Determination of several potential virulence factors in non-O1 Vibrio cholerae, Pseudomonas aeruginosa, faecal coliforms and streptococci isolated from Marrakesh groundwater

Hafsa Lamrani Alaoui, Khalid Oufdou and Nour-eddine Mezrioui

ABSTRACT

The dynamic, hemolytic and hemagglutination activities and the antibiotic resistance of non-O1 Vibrio cholerae, Pseudomonas aeruginosa, faecal coliforms (FC) and faecal streptococci (FS), isolated by standard membrane filtration methods from suburban and rural groundwater supplies, were carried out. Detectable non-O1 V. cholerae and P. aeruginosa was present in 81% and 88% of samples. The total occurrence of FC and FS during the period of study was 94%. The annual average densities of non-O1 V. cholerae were 4,903 MPN/100 mL. While, they were 206, 1,891 and 1,246 cfu/100 mL for P. aeruginosa, FC and FS respectively. Non-O1 V. cholerae strains had the highest percentage of hemolytic activities (α + β) (71.29%), whereas 20.71% of FS, 16.88% of FC and 9.13% of P. aeruginosa strains produced hemolysin. Bacterial strains isolated were found to be adhesive, with percentages of 63.09%, 65.09%, 84.06% and 87.98% respectively for non-O1 V. cholerae, FS, FC and P. aeruginosa. As for antibiotic resistance, the overall resistance of non-O1 V. cholerae strains was 79%, whereas it was 100% for the other bacteria. Non-O1 V. cholerae resistance was expressed towards sulfamethoxazole (75%), streptomycin (62%) and cephalothin (60%). Obtained results indicated correlation between bacteriological pollution and their public health implications.

Key words | antibiotic resistance, groundwater, hemagglutination, hemolysin, non-O1 Vibrio cholerae, virulence

INTRODUCTION

Vibrio cholerae is a natural inhabitant of the aquatic environment where water plays an important role in its transmission and epidemiology (WHO 1993; Chakraborty et al. 1997). This bacterium is widely distributed in bays, estuaries, coastal water, reservoirs, rivers and water supplies for human consumption (Pathak et al. 1992; Isaac-Marquez et al. 1998; Dumontet et al. 2000). Interest in examining the non-O1 serogroup of V. cholerae has been accentuated at an international level, given that some recent epidemic outbreaks in India and Bangladesh have been caused by non-O1 V. cholerae isolated in aquatic environment (Ramamurthy et al. 1995). Currently, it is recognized that non-O1 V. cholerae includes 150 serotypes and play an important role as the causative agents of sporadic cases of cholera-like disease and isolated outbreaks linked to the consumption of contaminated water (Yamamoto et al. 1983; Chakraborty et al. 1997). Non-O1 V. cholerae has also been implicated in extraintestinal infections, including wounds, ear, sputum, urine and cerebrospinal fluid (WHO 1993).

Several virulence factors such heat stable toxin (ST) (Arita et al. 1986), hemolysin (Yoh et al. 1986) and other cell-associated hemagglutinins (Banerjee et al. 1990) have
been identified in non-O1 *V. cholerae*. Production of hemolysin and surface hemagglutinins of pathogenic bacteria are important virulence determinants as they may serve as recognition and invasion molecules in cell-cell interaction affecting the host-pathogen relationship (Guhathakurta et al. 1999).

Among the other virulence factors, the resistance to antibiotics is more important. Several authors have noted that *V. cholerae* species are rapidly adapting to new drugs commonly used in medicine (Garg et al. 2000; Thiagarajah & Verkman 2005), becoming a potential risk to public health. In addition, molecular analyses demonstrated that resistance to antibiotics and the other virulence factors are chromosomally mediated (Yamamoto et al. 1995; Trucksis et al. 1998).

The objective of this work is to determine the occurrence and the potential virulence of non-O1 *V. cholerae* in supplying well waters in comparison with other bacteria: *Pseudomonas aeruginosa*, faecal coliforms (FC) and faecal streptococci (FS). The virulence of these bacteria was determined by their hemolytic and hemagglutination activities and susceptibility to antibiotics. To our knowledge, this is the first report on the incidence of hemolytic and hemagglutination activities and antibiotic resistance of bacteria isolated from rural and suburban well waters from Morocco and particularly in Marrakesh groundwater.

**MATERIALS AND METHODS**

**Study area and sample collection**

Sixteen wells covering two regions (Tensift and Jbilet) were studied. They are situated at the North of Marrakesh city (31°36′N, 08°02′W, Morocco). Schist dominated geological formations of the ten studied wells (W1, W2, W3, W4, W5, W6, W7, W8, W9, W10) situated at the Jbilet region. They are affected by very strong breaks generating a hydraulic continuity between them. Whereas, the superficial limestone formations and the very permeable alluvial deposits dominated the six prospected wells (W11, W12, W13, W14, W15, W16) located in Tensift region (Figure 1).

In both regions, the formations are permeable and groundwater is relatively little deep (6 to 30 m). In our preliminary research, the bacteriological and physico-chemical quality was studied in sixteen well waters. Since the degree of pollution in these wells was different, it appeared indispensable to choose some wells to study the dynamic of non-O1 *V. cholerae*. Indeed, we chose six wells according to their emplacements and relative importance for local populations and because the detection of this pathogen is onerous and laborious. Our choice was also focused on wells which may be affected by anthropogenic disturbance (Table 1). The well (W11) which was not affected by any sources of pollution, was taken as a control. For these reasons and after the determination of the fecal contamination degree, we chose the following wells to search for non-O1 *V. cholerae*: W2, W3, W5, W9, W11 and W14.

Spatial and temporal evolutions of non-O1 *V. cholerae*, *P. aeruginosa*, FC and FS densities were carried out over a year (April 2004 to April 2005). Samples were collected with weighted bucket (50 cm below the water well surface). The plastic bucket was washed and rinsed carefully with sterilized distilled water to remove all possible trace of disinfectant or contamination. It was then dried before sampling from each studied well. For some wells, samples were taken after pumping for 3 min. Samples water were collected in sterile one-liter flasks and immediately transported at 4°C in the icebox to the laboratory.

**Bacterial enumeration**

For non-O1 *V. cholerae*, the three tubes MPN technique was accomplished to search this bacterium according to the methodology described by Mezrioui et al. (1995) and

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**Figure 1** Map of the studied site; solid circles represent the studied wells (W).
100, 10 and 1 ml or dilutions of well water were concentrated through membrane filters with pore size of 0.45 μm. They were inoculated into three tubes using the MPN technique with 3 stages: (i) Enrichment by culture of 100, 10 or 1 ml of the sample from the series of three tubes of alkaline peptone water (1% peptone, 1% NaCl, pH 8.6) incubated at 37°C for 18 h. (ii) Isolation was performed by culture of 1 ml taken from the surface of each enrichment tube or from one of its dilutions on Thiosulfate-Citrate-Bile-Sucrose agar (TCBS), incubated at 37°C for 24 h [16, 9]. (iii) Identification of the colonies assumed to be those of *V. cholerae* non-O1 by means of the following screening tests: examination for cytochrome oxidase, D-glucose fermentation, sensitivity to the vibriostatic compound O/129 (2,4-diamino 6,7-diisopropylpteridine: 150 μg ml⁻¹), and agglutination to anti O1 *V. cholerae* serum. An enrichment tube was considered positive if one of the colonies isolated on the corresponding TCBS medium was confirmed to be *V. cholerae* non-O1, to be oxidase (+), to have fermentative metabolism of D-glucose (37°C, 24 h), to be sensitive to O/129 (37°C, 24 h) and to show no agglutination with anti O1 *V. cholerae* serum.

Densities of the other bacteria were determined by standard membrane filtration methods. Selective mediums were used to enumerate each bacterial group. 100, 10 and 1 mL of water samples were filtered (pore size: 0.45 μm). The filter was transferred on selective mediums. Cetrimid agar (Merck) was used for enumeration of *P. aeruginosa*. After incubation at 42°C for 24 to 48 h, the fluorescent green colonies were counted. 2, 3, 5-Triphenyl-tetrazolium chloride (TTC) and tergitol lactose agar (Biokar) was used as selective medium for FC. After incubation at 44.5°C for 24 h, the yellow colonies were counted. As for FS, the selective medium Slanetz agar (Pasteur Institute Production) was used. The pink colonies were counted after incubation at 37°C for 24 h. The enumeration of these bacteria was done by indirect count of colonies forming units (cfu).

**Hemolytic activity and hemagglutination assays**

Bacterial strains were isolated from Marrakesh groundwater during the period of study. Two to four colonies were arbitrarily chosen each month from each well. They were
streaked onto Trypticase Soy Agar (TSA) for purification. Thus, 317 strains of non-O1 *V. cholerae*, 208 strains of *P. aeruginosa*, 320 strains of FC and 338 strains of FS were collected from the prospected wells over the year.

The ability of all these isolates to adhere and destroy host cells was verified with human O erythrocytes. Quantification of hemolytic activity of bacterial cells was performed by the procedure described by Rahim & Sultan Aziz (1994). The production of hemolysin was determined by the demonstration of hemolysis zones around colonies on blood agar plates (Columbia agar, BioMerieux) containing 5% (v/v) human O blood after 18 to 24 h of incubation at 37°C. Green hemolysis was noted as α, clear hemolysis as β and no hemolysis as γ.

The hemagglutination test was realized according to the methodology described by Pal et al. (1992). Reactions were considered negative if no agglutination occurred within 10 min, and they were considered positive if agglutination occurred within 5–10 min of incubation. The degree of hemagglutination was scored from 2− to 1+ depending on intensity and time. If the reaction is complete and immediate, the agglutination is noted 2+. If the agglutination is not immediate and is achieved within 10 min, it is noted 1+.

**Antimicrobial susceptibility testing**

1,183 strains of non-O1 *V. cholerae*, *P. aeruginosa*, FC and FS, were examined for their antibiotic resistance. The plates were prepared by adding the antibiotic to molten Muller-Hinton agar, previously cooled to 45°C. Each plate was inoculated with the isolates using the multipoint inoculation method described by Oufdou et al. (1999) and Fars et al. (2005) and incubated at 37°C for 24 h. A control plate without antibiotic was prepared and similarly inoculated. An isolate was considered to be resistant to an antimicrobial agent if its growth in the medium containing the agent was similar to that obtained on the control plate. The concentrations (given in µg·mL⁻¹) of the antibiotics tested were: ampicillin (Amp: 30), amoxicillin (Amx: 30), amoxicillin-Clavulanic acid (Amx-Clav: 40), chloramphenicol (Chl: 30), cephalothin (Cfl: 30), cephamandole (Cfm: 30), cephoxatin (Cfx: 30), erythromycin (Ery: 15), gentamycin (Gm: 10), Imipenem (Imp: 10), kanamycin (Km: 20), streptomycin (Str: 20), nalidixic acid (Na: 50), novobiocin (Nv: 25), polymyxin B (PB: 37.5), tetracycline (Tc: 10), sulphamethoxazole (Smx: 100) and trimethoprim (Tpm: 5). These antibiotics were chosen for two reasons: (i) they have been used in the treatment of human and/or livestock illnesses; and (ii) they have been used in previous surveys of antibiotic resistance in aquatic environments (Kelch & Lee 1978; Mezrioui & Oufdou 1996; Oufdou et al. 1999; Fars et al. 2005).

**Statistical analysis**

The dynamics of non-O1 *V. cholerae*, *P. aeruginosa*, FC and FS were statistically analyzed using SPSS 10.0 for windows. The two–factor analysis of variance (ANOVA) was used for comparison of bacterial abundances.

Comparison between percentages of antibiotic resistance, hemolytic and hemagglutination activities was performed using the test of two proportions or frequencies described by Schwartz (1965). This test shows whether a difference between two frequencies: $f_1$ observed on $n_1$ samples and $f_2$ observed on $n_2$ samples, is significant. It will determine:

$$ t = \frac{f_1 - f_2}{\sqrt{f(1-f)(1/n_1 + 1/n_2)}} $$

If $|t| < 1.96$: the difference between $f_1$ and $f_2$ was not significant ($P > 0.05$).

If $|t| \geq 1.96$: the difference was significant ($P \leq 0.05$).

**RESULTS AND DISCUSSION**

Densities variation of non-O1 *V. cholerae*, *P. aeruginosa*, FC and FS are presented at each sampling station during a year in Figure 2. Detectable non-O1 *V. cholerae* was present in 81% of samples and the average abundances ranged from 0 to 11,100 MPN/100 mL. Detectable *P. aeruginosa* was present in 88% of samples and its abundances ranged from 0 to 1,670 cfu/100 mL. The total occurrence of FC and FS during the period of study was 94% and their densities varied respectively from a minimum of 0 cfu/100 mL to a maximum of 10,200 cfu/100 mL for FC and 6,700 cfu/100 mL for FS. The annual average densities
of non-O1 *V. cholerae* were 4,903 MPN/100 mL in all samples. Whereas, the annual average densities of *P. aeruginosa*, FC and FS were respectively 206 cfu/100 mL, 1,891 cfu/100 mL and 1,246 cfu/100 mL (Figure 2).

*P. aeruginosa* abundances were significantly lower (*P*, 0.05, ANOVA test) than those of FC and FS. The average densities of FC, FS, *P. aeruginosa* and non-O1 *V. cholerae* were very significantly (*P* < 0.05, ANOVA test) higher in the whole wells compared to the control well (W11).

The incidence of non-O1 *V. cholerae* isolated from the aquatic environment, as well as their pathogenic and virulence-associated properties, vary greatly according to the geographic region. Our results demonstrated that non-O1 *V. cholerae* and the other studied bacteria, are widely distributed in the studied wells water. These wells serve as

![Figure 2](https://iwaponline.com/wst/article-pdf/61/7/1895/448239/1895.pdf)
an important natural resource for drinking water, domestic water supply and recreation for rural and suburban populations. This fact could be responsible for potential health effects on populations using this groundwater. According to WHO standards, the studied wells are completely unsuitable for drinking water and other domestic uses.

The highest abundances of studied bacteria were detected at the wells located near malfunctioning septic systems or beside a high number of pollution sources such as infiltration of wastewater, septic tanks seepage, discharge leachates or human and animal faecal materials nearby the studied wells. Moreover, the majority of the studied wells are situated at 0 m to 400 m from pollution sources. These factors led to the contamination of the groundwater.

Based on the results of the present study, it is possible to conclude that groundwater can play an important role as a transmission vehicle of non-O1 V. cholerae and the other studied bacteria. Isaac-Marquez et al. (1998) considered that the presence of non-O1 V. cholerae in water supplies might be responsible for a proportion of diarrheic diseases among population of the city of Campeche and the rural locality of Becal (Mexico). Several reports have demonstrated that gastrointestinal and extraintestinal infections caused by non-O1 V. cholerae are linked with contaminated water and other activities in aquatic environments, and this bacterium could therefore pose a problem for public health (WHO 1993; Chakraborty et al. 1997).

The pathogenicity of non-O1 V. cholerae has been related to a number of putative virulence factors, including the production of enterotoxin CT (Spira & Fedorka-Cray 1983; Yamamoto et al. 1983), enterotoxin LT or ST (Arita et al. 1986; Ichinose et al. 1987), cytolsin LT commonly referred to “hemolysins” (Ichinose et al. 1987), adhesins (Kabir & Ali 1985; Booth & Finkelstein 1986), cytotoxin shiga (O’Brien et al. 1984). It has been demonstrated that non-O1 V. cholerae adheres and invades the epithelial cells of gut mucosa and starts its multiplication (Nishibuchi et al. 1983). This situation occurs only with expression of certain virulence factors as previously cited (Nishibuchi et al. 1983; O’Brien et al. 1984; Ichinose et al. 1987).

To characterize the virulence factors of the bacterial isolates in our study, hemolysis and hemagglutination with human erythrocytes were realized.

Hemolysin production of non-O1 V. cholerae, P. aeruginosa, FC and FS strains were different (Table 2). A greater percentage of non-O1 V. cholerae isolates (71.29%) showed hemolytic activities ($\alpha + \beta$). 20.71% of FS strains and 16.88% of FC strains produced hemolysin while only 9.13% of all the P. aeruginosa isolates had such activities.

Analysis of a total of 1,183 strains isolated from the studied wells revealed that non-O1 V. cholerae had the highest $\beta$ hemolytic activity (33.12%), while only 3.44% of FC and 4.44% of FS strains have this type of hemolysis. As for P. aeruginosa, $\beta$ hemolytic activity was very low (1.44%). FC, FS and P. aeruginosa strains isolated from Marrakesh groundwater expressed significantly lower hemolytic activity compared to non-O1 V. cholerae ($P < 0.05$, test of two proportions) (Table 3(a–c)).

Hemolysin of V. cholerae is suggested to be a virulence factor contributing towards pathogenesis (Nagamune et al. 1995). Guhathakurta et al. (1999) purified a bifunctional hemolysin-phospholipase C molecule from non-O1 V. cholerae (O139) showing enterotoxic activity, as shown by fluid accumulation in the ligated rabbit ileal loop and in the intestine of suckling mice (Pal et al. 1998).

The percentages of hemolytic isolates observed in this study are comparable to those reported by Begum et al. (2006). These authors found that 80% of the total non-O1 and non-O139 V. cholerae isolates were hemolysin positive.

### Table 2 | Percentages of hemolysis among non-O1 V. cholerae, P. aeruginosa, FC and FS isolates

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>No of tested strains</th>
<th>No (%) of hemolytic strains</th>
<th>Type $\alpha$</th>
<th>Type $\beta$</th>
<th>$\alpha + \beta$</th>
<th>Type $\gamma$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-O1 V. cholerae</td>
<td>317</td>
<td>121 (38.17)</td>
<td>105 (33.12)</td>
<td>226 (71.29)</td>
<td>91 (28.71)</td>
<td></td>
</tr>
<tr>
<td>P. aeruginosa</td>
<td>208</td>
<td>16 (7.69)</td>
<td>3 (1.44)</td>
<td>19 (9.13)</td>
<td>189 (91.87)</td>
<td></td>
</tr>
<tr>
<td>FC</td>
<td>320</td>
<td>43 (13.44)</td>
<td>11 (3.44)</td>
<td>54 (16.88)</td>
<td>266 (83.12)</td>
<td></td>
</tr>
<tr>
<td>FS</td>
<td>338</td>
<td>55 (16.27)</td>
<td>15 (4.44)</td>
<td>70 (20.71)</td>
<td>268 (79.29)</td>
<td></td>
</tr>
</tbody>
</table>
However, our results were lower than those obtained by Amaro et al. (1990). These authors showed that 97% of environmental non-O1 \textit{V. cholerae} strains displayed hemolytic activity for human blood.

Adhesion to the intestinal mucosa represents the first step in the infectivity of bacterial pathogens such as \textit{V. cholerae} (Booth & Finkelstein 1986). This process is mediated by non-specific (mainly hydrophobic) and specific (binding of the bacterial adhesin with its receptor on the epithelial cell) interactions (Kabir & Ali 1983). Agglutination of erythrocytes is among the most useful assays to test the attachment ability of potential pathogens.

Bacterial strains isolated from Marrakesh groundwater were found to be adhesive, with a range of hemagglutination activities varying from 63.09% for non-O1 \textit{V. cholerae} to 65.09% for FS, 84.06% for FC and 87.98% for \textit{P. aeruginosa} (Table 4).

Among 317 strains of non-O1 \textit{V. cholerae}, 60 strains (18.93%) were strongly adhesive (+2) and 140 (44.16%) were partially agglutinated (+1) to erythrocytes. On the other hand, 69.06% of FC strains and 62.02% of \textit{P. aeruginosa} expressed complete agglutination (+2) capacity, and respectively 15% and 25.96% of them agglutinated partially (+1) to erythrocytes.

Our findings are in agreement with previous studies on hemagglutination distribution in \textit{V. cholerae} (Amaro et al. 1990). These authors showed that 109 (78%) of the environmental non-O1 \textit{V. cholerae} strains assayed, possessed agglutinating capacity.

Determination of several potential virulence factors in \textit{Vibrio} spp. by Baffone et al. (2001) demonstrated that species were adhesive, with percentages ranging from 40% for \textit{V. fluvialis} to 55–80% for \textit{V. alginolyticus}, non-O1 \textit{V. cholerae} and \textit{V. parahaemolyticus}.

### Table 3a | Comparison between percentages of haemolytic activities (\(\alpha + \beta\)) of the studied bacteria using the test of two proportions

<table>
<thead>
<tr>
<th>Frequencies difference between</th>
<th>\textit{V. cholerae} non-O1 and FS</th>
<th>\textit{V. cholerae} non-O1 and FC</th>
<th>\textit{V. cholerae} non-O1 and \textit{P. aeruginosa}</th>
</tr>
</thead>
<tbody>
<tr>
<td>(</td>
<td>t</td>
<td>)</td>
<td>6.283*</td>
</tr>
</tbody>
</table>

* The difference is significant if \(|t| \geq 1.96; p < 0.05.  

### Table 3b | Comparison between percentages of \(\beta\) haemolytic activities of the studied bacteria using the test of two proportions

<table>
<thead>
<tr>
<th>Frequencies difference between</th>
<th>\textit{V. cholerae} non-O1 and FS</th>
<th>\textit{V. cholerae} non-O1 and FC</th>
<th>\textit{V. cholerae} non-O1 and \textit{P. aeruginosa}</th>
</tr>
</thead>
<tbody>
<tr>
<td>(</td>
<td>t</td>
<td>)</td>
<td>3.579*</td>
</tr>
</tbody>
</table>

* The difference is significant if \(|t| \geq 1.96; p < 0.05.  

### Table 3c | Comparison between percentages of multiresistance of the studied bacteria using the test of two proportions

<table>
<thead>
<tr>
<th>Frequencies difference between</th>
<th>\textit{V. cholerae} non-O1 and FS</th>
<th>\textit{V. cholerae} non-O1 and FC</th>
<th>\textit{V. cholerae} non-O1 and \textit{P. aeruginosa}</th>
</tr>
</thead>
<tbody>
<tr>
<td>(</td>
<td>t</td>
<td>)</td>
<td>3.478*</td>
</tr>
</tbody>
</table>

* The difference is significant if \(|t| \geq 1.96; p < 0.05.  

### Table 4 | Percentages of hemagglutination among non-O1 \textit{V. cholerae}, \textit{P. aeruginosa}, FC and FS isolates

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>No of tested strains (2+)</th>
<th>(1+)</th>
<th>No (%) of no hemagglutinating strains</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-O1 \textit{V. cholerae}</td>
<td>317</td>
<td>60 (18.93)</td>
<td>140 (44.16)</td>
</tr>
<tr>
<td>\textit{P. aeruginosa}</td>
<td>208</td>
<td>129 (62.02)</td>
<td>54 (25.96)</td>
</tr>
<tr>
<td>FC</td>
<td>320</td>
<td>221 (69.06)</td>
<td>48 (15)</td>
</tr>
<tr>
<td>FS</td>
<td>338</td>
<td>159 (47.04)</td>
<td>61 (18.05)</td>
</tr>
</tbody>
</table>

2+ : The agglutination is immediate; 1+ : The agglutination is not immediate and is achieved within 10 min.
The percentages of antimicrobial sensibility of non-O1 V. cholerae, P. aeruginosa, FC and FS isolates to the antibiotics, are shown in Tables 5 and 6.

Antibiotic susceptibility testing revealed that the overall resistance (resistance to at least one antibiotic) of non-O1 V. cholerae strains was 79%, while it was 100% for P. aeruginosa, FC and FS strains (Table 5). The multi-resistance level of non-O1 V. cholerae strains (69%) was significantly lower ($P < 0.05$, test of two proportions) (Table 3(c)) than that of FC and FS strains (95%), whereas 100% of P. aeruginosa strains were multiresistant. The monoresistance (resistance to one antibiotic) of non-O1 V. cholerae was 10% while it was 5% for FC and FS strains. Sixty six strains (21%) of non-O1 V. cholerae were susceptible to all antibiotics tested, while none of the isolates P. aeruginosa, FC and FS was susceptible to all antibiotics tested.

Our results showed that among non-O1 V. cholerae strains resistance was most commonly observed towards sulfamethoxazole (75%), followed by streptomycin (62%) and cephalothin (60%) and trimethoprim (49%). A smaller proportion of these isolates were resistant to erythromycin (18%), kanamycin and polymyxin B (12%), cefotaxim (8%), gentamycin (7%) and tetracycline (2%). All the 317 non-O1 V. cholerae isolates were susceptible to chloramphenicol, nalidixic acid and novobiocin. Amaro et al. (1988) have demonstrated that non-O1 V. cholerae environmental isolates were resistant to sulfanilamide (80%), to ampicillin (65%) and to amoxicillin (61%) and were susceptible to chloramphenicol, nalidixic acid, tetracycline, novobiocin and trimethoprim. Radu et al. (2002) showed that all V. cholerae isolates were susceptible to chloramphenicol and exhibited high rates of resistance to cephalothin (90.9%), streptomycin (87.9%) and tetracycline (77.79%).

Antimicrobial susceptibility of FC strains was most commonly expressed towards sulfamethoxazole (91%), followed by cephalothin (88%) and ampicillin (84%) (Table 6). More than 50% of the FC isolates were resistant to kanamycin, gentamycin, streptomycin, trimethoprim and tetracycline. FS isolates were resistant to polymyxin (87%), sulfamethoxazole (86%) and nalidixic acid (85%), cephalothin (82%) and to streptomycin (74%).

### Table 5 | Frequency of mono and multiple-antibiotic resistance among the studied bacterial strains

<table>
<thead>
<tr>
<th>No (%) of</th>
<th>Non-O1 V. cholerae</th>
<th>FC</th>
<th>FS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monoresistance</td>
<td>31 (9.78)</td>
<td>16 (5)</td>
<td>17 (5.0)</td>
</tr>
<tr>
<td>Multiresistance*</td>
<td>220 (69.40)</td>
<td>304 (95)</td>
<td>321 (95)</td>
</tr>
<tr>
<td>Overall resistance†</td>
<td>251 (79.18)</td>
<td>320 (100)</td>
<td>338 (100)</td>
</tr>
</tbody>
</table>

*Multiresistance: resistance to at least two antibiotics.  †Overall resistance: resistance to at least one antibiotic.

### Table 6 | Percentages of antibiotic resistance among non-O1 V. cholerae ($n = 317$), P. aeruginosa ($n = 208$), FC ($n = 320$) and FS ($n = 338$) strains

<table>
<thead>
<tr>
<th>Antibiotics*</th>
<th>Non-O1 V. cholerae</th>
<th>P. aeruginosa</th>
<th>FC</th>
<th>FS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amp</td>
<td>42</td>
<td>83</td>
<td>84</td>
<td>28</td>
</tr>
<tr>
<td>Amx</td>
<td>28</td>
<td>75</td>
<td>70</td>
<td>58</td>
</tr>
<tr>
<td>Amp-Clav</td>
<td>14</td>
<td>77</td>
<td>71</td>
<td>34</td>
</tr>
<tr>
<td>Chl</td>
<td>0</td>
<td>75</td>
<td>13</td>
<td>57</td>
</tr>
<tr>
<td>Cfl</td>
<td>60</td>
<td>95</td>
<td>88</td>
<td>82</td>
</tr>
<tr>
<td>Cfm</td>
<td>23</td>
<td>90</td>
<td>60</td>
<td>77</td>
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<td>Cfx</td>
<td>8</td>
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<td>35</td>
<td>40</td>
</tr>
<tr>
<td>Ery</td>
<td>18</td>
<td>75</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Gm</td>
<td>7</td>
<td>52</td>
<td>34</td>
<td>67</td>
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<tr>
<td>Imp</td>
<td>ND</td>
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*Ampicillin (Amp), amoxicillin (Amx), amoxicillin-clavulanic acid (Amx-Clav), chloramphenicol (Chl), cephalothin (Cfl), cephamandole (Cfm), cefotaxim (Cfx), erythromycin (Ery), gentamycin (Gm), kanamycin (Km), streptomycin (Str), nalidixic acid (Na), novobiocin (Nv), polymyxin B (PB), tetracycline (Tc), sulphanmethoxazole (Smx) and trimethoprim (Tpm), imipenem (Imp).

ND: not determined.
FC strains were less resistant to chloramphenicol (13%) and nalidixic acid (28%), while FS isolates were less resistant to ampicillin (28%) and amoxicillin-clavulanic acid (34%). *P. aeruginosa* strains were generally resistant to the antibiotics tested, whereas they were less resistant only to imipenem (12%).

The predominant resistance property observed was to β-lactam antibiotics, either alone or in combination with resistance to other antibiotics. Bell et al. (1983) have also noted ampicillin and cephalothin resistance exhibited by most faecal coliforms isolated from both rural and urban environments. McKeon et al. (1995) assayed resistance to sixteen antibiotics of 265 coliform and noncoliform isolates from rural groundwater. These authors concluded that resistance was most commonly towards novobiocin, cephalothin and ampicillin with nearly 70% or more of the strains expressing resistance to at least one of these antibiotics. They have also noted that more than 30% of the isolates were resistant to tetracycline and nitrofurantoin.

The dominant multiresistant profiles noted for non-O1 *V. cholerae* were to seven antibiotics; of 220 strains resistant to at least two antibiotics, 53 strains (24.09%) were resistant to seven antibiotics with six patterns: “Str, Tpm, Smx, Amp, Amx, Cfl, Cfm”, “Str, Tpm, Smx, Amx, Amx-Clav, Cfl, Cft”, “Str, Tpm, Smx, Amp, Cfl, Cft, PB”, “Str, Tpm, Smx, Amp, Amx, Amx-Clav, Cfl”, “Str, Tpm, Smx, Amp, Cfl, Cfm, Cft” and “Gm, Str, Km, Smx, Amx, Amx-Clav, Cfl”. The maximal multiresistance was to ten antibiotics with two profiles: “Gm, Str, Km, Tpm, Smx, Amp, Amx, Cfl, Cfm, Ery” and “Gm, Str, Km, Tpm, Smx, Tc, Amp, Amx, Cfl, Cfm”.

Of the antimicrobial resistant FC strains isolated, 80% were resistant to five or more antibiotics. The dominant multiresistant profile noted for FC was to eight antibiotics (11.6%). The maximal multiresistance was to fourteen antibiotics with two profiles: “Amp, Amx, Amx-clav, Cfl, Cfm, Cft, Chl, Gm, Km, Na, PB, Smx, Str, Tc, Tpm” and “Amp, Amx, Amx-clav, Cfl, Cfm, Cft, Gm, Km, Na, PB, Smx, Str, Tc, Tpm”.

Of the antimicrobial resistant FS strains isolated, 90% were resistant to five or more antibiotics. The dominant multiresistant profiles noted were to twelve antibiotics (19.5%). The maximal multiresistance was to fifteen antibiotics with one profile: “Amp, Amx, Amx-clav, Cfl, Cfm, Cft, Chl, Gm, Km, Na, PB, Smx, Str, Tc, Tpm”.

Of the antimicrobial resistant *P. aeruginosa* strains isolated from Marrakesh groundwater, 100% were resistant to ten or more antibiotics. The dominant multiresistant profiles noted were to fourteen antibiotics; of 208 strains resistant to at least ten antibiotics, 51 strains (24.5%) were resistant to fourteen antibiotics. The maximal multiresistance was to seventeen antibiotics with one profile: “Amp, Amx, Amx-clav, Cfl, Cfm, Cft, Chl, Ery, Gm, Km, Na, Nv, PB, Smx, Str, Tc, Tpm”.

**CONCLUSIONS**

In light of the occurrence of non-O1 *V. cholerae* in the studied wells and their positive reactions for virulence factors, the potential public health significance of non-O1 *V. cholerae* in Marrakesh groundwater will be taken into consideration. These wells serve as an important natural resource for drinking water, domestic water supply and recreation for rural and suburban populations which use this groundwater without any previous treatment. This fact might be responsible for a proportion of diarrheic diseases observed among population using this well water. The high numbers of these microorganisms constitute a serious public health risk for the consumers, since most of them are producers of hemolysins, hemagglutinins and are multiresistant to antibiotics. Pollution activities around the wells appeared to lead an increase of virulence and resistance to antibiotics in groundwater. The obtained data indicate that non-O1 *V. cholerae, P. aeruginosa*, FC and FS are able to survive in well water during the period of study.

Non-O1 *V. cholerae* and the other studied bacteria isolated from Marrakesh groundwater are virulent since most of them are producers of hemolysins, hemagglutinins and are multiresistant to antibiotics. These microorganisms may have important public health implications. Their role in several cases of gastro-enteric and systemic pathologies noted at the local population of Marrakesh area (Jbilet and Tensift region) deserve greater interest and attention. Sanitation problems due to the consumption of these wells water require an urgent reaction to apply adequate solutions such as the protection of wells, the disinfection of groundwater and the public awareness.
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