *V. cholerae* S by modified CTAB minipreparation methods (1,2) and by modified salting out (3) (1% agarose; 5 volt/cm; 1.5 hor electrophoresis)

The modification of both methods based on the ability of lysis buffer alone to degrade the cell wall and cause cell lysis without the need for lysozyme presence which is responsible for peptedoglycan degradation because the cell wall of gram negative bacteria consists mainly of asymmetric lipid bilayer the outer membrane which arises in part from the presence of LPS which is external to the peptedooglycan and acts as a permeability barrier. So lysis of gram negative bacteria requires the outer membrane to be permilibilized and release the LPS and this was done by substances including chelator of divalent cations the EDTA, polycationic species and various small molecules such as Tris which often used as buffer in lysis methods as described by [11,21] and with the presence of detergent making hydrophobic interaction with released lipids to form micells in aqueous solution , SDS or Triton X100 was used extensively as a selective solubilization of membrane and served as denaturation agent for protein in DNA extraction methods .

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