ORIGINAL ARTICLE

TREATED WASTEWATER REUSE IN IRRIGATION: DETECTION AND CHARACTERIZATION OF POTENTIALLY PATHOGENIC *VIBRIO* SPP

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ABSTRACT

Background: Agadir city (Morocco) is located in a semi-arid area, surface water resources are scarce and ever-increasing demand for irrigation water leads to the overpumping of groundwater. The use of treated wastewater for irrigation of agricultural lands and urban landscapes contribute to the preservation of freshwater reservoirs. However, this practice could result in a microbiological contamination risk to soil and vegetation. Pathogenic Vibrios frequently detected in wastewater has emerged as a serious threat to human health. The present study was initiated to evaluate the eventual occurrence of potentially pathogenic. **Methods**: *Vibrio* species were searched in a golf course (GW) irrigated with treated wastewater. In order to better assess risks due to the use of treated wastewater, another golf course (GG) irrigated with groundwater was served as the control. From January to August 2016, groundwater, treated wastewater, turfgrass, and soil samples were collected and analyzed. 162 presumptive *Vibrio* organisms were isolated on TCBS agar plates and tested by PCR. **Results**: The result showed that all positive *Vibrio* strains were isolated from GW and from the wastewater treatment plant, while all samples taken from GG were negative for the species studied. 64 (67%) isolates were positive for *V. cholerae*, 30 (31%) for *V. alginolyticus*, and 2 (2%) for *V. parahaemolyticus*. *Vibrio* strains were detected much higher in treated wastewater (44%) than in the soil (27%), turfgrass (18%), and groundwater (11%). All *V. cholerae* strains were negative for the ctxA gene. Likewise, *V. parahaemolyticus* strains were also negative for tdh and trh genes. **Conclusion**: In conclusion, the irrigation of GW with treated wastewater has led to contamination of soil and turfgrass with *Vibrio* spp. The contamination was seemingly due to the presence of these bacteria in irrigation water.

Keywords: *Golf course, Irrigation, Treated wastewater, Pathogenic Vibrio*

1. INTRODUCTION

Many countries of the Middle East and North African (MENA) are affected by water scarcity. Morocco is classified among those countries with high water demand and limited water availability. In southern Morocco, particularly in the Souss-Massa Basin, agriculture is one of the most important economic activities in the region. Overpumping combined with drought impact caused a reduction in the piezometric level of the local aquifer system; the deficit varies from 100 to 370 million m3/yr for Souss aquifer and 60 million m3/yr for Chtouka aquifer [1], and according to the ABHSMD Data 95% of all the water resources go towards irrigation uses. In Agadir city, there are approximately 878 hectare urban green areas, which consume about 8 million m3/yr of water. 268 hectares of the total area of green spaces are occupied by golf courses with an estimated water need of 3.2 million m3/yr.

The reuse of treated wastewater for irrigation could be an alternative solution as appears to be the case in certain Mediterranean countries [2]. This practice is considered a realistic way of managing water resources, and contributes to the preservation of the volume of freshwater available for human consumption, especially in regions that groundwater is used for irrigation. However, treated wastewater used in agriculture can be a source of microbial pathogens that are often associated with health and environmental risks. The presence of pathogenic microorganisms in wastewater has been reported previously [34, 35].

In many studies, the genus *Vibrio* has been isolated from wastewater [25, 35]. This genus includes about 100 species [28], all species are gram-negative bacteria, generally oxidase-positive and possess respiratory nitrate reductase. *Vibrio* is omnipresent in the estuarine and marine environment, with some freshwater examples. Factors such as salinity, pH, and temperature affect the growth of *Vibrio* [12]. Several *Vibrio* species are described as human and animal pathogens [32], amongst these species, *V. cholerae, V. parahaemolyticus, V. alginolyticus, V. vulnificus and V. mimicus.*

In the Agadir region, the majority of the studies have focused on assessing the performance of the M'zar wastewater treatment plant (M'zar WWTP) [13, 14]. Eddabra (2011) described the occurrence of different potentially pathogenic *Vibrio* in treated/untreated wastewater of M'zar WWTP [13]. Starting in August 2010, the treated wastewater of M'zar WWTP origin has been used for irrigation of a golf course located in Agadir city. However, risks to public health related to the eventual occurrence of *Vibrio* in irrigation water and golf course were not addressed. In this context, this study is the

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first to report the isolation of *Vibrio* pathogen strains from green spaces irrigated with treated wastewater originating from the M'zar WWTP.

2. MATERIELS AND METHODES

2.1. Sampling sites and sampling

The study was conducted in Agadir city. The region of Agadir is experiencing strong growth in urbanization, population, tourism, and agricultural activities, inducing more demand for water. In this city, golf courses are irrigated with groundwater. In order to protect and conserve groundwater, local authorities promote the reclaimed water reuse for irrigation, However, so far only one golf course is using treated wastewater originating from M'zar wastewater treatment plant (WWTP).

M'zar WWTP is the largest treatment plant in the region of Agadir; it is located on the coastal dunes of M'zar within the Souss-Massa National Park at about 5 km from the city center of Agadir. The plant collects the entire town's wastewater. The treatment technique is based on the principle of infiltration-percolation on dune sand (secondary treatment). The tertiary stage of the treatment system is disinfection using ultraviolet (UV) light. Tertiary-treated wastewater is pumped and stored before use in a large pond located in the golf course GW.

The GW is located in Agadir city, approximately 3 km city center, and adjacent to the forest of Bensergao. The golf course (GW) was built in 2009 and extends over 100 ha, with a seven years history of treated wastewater use in irrigation. In order to better understand the impact of irrigation with treated wastewater, a golf course (GG) was served as the control because is irrigated with groundwater local.

From January to August of 2016, composite samples were taken from GW (water irrigation, turfgrass, soil, and groundwater samples), from GG (turfgrass, soil and groundwater samples), and at the output of the M'zar WWTP. All samples were tested for the presence of *Vibio* spp.

2.2. Microbiological analysis

For isolation of *Vibrio* strains from treated wastewater and groundwater samples, a volume of 450 mL was filtered through a nitrocellulose 0.45 µm filter. The membrane was placed in 50 mL of alkaline buffered peptone water (ABPW), pH 8.6 ± 0.2) and incubated for 18–20 h at 36 ± 2 °C. A portion of the suspension of ABPW was spread on Thiosulfate Citrate Bile Salts Sucrose (TCBS) agar plates and then was incubated at 36 ± 2 ◦C for 24 h ± 3 h. The yellow or green colonies were selected as presumptive *Vibrio* strains colonies and transferred to saline nutrient agar plates and then incubated at 37 °C for 18–24h. The pure cultures obtained were tested for oxidase activity.

As for the soil and turfgrass samples, a 25g portion of each sample was weighed and homogenized in 225 mL of ABPW, pH 8.6 ± 0.2 and incubated for 18–20 h at 36 ± 2 °C. Then, enrichment cultures parts were taken and plated onto TCBS agar and incubated at 37°C for 24 h. The green or yellow colonies considered to be presumptive *Vibrio* strains were transferred to saline nutrient agar plates and then incubated at 37 °C for 18–24. The pure cultures obtained were tested for oxidase activity.

Molecular characterization of presumptive *Vibrio* strains was performed in the Center for Vibrios and Cholera at the Institut Pasteur (Paris, France). Bacterial colonies (oxidase-positive isolates) were suspended in 1 mL of sterile water and centrifuged. The pellet was suspended in 200 µL of InstaGene Matrix (Bio Rad laboratories, Marnes La Coquette, France) and incubated at 56 °C for 20 minutes. Then the suspensions were mixed by vortex at a high speed for 10 s and placed in a 100 °C heat block for 8 minutes. The lysates were vortexed at high speed for 10 seconds and centrifuged at 12 000 rpm for 3 min. The InstaGene DNA preparations were stored at−20 °C.

The polymerase chain reaction (PCR) was used to confirm the identities of *Vibrio* species. A total of 162 presumptive *Vibrio* isolates obtained using TCBS agar were analyzed, including 98 strains isolated from water, 37 from the soil, and 27 from turfgrass. *V. cholerae* was identified using specific primers targeting specific 16S–23S rRNA intergenic sequence as described by 6. Chun et al., (1999) [6]**.** For *V. parahaemolyticus*, specific primers targeting the pR72H gene were used as described by [20]**.** The *V. vulnificus* and *V. alginolyticus* species were identified by targeting specifically the cytotoxin-hemolysin gene for *V. vulnificus* [16] and collagenase gene for *V. alginolyticus* [10] respectively. The detection of ctxA in the *V. cholerae* strains was performed as previously described by Fields et al., (1992) [15]**.** *V. parahaemolyticus* positive strains were tested for the presence of tdh and trh genes, using primers as described by Bej et al., (1999) [3]**.** The primers, annealing temperatures, and the number of cycles are presented in Table 1.

Table 1: Primers used for amplification of each *Vibrio* species and virulence genes.

Species	Target gene	Primers	Amplicon size (bp)	References
V. cholerae	ISR 16 S/23 S	pr VC-F: TTAAGCSTTTTCRCTGAGAATG pr VCM-R: AGTCACTTAACCATACAACCCG	300	[1]
	CtxA	pr CTX2: CGGGCAGATTCTAGACCTCCTG pr CTX3: CGATGATCTTGGAGCATTCCCAC	563	[15]
V. parahaemolyticus	pR72H	pr Vp32 : CGAATCCTTGAACATACGCAGC pr Vp33 : GCGAATTCGATAGGGTGTTAACC	320 pb	[20]
	Thermostable direct hemolysin (TDH)	pr L-tdh: GTAAAGGTGTCTGACTTTTTGAC pr R-tdh: TGGAATAGAACCTTCATCTTCACC	849 bp	[3]
	TDH-related hemolysin (TRH)	pr L-trh : TTGGCTTCGATATTTTCAGTATCT pr R-trh: CATAACAAACATATGCCCATTTCCG	500 bp	
V. vulnificus	Cytotoxin-hemolysin	pr VVp1: CCGGCGGTACAGGTTGGCGC pr VVp2: CGCCACCCACTTTCGGGCC	519	[16]
V. alginolyticus	Collagenase	pr VA-F: CGAGTACAGTCACTTGAAAGCC pr VA-R: CACAACAGAACTCGCGTTACC	737 bp	[10]

3. RESULTS

The aim of this study was to identify the occurrence or *Vibrio* species pathogenic for humans. A total of 162 presumptive *Vibrio* isolates were collected from Golf course GW, Golf course GG, and from the M'zar WWTP. All presumptive *Vibrio* isolates were tested by conventional PCR. The results showed that 96 strains isolated were confirmed to belong to three species, including *V. parahaemolyticus*, *V. cholerae* and *V. alginolyticus* (Table 2).

Table 2 Distribution of *Vibrio* strains isolated according species.

***Vibrio* strains (n = 96)**	**Number**	**Percentage (%)**
V. cholerae	64	67
V. alginolyticus	30	31
V. parahaemolyticus	2	2

n = number of srains

88 (91%) strains were detected in GW and 8 (9%) strains at the output of the WWTP whereas; all the isolates taken from GG were negative. *Vibrio* species were isolated much higher from the treated wastewater storage pond (36%) than from the output of the WWTP (8%). 26 (27%) *Vibrio* strains were detected from the soil, 17 (18%) from the turfgrass, while 10 (11%) from groundwater sampled from the GW (Table 3).

Table 3 Distribution of *Vibrio* srains isolated from the WWTP and the golf courses.

***Vibrio* strains (n = 96)**	**M'zar WWTP**	**Golf course irrigated with treated wastewater (GW)**			**Golf course irrigated with groundwater (GG)**		
	Treated wastewater at the output of the plant	Treated wastewater storage pond	Groundwater	Turgrass	Soil	Groundwater	Turgrass	Soil
	8 (8%)	35 (36%)	10 (11%)	17(18%)	26 (27%)	Not detected	Not detected	Not detected

n = number of srains.

Out of the 96 *Vibrio* strains, 64 (67%) were positive for *V. cholerae* (Table 4). 31 (48%) *V. cholerae* strains were isolated from the treated wastewater (24 strains from treated wastewater storage pond and 7 strains at the output of the M'zar WWTP) (Table 4). The occurrence of *V. cholerae* in the soil and turfgrass irrigated with treated wastewater was 14 (26%) and 9 (17%) strains, respectively (Table 4). Our finding revealed also the presence of 10 (16%) *V. cholerae* strains in groundwater samples (GW). PCR detection results of ctxA gene showed that all the isolates were identified as non-O139/non-O1 *V. cholerae* and they did not possess the ctxA gene (Table 4). 30 (31%) strains *Vibrio* isolated were *Vibrio alginoliticus* (Table 4). *V. alginolyticus* was detected in similar percentages (37 %) from the soil and the treated wastewater storage pond, while 7 (23%) strains were isolated from the turfgrass and one strain (3%) from the treated wastewater at the output of the WWTP (Table 4). Two strains (2%) of *V. parahaemolyticus* were isolated from all

samples analyzed, one each from the soil and the other from the turfgrass irrigated with treated wastewater (Table 4). Ours results showed that strains of *V. parahaemolyticus* detected were negative for trh and tdh genes (Table 4).

Table 4 Distribution of *Vibrio* strains isolated from the M'zar WWTP and the golf courses.

***Vibrio* srains (n = 96)**	**M'zar WWTP**	**Golf course irrigated with treated wastewater (GW)**				**Golf course irrigated with groundwater (GG)**		
	Treated wastewater at the output of the plant	Treated wastewater storage pond	Groundwater	Turgrass	Soil	Ground-water	Turgrass	Soil
***V. cholerae* (n = 64)**	7 (11%)	24 (37%)	10 (16%)	9 (14%)	14 (22%)	ND	ND	ND
***V. cholerae serovar* O1 and O139**	ND	ND	ND	ND	ND	ND	ND	ND
***V. alginolyticus* (n = 30)**	**1 (3%)**	**11 (37%)**	ND	**7 (23%)**	**11 (37%)**	ND	ND	ND
***V. parahaemolyticus* (n = 2)**	ND	ND	ND	**1 (50%)**	**1 (50%)**	ND	ND	ND
tdh- and trh-positive *V. parahaemolyticus*	ND	ND	ND	ND	ND	ND	ND	ND

n = number of strains; **ND** = Not detected.

4. DISCUSSION

Contaminated vegetables and plants have been associated with treated wastewater irrigation. Rai and Tripathi (2007) isolated *Vibrio* spp from vegetables irrigated with partially treated wastewater [29]. *Vibrio* spp. was also among the pathogens detected in irrigated vegetables and in the wastewater used for irrigation [26]. Likewise, Dahiru and Enabulele (2015) have reported the occurrence and diversity of seven *Vibrio* species on lettuce and wastewater used in irrigation [8].

V. cholerae is a human pathogen that has been associated with cholera epidemics in many developing countries [21]. It's a Gram-negative bacterium and classified into approximately 200 distinct serogroups. Among them, the serogroups O1 and O139 that are established as the main agents of cholera [7]. Our findings were in agreement with those found by Eddabra, (2011), *V. cholerae* were the species most isolated by Eddabra, (2011) who studied the occurrence of *Vibrio* spp in secondary treated wastewater of M'zar WWTP. isolated V. cholerae from vegetable samples irrigated with treated wastewater [17]. Furthermore, V. cholerae strains were also found in lettuce samples irrigated with wastewater [8].

Groundwater contamination by *V. cholerae* has previously been described [31]**.** The use of treated wastewater in irrigation can be at the origin of the *V. cholerae* contamination of groundwater. Downs et al., (1999) reported that detection of *V. cholerae* non-01 in surface waters at sites irrigated with treated wastewater suggested potential *Vibrio* contamination of near-surface groundwater [11]. The contamination by *V. cholerae* of groundwater (GW) can be also attributed to the intrusion of seawater. Unno et al., (2015) reported that seawater-intrusion can significantly modify the groundwater bacterial community [33]. In other studies, *V. cholerae* non-O1 and non-O139 strains were isolated from seawater samples [30]. The absence of ctxA gene in environmental strains has been reported in several studies [9, 18]. However, intestinal and extra-intestinal infections have been linked to non-O1 and non-O139 *V. cholera* [18].

V. alginolyticus is a bacterium found mainly in marine and estuarine waters [24]**.** It's an opportunistic pathogen of marine animals [5]. However, the species is an emerging pathogen of humans [4]**.** Previous studies have reported the occurrence of *V. alginolyticus* in treated wastewater. Among the 58 *Vibrio* spp isolated from secondary treated wastewater of M'zar WWTP, (29%) were identified as *V. alginolyticus* (Eddabra, 2011). Khouadja et al., (2014) has also isolated more frequently *V. alginolyticus* strains from treated wastewaters [19].

V. parahaemolyticus is widely distributed in marine and estuarine environments around the world [36]. This species is a food-borne pathogen most frequently associated with gastroenteritis in humans [23]. The pathogenesis of *V. parahaemolyticus* depends on many virulence factors including, secretion systems (T3SS1 and T3SS2), thermostable direct hemolysin (tdh) and the thermostable direct hemolysin-related hemolysin (trh) [22]. Molecular identification of Vibrio strains isolated from final effluents of wastewater treatment revealed that *V. parahaemolyticus* strains were dominant [27]. Also*, V. paraheamolyticus* strains were detected in from lettuce irrigated with wastewater [8]. In this study, no treated wastewater sample was positive for *V. parahaemolyticus,* similar results were obtained in the study carried out by (Eddabra, 2011).

5. CONCLUSION

In the Agadir region, the use of unconventional wastewater sources is a strategic solution for the preservation of fresh water. The present study was carried out to assess the occurrence of pathogenic *Vibrio* in treated wastewater used in irrigation and its possible public health risks. According to guidelines Moroccan for reuse of treated wastewater in irrigation, *V. cholerae* O1 and O139 must be absent in 450 mL of irrigation water. Our results show that all strains *V. cholerae* isolated from treated wastewater and GW were negative for ctxA gene. However, other human pathogenic *Vibrio* spp., including *V. cholerae* non-O1 and non-O139, *V. parahaemolyticus*, and *V. alginolyticus* were detected in the treated wastewater and the two golf courses. Therefore, the environment may be important as reservoirs, and resistant *Vibrio* strains may pose a serious threat to human health. Irrigation with treated wastewater could be a source of green spaces contamination by *Vibrio* spp. In order to ensure the reduction of pathogenic *Vibrio* pollution, tertiary treatment adopted in M'zar WWTP and treated wastewater storage pond that installed in the golf course GW must be well managed.

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