# Molecular study of *Vibrio cholerae* O1 serotype Ogawa and non-O1/139 isolated from the environment in Tunisia

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## ABSTRACT

**Objective:** *Vibrio cholerae* represents an enormous public health burden, especially in developing countries. In this study we report for the first time the incidence of *V. cholerae* isolated in Tunisia during a 6-year period (2007-2012).
**Methods:** Forty-six *V. cholerae* isolates were isolated from wastewater, patients and aquatic environment. The isolates were identified as O1 Ogawa (n=16) and non-O1/non-O139 (n=30) and were investigated by determining the antibiotic susceptibility, virulence genes, plasmid content, and genetic relationship by PFGE.
**Results:** 84.7 % of isolates were susceptible to all antibiotics, 8.7 % and 6.5% were resistant to 2 and 3 antibiotics, respectively. Fifteen non-O1/139 isolates harbored plasmids and a common ca-7kb plasmid was observed. The PCR for virulence-associated genes within the CTX element and the VPI gene cluster showed that *ctxA*, *zot* , and *tcpA* genes were detected each in 75 % of isolates; the *ace* gene in 50% of isolates. In non-O1/139 isolates, *ace* gene was amplified in 43.3% of isolates and *ctxA*, *tcpA*, and *zot* genes were found each in 6.6% of isolates. The clinical non-O1 isolates were negative for tested genes. For *V. cholerae* O1 isolates, three virulence-genes combination were detected: *ace-ctxA- tcpA-zot* (7 isolates), *ctxA- tcpA-zot* (5 isolates), and *ace-ctxA* (one isolate). PFGE owed five pulsotypes, among which the majority of isolates clustered in the pulsotype A.
**Conclusions:** The occurrence of toxigenic *V. cholerae* isolates in treated wastewater and aquatic environment is worrisome and warrant for a risk of possible biodiversity and human infection in the future.

**KEY WORDS:** *Vibrio cholerae* O1
Tunisia
Wastewater
Virulence genes

## Introduction

*Vibrio cholerae* is the causative agent of cholera. Seven documented cholera pandemics have claimed millions of lives and the disease continues to affect 3-5 million people annually [1,2]. *V. cholerae* is subdivided into more than 208 somatic O-antigen serogroups, but only two serogroups, O1 and O139, are recognized as pathogenic for man and as responsible for epidemic and pandemic cholera [3]. There are two biotypes of *V. cholerae* O1; classical and El Tor, which are believed to have evolved from separate lineages [4]. The El Tor biotype have better adaptability to survive in the environment and in the human host, whereas the Classical biotypes are more toxigenic and cause severe form of illness than El Tor strains [5]. Non-O1 and non-139 serogroups are ubiquitous in the aquatic environment and comprise a heterogeneous group of organisms whose clinical association with human is inadequately understood [6]. Historically, O1 strains have been responsible for all major epidemics, including seven pandemics [7]. In 1992, an epidemic clone of serogroup non-O1/O139 (Bengal) appeared in southern India [8].

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In contrast, non-O1/non-O139 serogroups of *V. cholerae* may cause sporadic diarrheal episodes ranging from mild to severe morbidity [4]. In *V. cholerae*, the major virulence genes appear to exist in clusters, and there are at least two regions of the *V. cholerae* chromosome in which genes encoding virulence factors are clustered [9]. These include the

CTX element, which has now been shown to be the genome of a filamentous bacteriophage, and the toxin-coregulated pilus (TCP)-accessory colonization factor (ACF) gene cluster, referred to as the toxin-coregulated pilus (TCP) pathogenicity island [4]. The CTX element is a 4.5-kb core region mainly identified in toxigenic *V. cholerae* O1 and O139, but is not found in non-toxigenic strains. It carries at least six genes, including the cholera toxin (CT) (ctxAB encoding the A and B subunits of CT), zot (encoding zonula occludens toxin), cep (encoding core-encoded pilin ace (encoding accessory cholera enterotoxin and orfU (encoding a product of unknown function, but possibly implicated in CTXΦ morphogenesis) [4]. The TCP, type IV pilus, is the most important colonization factor required for intestinal colonization. Structurally, the TCP is thought to be a polymer composed of a single structural subunit, TcpA, and also serves as the receptor for the CTXΦ bacteriophage. The genes required for TCP synthesis, including tcpA, as well as the acf genes and the genes encoding the transcriptional activators ToxT and TcpP are located on a ca.40-kb Vibrio Pathogenicity Island (VPI) [4].

Our laboratory, the National Centre of Salmonella, Shigella and *Vibrio* spp-Pasteur Institute of Tunisia, received many samples of wastewater from the Regional Hygiene of Ben Arous, during the period from July 2012 - August 2012, as part of systematic analysis of wastewater performed for surveillance and monitoring. From those wastewater samples, *V. cholerae* O1 serotype Ogawa was isolated in our laboratory. However, there were no reports of human illness due to cholera. The Tunisian Ministry of Public Health issued a statement on Thursday, August 3rd, 2012 assuring Tunisians that no cases of human illness due to cholera have been found. Thus, this finding presented no risk to the public health, as there have been no cases of human in Tunisia since 1982.

The aim of this study was to investigate the existence of *V. cholerae* in wastewater, aquatic environments and cases of human infections in Tunisia during a 6-year period (2007-2012). Phenotypic and genotypic characterizations were undertaken by studying antibiotic susceptibility patterns, virulence gene profiling, plasmid contents, and genetic fingerprinting using Pulsed Field Gel Electrophoresis (PFGE).

## Materials and Methods

### Isolation and identification of bacterial strains

During the period of 2007-2012 a total of 46 *V. cholerae* isolates were isolated from three patients and from aquatic environment from different regions in Tunisia (Figure1, Table 1): Sousse, Nabeul, Ariana, Rades, Mellasine (a province of Tunis, near Lake Sijoumi), Hammam Chat, Sfax, and Nouvelle Madina. These regions, located all along the Mediterranean Sea, are areas for factories and tourism, also point for wastewater treatment so it can be used for the irrigation of golf course, green way or park. If it is sufficiently clean, it can also be used for groundwater recharge or agricultural purposes.

These strains were conserved at the National Center of *Salmonella*, *Shigella* and *Vibrio* spp. - Pasteur Institute of Tunisia. Two closely related bacteria *Aeromonas hydrophila* (2 strains) and *Aeromonas sobria* (1 strain) were also used as control strains. Only presumptive *V. cholerae* (n= 46) isolates were investigated in this study. Frozen stock cultures were subcultured on Luria-Bertani (LB) broth (Sigma), then streaked onto thiosulfate-citrate-bile salt-sucrose agar plate (TCBS) (Oxoid) and incubated overnight 37°C to verify purity. Pure cultures of the bacterial isolates were subjected to standard morphological, biochemical tests, Gram staining, oxidase test (BioRad-France), morphology and motility test. At the same time, the Vibrio isolates were subjected to biochemical identification using a commercial API 20E kit (BioMérieux, France). API Web software was used to identify the strains biochemically. All the strains were serotyped using polyvalent O1, monospecific Inaba and Ogawa antisera and also monoclonal O139 antiserum (Bio-Rad, France) according to the manufacturer's specification.

**Table 1.** Phenotypic and genotypic characteristics of *Vibrio cholerae* strains isolated in Tunisia during the period 2007 – 2012.

| Isolation Date | Code | Isolate | Source | Locality | Resistance profile | Virulence genes | PFGE | Plasmid profile |
|---|---|---|---|---|---|---|---|---|
| 12/2/2007 | 170 | *V.cholerae* non O1 | Treated Wastewater | Ariana | - | - | C | 7 |
| | 171 | *V.cholerae* O1-Ogawa | Untreated Wastewater | Ariana | AMP, CEF | - | C | - |
| | 188 | *V. cholerae* non O1 | Untreated Wastewater | Ariana | - | *ace* | A | - |
| 12/6/2007 | 761 | *V.cholerae* non O1 | Lake water | Nabeul | AMP, CEF ,AMC | - | A | 7 |
| | 762 | *V. cholerae* non O1 | Lake water | Nabeul | – | - | A | - |
| 28/6/2007 | 857 | *V.cholerae* non O1 | Untreated Wastewater | Ariana | – | - | D | 7 |
| | 858 | *V.cholerae* O1-Ogawa | Untreated Wastewater | Ariana | - | - | E | - |
| 3/7/2007 | 883 | *V.cholerae* O1-Ogawa | Untreated Wastewater | Ariana | – | - | A | - |
| | 884 | *V.cholerae* non O1 | Treated Wastewater | Ariana | – | *ace* | A | 7 |
| | 885 | *V.cholerae* non O1 | Treated Wastewater | Ariana | - | - | A | - |
| | 886 | *V.cholerae* non O1 | Treated Wastewater | Ariana | TET | *ace* | A | 7 |
| | 887 | *V.cholerae* non O1 | Treated Wastewater | Ariana | - | - | A | - |
| | 888 | *V.cholerae* non O1 | Treated Wastewater | Ariana | - | - | A | - |
| | VcNA | *V.cholerae* non O1 | Non diarrheagenic stool | Mellasine | - | - | A | - |
| 18/7/2007 | 1V1(T) | *V.cholerae* non O1 | Sea water | Rades | – | *Ace/ ctxA/ tcpA/ zot* | C | 7 |
| | 1V2(T) | *V.cholerae* non O1 | Sea water | Rades | – | *ace / ctxA/ tcpA/ zot* | A | 7 |
| | 2V1(T) | *V. cholerae* non O1 | Sea water | Rades | – | - | C | 7 |
| | 2V2(T) | *V. cholerae* non O1 | Sea water | Rades | – | *ace* | A | 7 |
| | 3V2(R) | *V. cholerae* non O1 | Sea water | Rades | - | - | A | 53, 8, 7, 5.5, 5, 4 |
| | 4V(R) | *V. cholerae* non O1 | Sea water | Rades | - | *ace* | A | 7 |
| 28/8/2007 | 7V(R) | *V. cholerae* non O1 | Sea water | Rades | AMP, CEF, AMC | - | A | - |
| 25/9/2007 | 11V1(T) | *V. cholerae* non O1 | Sea water | Rades | KAN | - | A | 7 |
| 3/1/2008 | 19V(R) | *V. cholerae* non O1 | Sea water | Rades | AMP, CEF, CHL | *ace* | A | - |
| 4/2/2008 | 25V1(R) | *V. cholerae* non O1 | Sediment | Rades-Tunis Gulf | – | *ace* | A | - |
| | 25V2(R) | *V. cholerae* non O1 | Sediment | Rades-Tunis Gulf | – | *ace* | A | 7 |
| 20/5/2008 | 33V1(R) | *V. cholerae* non O1 | Sediment | Rades-Tunis Gulf | - | *ace* | A | - |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 33V2(R) | *V. cholerae* non O1 | Sediment | Rades-Tunis Gulf | AMP, CEF | - | A | - |
| 11/6/2008 | 894 | *V. cholerae* non O1 | Wastewater | Ariana | – | - | A | - |
| | 895 | *V. cholerae* non O1 | Wastewater | Ariana | – | - | A | - |
| 16/7/2008 | 1078 | *V. cholerae* non O1 | Blood culture | Sfax | – | - | B | - |
| 13/10/2008 | 1494 | *V. cholerae* non O1 | Blood culture | Sfax | – | - | A | - |
| | 1495 | *V. cholerae* O1-Ogawa | Wastewater | Sousse | – | *Ace* / *ctxA*/ *tcpA*/ *zot* | A | - |
| 29/09/2010 | 1730 | *V. cholerae* O1-Ogawa | Untreated Wastewater | Hammam Sousse | AMP, CEF | *Ace* / *ctxA*/ *tcpA*/ *zot* | A | - |
| 24/09/2010 | 1729 | *V. cholerae* O1-Ogawa | Wastewater | Hammam Sousse | – | *ctxA*/ *tcpA*/ *zot* | A | - |
| 30/4/2011 | 584 | *V. cholerae* O1-Ogawa | Sea water | Sousse | – | *ace* / *ctxA*/ *tcpA*/ *zot* | B | - |
| | 647 | *V. cholerae* O1-Ogawa | Treated Wastewater | Hammam Sousse | – | *ace* / *ctxA*/ *tcpA*/ *zot* | B | - |
| 9/5/2011 | 580 | *V. cholerae* non O1 | Pond in golf yard | Sousse- Kantaoui | TET | *ace* | A | 7 |
| | 581 | *V. cholerae* non O1 | Pond in golf yard | Sousse- Kantaoui | TET | *ace* | A | 7 |
| 13/5/2011 | 585 | *V. cholerae* O1-Ogawa | Sea water | Sousse | – | *ace* / *ctxA*/ *tcpA*/ *zot* | B | - |
| 26/5/2011 | 649 | *V. cholerae* O1-Ogawa | Sea water | Sousse | – | *ace* / *ctxA*/ *tcpA*/ *zot* | B | - |
| 2/6/2011 | 648 | *V. cholerae* O1-Ogawa | Treated Wastewater | Sousse- Sidi Bou ali | – | *ctxA*/ *tcpA*/ *zot* | B | - |
| 9/6/2011 | 714 | *V. cholerae* O1-Ogawa | Untreated Wastewater | Sousse- Sidi Bou ali-Oued Essoud | – | *ctxA*/ *tcpA*/ *zot* | A | - |
| 29/6/2011 | 799 | *V. cholerae* O1-Ogawa | Treated Wastewater | Hammam Sousse | – | - | B | - |
| 13/7/2011 | 1162 | *V. cholerae* O1-Ogawa | Treated Wastewater | Hammam Sousse | – | *ace* / *ctxA* | B | - |
| 21/7/2011 | 1163 | *V. cholerae* O1-Ogawa | Treated Wastewater | Sousse-South | – | *ace* / *ctxA*/ *tcpA*/ *zot* | B | - |
| 12/6/2012 | 923 | *V. cholerae* O1-Ogawa | Untreated Wastewater | Nouvelle medina-Mé-llien | AMP, CEF | *ctxA*/ *tcpA*/ *zot* | A | - |

**Genomic DNA extraction**

DNA was extracted from bacterial cells by boiling method as described previously by Al-Gallas et al [10].

**PCR assays for16S-23S IGSs and virulence genes**

The PCR primers used for the amplification of DNA fragments spanning the 16S-23S IGSs were targeted at highly conserved regions of the 3' and 5' ends of the 16S and 23S rDNA, respectively. Primers and PCR amplification conditions were as described [11]. Non- *V. cholerae* isolates (*V.* cludens toxin gene), and *tcpA* (toxin co-regulated pilus gene) [12]. PCR was performed with a thermal cycler (Perkin-Elmer). The cycling profile was as follows: initial denaturation at 94°C for 5 minutes, followed by 30 cycles consisting of 94°C for 1 minutes, 60°C for 1 minutes, and 72°C for 1 minutes and a final extension step of 72°C for 10 minutes. The *ace* gene (accessory cholera enterotoxin) was investigated by simplex PCR. The amplification program for the *ace* gene began with denaturation at 94°C for 1minutes, which was followed by 29 cycles consisting of *alginolytica*, *V. mimicus*, *V. vulnificus*, *V. metschnikovii*, and *V. parahaemolyticus*) from our collection were used as control in order to compare IGS profiles of *V. cholerae* and non- *V. cholerae* isolates. *Aeromonas hydrophila* (2 strains) and *Aeromonas sobria* (1 strain) were used as negative control strains.

Multiplex PCR assay was performed to detect the presence of toxin genes *ctxA* (cholerae toxin gene), *zot* (zonula oc

94°C for 30s, 55°C for 30s, and 72°C for 30s and a final extension step of 72°C for 5 minutes [12]. Amplified products were separated on 1% agarose gel, stained with ethidium bromide, and photographed. The following reference strains were used as positive control: *V. cholerae* O1 MTCC 3906 ElTor (MTCC 3906), *V. cholerae* O1 Classical 569B (Classical), *V. cholerae* nonO1-nonO139 NT5394 (NT5394). They were obtained from Institut National des Sciences et Technologies de la Mer (INSTM), Salammbô, Tunisia.

**Figure 1.** (A) Map of Tunisia indicating the major regions. (B) Sites where Vibrio strains were isolated are circulated: Capital Tunisia with the provenince( Rades, Hammam chat, Ezzahra, Mellasine), Ariana, Nabeul, Sfax and Sousse.

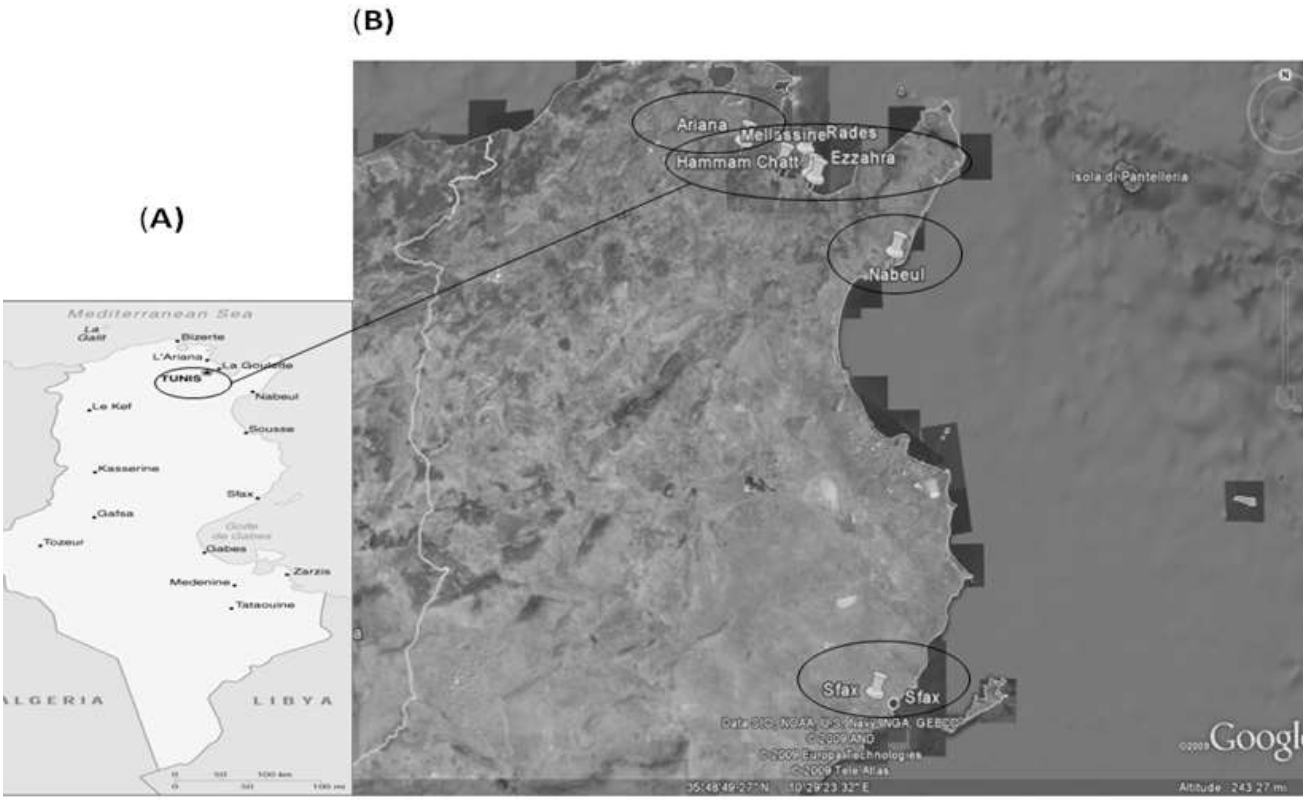
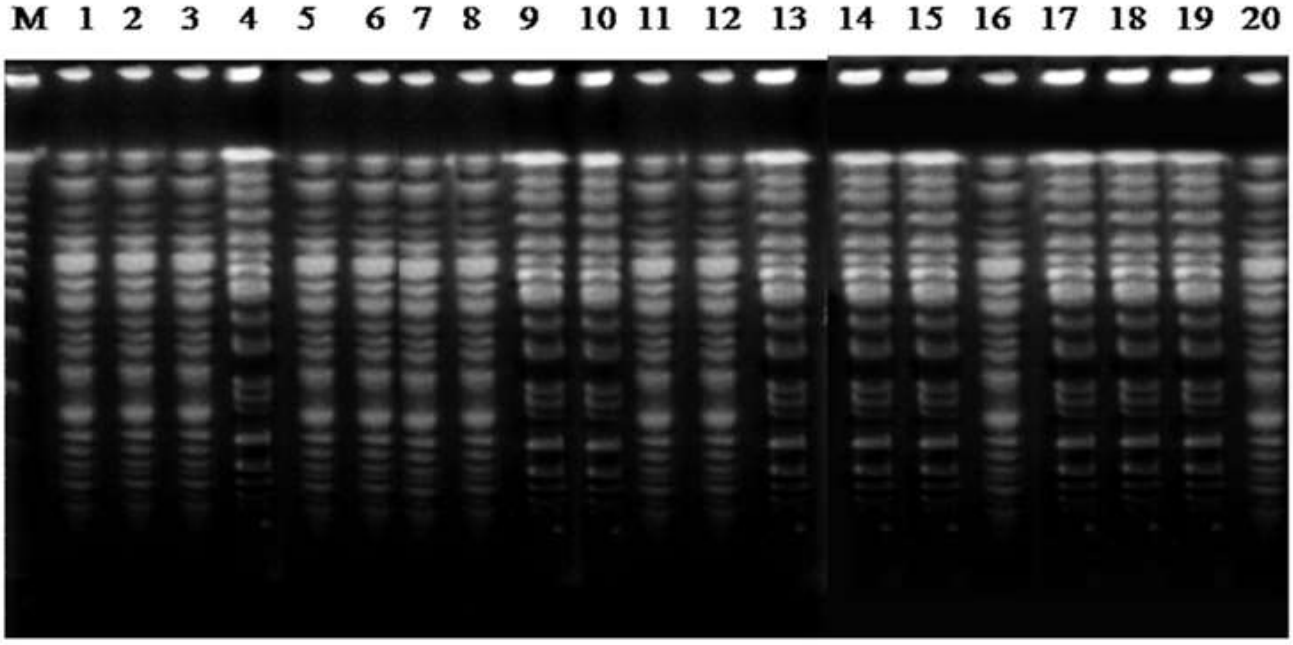


**Figure 2**. PFGE fragment patterns of *NotI*-digested total cellular DNAs from *V. cholerae* isolates. M: Standard Lambda ladder (Bio-Rad, France); lanes (pulsotypes), 1 : 33V2 (A); 2:894(A) ; 3:895 (A) ; 4: 1078 (B) ; 5: 1494 (A) ; 6: 1495 (A) ; 7: 1730 (A) ; 8: 1729 (A); 9: 584 (B); 10: 647 (B); 11: 580 (A) ; 12: 581 (A) ; 13: 585 (B); 14: 649 (B) ; 15: 648 (B) ; 16: 714 (A); 17: 799 (B) ; 18: 1162 (B); 19: 1163 (B); 20: 923 (A)




**Plasmid analysis**

Plasmid DNA was isolated, for the *Vibrio* strains, by the alkaline lysis method as described [13]. The approximate molecular sizes of plasmids were determined by comparison with plasmids of known size from *Escherichia coli* K-12 V517 (53.7, 7.2, 5.4, 5, 4, 3, 2.6 and 2 kb) as described [13].

**Antibiotic susceptibility testing**

Antimicrobial susceptibility was determined by the disc diffusion method on Mueller Hinton agar according to the recommendations of the Antibiogram Committee of the French Society of Microbiology(CA-SFM) [14].The following antimicrobial agents (Bio-rad) were tested (µg/disc): ampicillin (AMP, 10µg), cephalothin (CEF, 30µg); cefotaxime (CTX, 30µg); amoxicillin-clavulanic acid (AMC, 20/10µg), trimethoprim-sulfamethoxazole (SXT, 1.25/23.5µg ); kanamycin, (KAN, 30UI), gentamicin (GEN,10µg), erythromycin (15µg), streptomycin (STR, 10UI), nalidixic acid (NAL, 30µg ), ciprofloxacin (CIP,5µg ), ofloxacin (OFX, 5µg), tetracycline (TET, 30µg), chloramphenicol (CHL, 30µg), and the vibriostatic agent O/129 (10µg). *Salmonella cholerea suis* ATCC14028 was used as a control strain. Classification of isolates as susceptible, intermediately resistant, or resistant was determined by OSIRIS Software-Version 3X (Bio-Rad).

**Genomic fingerprinting by Pulsed field gel electrophoresis (PFGE)**

Pulsed field gel electrophoresis (PFGE) analysis was performed according to a PulseNet standardized protocol for *Vibrio cholerae* subtyping [15]. PFGE banding patterns were analyzed visually and Patterns differing by 3 bands or more were considered as separate pulsotypes [16].

## Statistical analysis

Data entry and analysis were performed using SPSS version 16. The data were expressed as mean ± SD and percentages.

## Results

**Isolates confirmation by 16S-23S rDNA intergenic spacers (IGSs)**

The presumptive 46 *V. cholerae* isolates were identified by biochemical tests and were serotyped. Isolates were classified as: *V. cholerae* O1 (16 isolates) and *V. cholerae* non-O1/non-O139 (30 isolates). All *V. cholerae* O1 isolates were found to be El Tor biotype serotype Ogawa (Table 1),

14 of them were from wastewater in the treatment operation stations which supply water for irrigation of golf courses, green ways or parks. If it is sufficiently clean, it can also be used for groundwater recharge or agricultural purposes. The remaining two isolates were from sea water and lakes. Regarding the origin of *V. cholerae* non-O1/non-O139 isolates, three were from human origin. One isolated from a child who came to the health center for a non-diarrheal illness and had been included in the control group to study the etiology of acute diarrhea in Tunisia [18]. The other two strains isolated from blood cultures of two patients (one due to heart attack and the other for brain damage) in the ICU of Sfax Hospital. These strains sent to our National Center for identification and confirmation. Unfortunately, they didn't provide us with any other information about these two patients. The remaining isolates were from aquatic environments. The IGS profiles showed a unique IGS type. *V. cholerae* O1 and non-O1/non-O139 isolates gave a profile with DNA bands ranging from 700 to 1000 bp in size. The control non- *V. cholerae* isolates gave rise to a single 1000 bp DNA fragment. These profiles of DNA fragments were corresponded to the predicted size of the 16S-23S rDNA genes. PCR was carried out on total DNA of *Aeromonas hydrophila* (2 strains) and *Aeromonas sobria* and the results were negative.

**Virulence genes detection by PCR**

Amplification of the virulence genes *ctxA*, *zot* and *ace* located on the CTX cassette and *tcpA* from the pathogenicity island (VP1) were analysed in all isolates (Table 1). For *V. cholerae* O1 isolates, *ctxA*, *zot* , and *tcpA* (El Tor biotype) genes were detected each in 12 (75 %) isolates; the *ace* gene in 8 (50%) isolates. However, these frequencies are much lower for non-O1 isolates, except for *ace* gene. Indeed, *ace* gene was amplified in 13 (43.3%) isolates whereas *ctxA*, *tcpA*, and *zot* genes were found each in two (6.6%) non-O1 isolates. Investigated genes were not detected in 56.6% (17/30 isolates) and 25% (4/16 isolates) of isolates of non-O1 and *V. cholerae* O1 isolates, respectively. It is worthy to note that clinical non-O1 isolates were negative for tested genes.

For *V. cholerae* O1 isolates, three virulence-genes combination were detected: *ace-ctxA- tcpA-zot* (7 isolates), *ctxA-tcpA-zot* (5 isolates), and *ace-ctxA* (one isolate). Whereas for non-O isolates only the combination *ace-ctxA-tcpA-zot* (one isolates) was detected. Remarkably, for non-O1 isolates the *ace* gene occur alone in 11 isolates in contrast to O1 isolates where this gene, if detected, never found alone.

**Antibiotic resistance patterns**

Antibiotic susceptibility testing showed that 84.7 % (39 isolates) were susceptible to all antibiotics, 8.7 % (4 isolates) were resistant against 2 antibiotics, and 6.5 % (3 isolates) were resistant against 3 antibiotics (Table 1). Low rates of resistance were observed for tetracycline (3 isolates), ampicillin (7 isolates), cephalotin (7 isolates), and chloramphenicol (1 isolate), and kanamycin (1 isolate). No resistance was observed against streptomycin, gentamicin, nalidixic acid, ciprofloxacin, ofloxacin, erythromycin, the vibriostatic agent O/129, or trimethoprim-sulfamethoxazole. Among 16 *V. cholerae* O1-Ogawa isolates, only three isolates were resistants to antibiotics, being ampicillin and cephalotin. The clinical non-O1 isolates were susceptible to all antibiotics tested. These finding show that non-O1 isolates were more resistant than O1 Ogawa isolates. No correlation was found among antibiotic susceptibility patterns and the year and place of isolation of the strains.

**Plasmid profiles**

Fifteen of the *V. cholerae* non-O1 isolates harboured plasmids. A unique ca-7kb plasmid was observed in 14 isolates and one harboured six plasmids with approximate molecular sizes ranging from 7 to 53 Kb (Table 1). It is worthy to note that human *V. cholerae* non-O1 isolates were plasmid free, in addition all the *V. cholerae* O1 isolates were also plasmid free. No clear association between plasmid occurrence and antibiotic resistance was observed and strains with the same antibiotic resistance profile were plasmid container or plasmid-free, moreover, the multi-plasmid bearing isolate was pan-susceptible.

**Genetic relationship by Pulsed field gel electrophoresis (PFGE)**

To investigate the genetic relationship of our collection PFGE was used. Within the 46 isolates, we found 5 pulsotypes called arbitrarily A, B, C, D, and E, presenting 31, 9, 4, 1, and 1 isolates, respectively (Figure 2, Table 1 ). In *V. cholerae* O1, the predominant pulsotypes were B and A found in 8 and 6 isolates, respectively, and pulsotypes C and E where found each in one isolates. Non-O1 isolates showed predominance of pulsotype A presenting 25 isolates, the pulsotypes C, D and B were detected in 3, 1, and 1 respectively. It is of interest to highlight that the non-O1 isolated from blood culture (Strain 1494) and from stool culture (VcNA) were of pulsotype A, the third clinical isolate (Strain 1078) was of pulsotype B.

## Discussion

This study is the first scientific report from Tunisia characterizing *V. cholerae* at the molecular level, the last clinical report of *V. cholerae* non-O1 has been in 1993 reporting a septicaemia due to non-O1 isolate in old women suffering of carcinoma [17]. However, it is worthy to note that cholera is not a public health hazard in Tunisia owing to developed sanitary systems in Tunisia as well as the absence of many risk factors of cholera dissemination. In this report we try to characterize a collection of *V. cholerae* isolates during 2007-2012 in order to know the real epidemiological situation of *V. cholerae* in our country as well as to characterize the genetic background of these isolates.

In Tunisia, wastewater treatment consists of applying known technology to improve or upgrade the quality of wastewater. Usually wastewater treatment will involve collecting the wastewater in a central, segregated location (the Wastewater Treatment Plant) and subjecting the wastewater to various treatment processes. Most often, since large volumes of wastewater are involved, treatment processes are carried out on continuously flowing wastewaters (continuous flow or "open" systems) rather than as "batch" or a series of periodic treatment processes in which treatment is carried out on parcels or "batches" of wastewaters. Wastewater treatment can be categorized by the nature of the treatment process operation being used; for example, physical, chemical or biological in many stages. Chlorination used at all stages in treatment, it is considered to be a method by itself especially for disinfection. It involves the application of chlorine to the wastewater for the following purposes: Disinfection or destruction of pathogenic organisms, Prevention of wastewater decomposition, odor control [18]. The hygienic laboratories from all over the country involved in the routine control for pathogenic organisms in the wastewater before and after treatment.

During the period of 2007-2012 we collected 46 isolates of *V. cholerae*. This collection was done during routine surveys of wastewater from different point of treatment and different environmental sites; also from aquatic environment along the East Mediterranean border of Tunisia. In addition, three isolates were received from two hospitals for identification confirmation. We found 16 *V. cholerae* O1 biotype El Tor isolates of Ogawa serotype and 30 *V. cholerae* non-O1/non-O139 including three clinical isolates. In addition to be the first report of *V. cholerae* isolation in Tunisia since more than 30 years ago, this is also the first report of Ogawa serotype in Tunisia from an environmental source, indeed others teams have previously reported only non-cholerigenic vibrios as *V. parahaemolyticus*, *V. fluvialis* and *V. alginolyticus* from aquatic environments [19, 20].

Antimicrobial susceptibility study showed that 84.7 % were susceptible to all antibiotics, 8.7 % were resistant against 2 antibiotics, and 6.5 % were resistant against 3 antibiotics. These findings showed the general susceptibility to antibiotics of *V. cholerae* isolates, regardless of serotype, and it is important to note the low resistance rates or the absence of resistance against the most common antibiotics used in the treatment of cholera (e.g. tetracycline, erythromycin, quinolones, and trimethorime-sulfamethoxazole). Many studies have reported high rates of antimicrobial resistance especially against ampicillin, erythromycin, quinolones, and trimethoprim-sulfamethoxazol [21, 22, 23] in contrast to our findings.

However, it seems that antibiotic susceptibility of *V. cholerae* varied geographically due to the source of the studied isolates (clinical or environmental isolates) or differences in antibiotics usage in different parts of the world. It is also interesting to highlight that *V. cholerae* O1-Ogawa isolates were more susceptible than non-O1 isolates, only three were resistant and with the same resistance pattern (ampicillin + cephalotin). The three clinical isolates were also pan-susceptibles; unfortunately, we have no clinical data about these patients in order to know their clinical evolution. In *V. cholerae* resistance to antimicrobial agents can arise through many mechanisms such as spontaneous mutations in the bacterial chromosome (resistance to quinolone), horizontal gene transfer via self-transmissible mobile genetic elements, including SXT elements, mobile integrons, and conjugative plasmids [24]. Owing to many limitations, we studied only the plasmid content of our isolates. We found that only 15 isolates, all non-O1, harboured plasmids. The remarkable trait is the occurrence of a ca.7 Kb in all these plasmid-bearing non-O1/139 isolates regardless of antibiotic resistance profile, virulence genes content, or geographic origin. Therefore, it seems that this plasmid did not harbor genes encoding antibiotic resistance or virulence factors; however, it is plausible to harbor genes encoding degradation of organic products or heavy metals [25, 26]. In addition, the ca. 7kb plasmid might be also a cryptic plasmid that do not encoded any known function, indeed cryptic plasmids of low molecular weights have been reported previously in *V. cholera* isolates [27, 28]. In *Vibrio* species, as in other bacteria, cryptic plasmids might constitute a key precursor platform of genes capture and promoting dissemination of virulence-, and antibiotic resistance encoding genes or genes mediated environmental adaptive proteins [29]. Concerning the O1 El Tor Ogawa isolates, it is also of interest the absence of plasmid; however, it is not surprising regarding their susceptibility to antibiotics. Indeed, plasmids occurrence in *V. cholerae* O1 and non-O1 of clinical and, rarely, of environmental origins is common in resistant isolates [30].

Amplification of the virulence genes *ctx*A, *zot* and *ace* located on the CTX cassette and *tcp*A from the *Vibrio* pathogenicity island (VPI) differed among the 16 *V. cholerae* O1 El Tor Ogawa and the 30 *V. cholerae* non-O1 isolates analyzed. This confirms previous observations on the heterogeneity of virulence factors in environmental *V. cholerae* isolates [31]. Amongst 16 *V. cholerae* O1 isolates 12 (75 %) contained the *ctx* gene, this finding is much higher than previously reported rate of 16.5% in environmental O1 isolates in some Asiatic countries but similar (88.6%) to clinical isolates [7]. The occurrence of *zot*, *ace* and *tcpA* gene were also prevalent, in contrast to previously reported rates for environmental *V. cholerae* isolates [7]. Although rare, combinations containing natural deletions of either *zot*, and/or *ace* genes were also detected among O1 strains. According to the literature this finding is unexpected since the three genes (*ctxA*, *ace*, and *zot*) are part of the CTX genetic element [32]. This would indicate that deletions within the core dynamic sector of CTX element occur naturally [33] and it would be interesting to examine the core region of these strains and to study their pathogenesis power in experimental models. It is noteworthy, that occurrence of the *zot* or *ace* genes independent of the *ctx* gene has not been found in our isolates. However, the presence of the *zot* gene and the absence of the *ctx* gene in *V. cholerae* strains has been reported [34] contrarily to what reported else were [35] indicating that *zot* gene does not occur independently from *ctx* gene in *V. cholerae*. Therefore, a debate has been evocated about the real role of ZOT in the pathogenesis of *V. cholerae* [31]. It was also hypothesized that non toxigenic strains harboring *zot* and/or *ace* genes are possible progenitors or intermediates in the origination of toxigenic strains as they possess the *tcpA* gene and are capable of acquiring CTXΦ by horizontal gene transfer [36]. The failure to amplify *tcpA* in one toxigenic isolate might be due to genetic polymorphism which could not be detected by primers specific to El Tor and the classical type of *tcpA*. Several variants of *tcpA* have been reported previously with divergence of sequence in the carboxy-terminal segment [37]. Only four O1 isolates were free for any virulence genes investigated, similar results have been reported worldwide [34]. Indeed, as mentioned above not all environmental O1 strains are toxigenic since the toxin cassette can be lost by recombination between the left and right RS

sequence, excising the entire CTX phage DNA. Taken together, these results of occurrence of virulence genes in our environmental *V. cholerae* O1- Ogawa isolates showed the trait of clinical isolates rather than environmental 'harmless' isolates previously reported by many investigators [36,34,38].

Virulotypes of *V. cholerae* non-O1 were different to the aforementioned virulotypes in *V. cholerae* O1 isolates. The *ctxA* gene was found in two non-O1 isolates obtained from sea water in one location (Rades city) during the same day of collection. Several authors had pronounced that the environmental *V. cholerae* strains do not possess the potential for causing cholera [39,36,4]. However, it was shown that 0.5 % of *V. cholerae* non-O1 isolates produced cholera toxin in the collection of *V. cholerae* non-O1 isolated from the aquatic environment of Calcutta [40]. The combination of virulence genes *ace-ctxA-tcpA-zot* was found in the toxigenic isolates; whereas 17 (56.6%) isolates were free for any virulence genes. Moreover, the *ace* gene was found alone, despite many attempts of amplification, in 11 non-O1 isolates. This finding is unexpected, since the *ace* gene is part of the CTX element and would been occurred at least with *ctx* or *zot* genes. We can speculate that this gene might be located on a genetic structure other the CTX element, or that the genomic plasticity in the vicinity of the active core of CTX has led to a truncated structure lacking the principal parts of the CTX element. Nevertheless, genomic mapping strategy might be necessary to understand the real scenario. Genetic investigation of the 46 isolates by PFGE showed five pulsotypes (A, B, C, D, and E) with predominance of PFGE pattern A in general. However, the majority of O1 isolates segregated in the patterns B (8 isolates) and A (6 isolates), and the majority of non-O1/139 (25 isolates) segregated in the PFGE pattern A. These findings are in contrast to previously reported results; indeed, molecular typing methods have revealed that environmental *V. cholerae* represents an extensively heterogeneous population [41]. Interestingly, several *V. cholerae* O1 and non-O1/139 clustered within the same pulsotypes, evocating a likelihood evolution of the majority of *V. cholerae* O1 serotype Ogawa and non-O1/O139 from a unique ancestral clone. This might be explained by the phenomenon of 'serotype conversion' [42] that explained, in part, the emergence of epidemic *V. cholerae* O139 serogroup strains that have evolved from El Tor strains [43]. Part of the genomic homogeneity between several O1 and non O1/139 isolates shows the extraordinary ability of *V. cholerae* to persist in the environment for a long years and the large distribution of their pandemic clones that reported worldwide. It is likely that horizontal and vertical genetic transfer of phages, pathogenicity islands and plasmids as well as genomic rearrangements would have played a crucial role in the phenotypic and genotypic diversity of *Vibrio* isolates [3].

In conclusion, we report for the first time the occurrence of *V. cholerae* isolates in Tunisia, these isolates were of serotypes O1 Ogawa and non O1/O139. Antibiotic susceptibility, plasmid and virulence genes contents, and genetic homogeneity of the reported isolated suggested a possible common ancestral lineage. This ancestral might has been slightly evolved under genetic gain and pert of mobile genetic elements and environmental stress. Antibiotics susceptibility showed an overall susceptibility of the isolates, thus our study can be used as a guideline of cholera treatment in case of human infection in our country Tunisia. The occurrence of toxigenic *V. cholerae* isolates in the treated wastewater and aquatic environment is worrisome and warrant for a risk of possible biodiversity and human infection in the future. Therefore, through investigations of more aquatic environment in the vicinity of Human agglomerations and agriculture area are urgently needed. Also, systematic Vibrio surveillance system is recommended.

## Conflict of Interest

We declare that we have no conflict of interest.

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