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Occurrence and antibiotic resistance of *Vibrio parahaemolyticus* isolated from the Tunisian coastal seawater

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ABSTRACT

Vibrio parahaemolyticus is a gram-negative bacterium ubiquitous in seawater or estuarine water throughout the world. It is a major cause of seafood gastroenteritis complications. In this study, the presence of *V. parahaemolyticus* was investigated in 66 seawater samples collected during 2018 from 15 stations spread along the Tunisian coast using selective media including CHROMagar Vibrio media. The results show that only eight samples contained *V. parahaemolyticus*. However, while *Vibrio alginolyticus* was detected in all samples; both *Vibrio cholerae* and *Vibrio vulnificus* were not found. Nine of the presumed *V. parahaemolyticus* colonies were purified on tryptic soy agar from eight positive samples then identified by the API 20E biochemical test and confirmed by the presence of a specific target *toxR* gene. The detection of virulence genes, thermostable direct haemolysin (*tdh*) and thermostable-related haemolysin (*trh*), by the polymerase chain reaction (PCR) showed the presence of only two *trh*-positive isolates. The assessment of antibiotic susceptibility of the *V. parahaemolyticus* isolated revealed a complete resistance to colistin, amikacin, penicillin and cefotaxime and a total sensitivity to chloramphenicol, nitrofurantoin and sulfamethoxazole-trimethoprim with a multiple antibiotic resistance index (MAR) ranging from 0.4 to 0.5.

Key words: antibiotic resistance, seawater, *Vibrio parahaemolyticus*, virulence gene

HIGHLIGHTS

- This study represents the first spatiotemporal evaluation of *V. parahaemolyticus* in the Tunisian coastal seawater.
- It shows the low presence of *V. parahaemolyticus*.
- It provides an idea of the degree of antibiotic resistance of this species.
- It could indicate the possibility of the emergence of multiresistant strains.
- The presence of virulent strains suggests further investigations of this bacterium in recreational waters and marine products.

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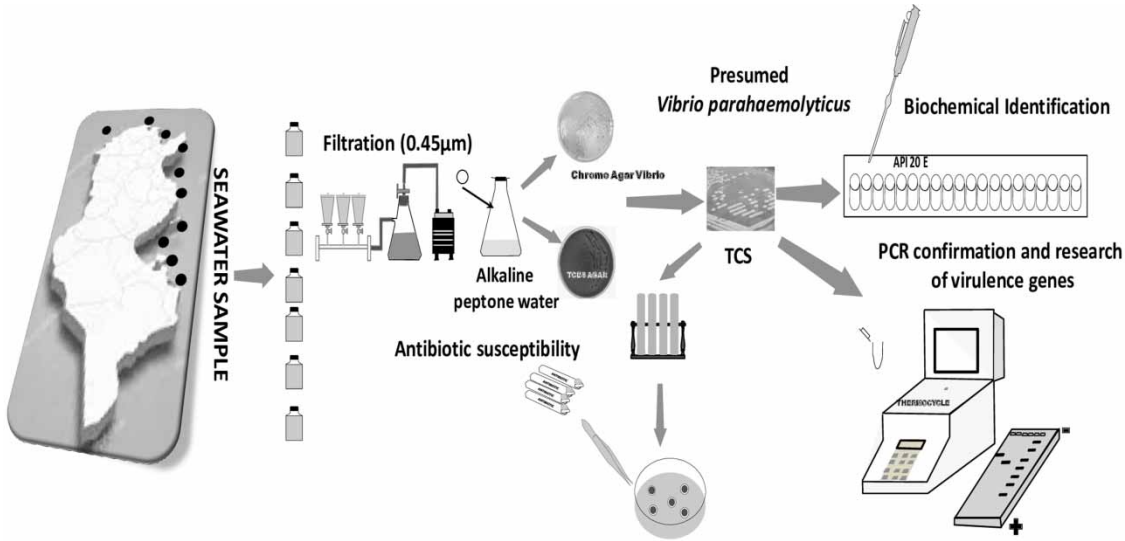
GRAPHICAL ABSTRACT



INTRODUCTION

Vibrio parahaemolyticus is a natural halophilic bacterium found in estuarine and in shore marine water throughout the world (Letchumanan *et al.* 2015; Lopez-Joven *et al.* 2015). Since its isolation about 72 years ago (Fujino *et al.* 1953), infection by this bacteria has frequently been reported and has been closely associated with the consumption of raw or insufficiently cooked seafood or through wounds (Oliver 2005; Lopez-Joven *et al.* 2015). *V. parahaemolyticus* is recognized as a cosmopolitan bacterium frequently isolated from the USA, Canada (Newton *et al.* 2012; Taylor *et al.* 2018), Asia (Letchumanan Chan & Lee 2014; Yang *et al.* 2017), European countries (Cantet *et al.* 2013; Passalacqua *et al.* 2016), South America (Martinez-Urtaza *et al.* 2013; Raszl *et al.* 2016) and Africa (Malainine *et al.* 2013).

Some *V. parahaemolyticus* strains are pathogenic to humans and were responsible for 25% of seafood-borne diseases (Martinez-Urtaza *et al.* 2013; Tran *et al.* 2018). This virulence was associated especially with the production of thermostable direct haemolysin (*tdh*) and/or thermostable-related haemolysin (*trh*) (Wang *et al.* 2018), also known as the Kanagawa phenomenon (KP) (Leoni *et al.* 2016). Nevertheless, virulence was reported in some negative *tdh* and *trh* strains (Ottaviani *et al.* 2012; Chung *et al.* 2016). Some specific clones of *V. parahaemolyticus* were recognized as responsible for pandemic episodes such as O4:K68, O3:K6, and O3:K69 serovars (Haendiges *et al.* 2015; Han *et al.* 2017). Apart from its pathogenesis in human beings, this bacterium was previously reported in aquaculture industry outbreaks worldwide (Khouadja *et al.* 2013b; Soto-Rodriguez *et al.* 2015).

Several factors influence the distribution and abundance of *V. parahaemolyticus* in the sea or in estuarine ecosystems including water temperature (De Paola *et al.* 2003; Konrad *et al.* 2017), salinity (Raszl *et al.* 2016), oceanic circulation, zooplankton density, harmful algal (Vezzulli *et al.* 2016; Han *et al.* 2017) and faecal pollution (Nongogo & Okoh 2014; Okeyo *et al.* 2018). Overall, most studies agree that warmer water along with low salinity constitutes suitable conditions for *V. parahaemolyticus* presence and abundance (Martinez-Urtaza *et al.* 2008; Urquhart *et al.* 2016).

The large use of antibiotics in public health or on farm animals is partly responsible for the emergence of multidrug-resistant strains in common environmental bacteria (Letchumanan *et al.* 2015; Park *et al.* 2018). Generally, the development of antibiotic resistance in bacteria is controlled by a variety of mechanisms, most commonly gene transfer, mutation (Dalsgaard *et al.* 2000; Bengtsson-Palm *et al.* 2018) and cell membrane modification.

While numerous foodborne infections are associated with *V. parahaemolyticus* worldwide (Wang *et al.* 2017), only a limited effort has been made to investigate this bacterium in the Tunisian seawater; however, most research has focused on the analysis and characterization of the *Vibrio alginolyticus* (Ben Kahla-Nakbi *et al.* 2009; Lajnef *et al.* 2012). To date, important data gaps are identified regarding the prevalence and the distribution of *V. parahaemolyticus* in the Tunisian coastal seawater.

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The few studies carried out on this bacterium reported a number of fish farm outbreaks caused by *V. parahaemolyticus* (Khouadja *et al.* 2013b).

In the present study, we provide information on the occurrence, the antimicrobial susceptibility and the potential virulence genes (*tdh* and *trh*) of *V. parahaemolyticus* isolated from 15 stations situated along the Tunisian coastal seawater.

MATERIALS AND METHODS

Site's location and sample's collection

During 2018, 66 surface seawater samples from 15 stations located along the Tunisian coast were collected and analysed specifically for the presence of *V. parahaemolyticus* while reporting the presence of *V. alginolyticus*, *Vibrio vulnificus* and *Vibrio cholerae* (Figure 1). Simultaneously, parameters including geographical coordinates, period of sampling, temperature (°C) of the water samples and the vocation of the sampling area were determined (Table 1). The sampling frequency varies from a minimum of twice a year to once a month depending on the logistics and the distance of each station.

Sampling stations were located in the coastal area delimited among 40–500 m from the coastline (Figure 1). The geographical coordinates of each station were obtained by a Garmin 60 GPS. Water samples were collected between 9 and 10 pm at 0.3 m depth in a 500 ml sterile glass bottle and then conserved in the darkness at 10 °C approximately, which is considered a non-stressful condition for *Vibrio* species (Shen *et al.* 2009; Wang *et al.* 2018). According to the proximity of the sampling stations to the laboratory, samples were processed within 7 min to 20 h after sampling.

Physical and chemical characteristics

Simultaneously to sampling, temperature (°C), salinity psu (practical salinity unit) and pH were recorded *in situ* using a thermo conductive probe (Thermo-salinometer WTW 320) and a pH-meter (WTW pH 3310). The turbidity expressed in NTU (Nephelometric Turbidity Unit) was measured in the laboratory using a turbidity meter Hach model Ratio XR 43900.

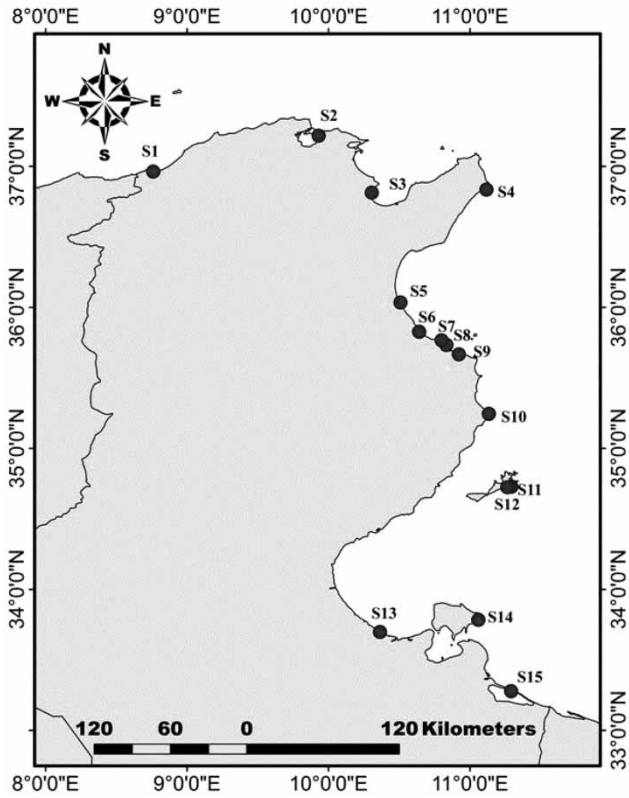

Figure 1 | Tunisian map showing the geographical emplacement of sampling sites.

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Table 1 | Sampling data and characterizations of some physicochemical parameters of sampling stations

Site	Month	Temp. (°C)	Salinity (psu)	Turbidity (NTU)	pH	Presumed *V. parahaemolyticus* isolates denomination	Water use	Latitude and longitude
Station1	December	13.3	36.8	0.698	8.21		Aquaculture use	36° 57′45.11″N 8° 45′40.28″E
	March	16.2	36.2	1.259	8.38			
Station 2	**May**	19.2	38.7	2.15	8.41	*V. parahaemolyticus* (Isol 9)	Shellfish culture	37°12′59.47″N 9°55′52.14″E
	June	24.1	38.0	1.149	8.51			
Station 3	February	11.2	38.2	1.589	8.35		Recreational area	36°48′47.49″N 10°18′23.67″E
	March	15.8	37.8	2.01	8.24			
	April	16.9	37.9	2.51	8.35			
	May	20.2	38.2	2.15	8.61			
Station 4	May	20.1	37.6	0.521	8.05		Recreational area	36°50′4.21″N 11° 7′4.15″E
	June	23.0	37.8	0.356	8.01			
Station 5	**November**	22.5	37.8	0.154	7.90	*V. parahaemolyticus* (Isol 6 and Isol 7)	Aquaculture area	36° 2′3.44″N 10°30′35.13″E
	December	17.3	37.8	0.225	7.89			
	April	18.7	37.8	0.321	8.11			
Station 6	**April**	18.4	39	1.254	8.55	*V. parahaemolyticus* (Isol 8)	Fishing harbour	35°49′33.30″N 10°38′31.35″E
	June	22.2	39	2.19	8.65			
	October	25.2	38.7	1.569	8.51			
Station 7	**January**	15.8	38.5	0.229	8.24	*V. parahaemolyticus* (Isol 3) *V. parahaemolyticus* (Isol 4)	Aquaculture area	35°44′50.14″N 10°49′49.37″E
	February	14.9	38.4	1.270	8.24			
	March	16.6	38.6	0.763	8.25			
	April	19.9	38.5	0.274	8.21			
	May	22.3	38.9	0.441	8.22			
	June	24.9	39.2	0.584	8.10			
	July	28.5	41.5	1.816	8.53			
	August	29.3	41.1	2.75	8.04			
	September	26.4	38.2	1.264	8.22			
	October	22.8	38.7	1.349	8.05			
	November	17.5	38.2	1.782	8.03			
	December	14.2	38.5	0.945	8.3			
Station 8	**January**	13.8	38.2	0.716	8.46	*V. parahaemolyticus* (Isol 5)	Aquaculture area	35°44′39.68″N 10°49′29.74″E
	February	12.6	38.4	0.961	8.17			
	March	16.5	38.2	1.767	8.35			
	April	18.4	38.3	0.247	8.27			
	May	24.8	38.2	0.971	8.19			
	June	28.4	39.2	0.798	8.39			
	July	29.3	41.2	0.975	8.62			

(*Continued.*)

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Table 1 | Continued

Site	Month	Temp. (°C)	Salinity (psu)	Turbidity (NTU)	pH	Presumed *V. parahaemolyticus* isolates denomination	Water use	Latitude and longitude
	August	28.7	42.1	1.658	8.46			
	September	25.4	39.2	1.256	8.05			
	October	22.8	38.5	1.684	8.44			
	November	16.2	38.2	1.313	8.13			
	December	13.1	38.5	0.328	8.31			
Station 9	March	18.4	38.6	1.441	8.45		Recreational area	35°39′58.88″N 10° 55′21.85″E
	May	24.7	39.7	1.887	8.62			
	June	24.7	41.1	0.961	8.52			
	July	31.2	43.1	1.571	8.50			
	September	26.0	41.0	1.581	8.43			
	October	24.6	39.9	2.13	8.10			
	November	20.2	38.9	1.522	8.51			
Station 10	Apr	18.3	38.1	0.356	8.11		Recreational area	35°14′39.12″N 11° 8′5.63″E
	June	23.3	38.3	0.256	8.09			
	Sep	27.1	38.7	0.456	8.12			
Station 11	**February**	16.7	38.0	0.722	8.05	*V. parahaemolyticus* (Isol 1)	Fishing area	34°43′37.34″N 11°17′37.71″E
	May	21.4	38.1	0.220	8.12			
	June	27.7	38.2	0.141	8.22			
	October	24.8	38.1	0.325	8.17			
Station 12	**February**	16.7	38.1	0.735	8.16	*V. parahaemolyticus* (Isol 2)	Fishing area	34°43′31.72″N 11°16′58.49″E
	May	21.4	38.1	0.232	8.18			
	June	27.7	38.2	0.165	8.25			
	October	24.8	38.0	0.319	8.19			
Station 13	January	13.5	39.3	0.541	8.24		shellfishing	33°41′23.87″N 10°22′7.01″
	February	14.1	37.8	0.741	8.22			
	March	16.2	36.5	0.589	8.54			
	April	23.1	39.6	1.113	8.48			
Station 14	February	13.8	38.5	0.214	8.01		Recreational area	33°46′58.42″N 11° 3′37.23″E
	March	18.1	38.8	0.351	8.21			
Station 15	April	24.5	39.2	0.524	8.36		Fishing area	33°16′36.64″N 11° 17′31.73″E
	June	29.2	41.1	0.689	8.51			

Months in bold indicate the presence of presumptive isolates *V. parahaemolyticus*.

Detection of *Vibrio* species by cultural methods

For *Vibrio* detection, 100 ml of seawater for each sample were filtered under sterile conditions through a 0.45 μm pore size cellulose nitrate filter (Whatman 7184-004). The filter was transferred in 200 ml of double concentrated alkaline saline peptone water (APW-salt 3% (w/v) NaCl, pH 8.4). After 18 h of incubation at 37 °C under shaking at 25 RPM (revolutions per

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minute), a loopful of each enrichment was streaked on *Vibrio* plates (CHROMagar™ Vibrio Microbiology, France), used for the selective isolation of some *Vibrio* species. The presence of presumptive colonies of *V. parahaemolyticus*, *V. alginolyticus*, *V. cholerae* and *V. vulnificus* was examined in the CHROMagar plate after 18 h of incubation at 37 °C based on their colours.

Only typical purple colonies of *V. parahaemolyticus*, in CHROMagar, were selected and plated onto thiosulphate citrate bile salt sucrose (TCBS, SCHARLAU, Spain) for further control. A typical colony with a purple colour in CHROMagar and a green one in TCBS is considered as a presumptive *V. parahaemolyticus*. Only eight samples from 66 showed the presence of purple colonies. In total, nine presumptive colonies were selected and purified on trypticase soy agar (TSA) (BIORAD) supplemented with 2% NaCl, then stored in the CRYO-BEADS (AES Laboratoire) at −80 °C for further analysis.

Biochemical identification of presumptive *V. parahaemolyticus* isolates

Catalase and cytochrome oxidase activities were tested, respectively, with hydrogen peroxide (30% volume) and oxidase discs (HIMedia product), while the Gram staining and motility were examined microscopically. The sensitivity to vibriostatic compound was tested by using O_{129} (150 μg/disk, Oxoid) in TSA supplemented with 2% NaCl (BIORAD). Furthermore, the identification of each presumptive *V. parahaemolyticus* isolate was performed by commercially available miniaturized identification systems API 20E (API–bioMerieux, Marcy l'Etoile, France) following the manufacturer's instructions. The inocula were prepared in sterile distilled water supplemented with 1% NaCl. Positive results were recorded according to colour change and identified by using the APIWEB identification software (APIWEB™ identification software bioMérieux). The results of identification were presented with the aforementioned acceptability provided by the software. All biochemical tests were made in duplicate.

Antibiotic susceptibility

The antibiotic susceptibility analysis of *V. parahaemolyticus* was carried out using the disc diffusion method (Bonnet *et al.* 2018) on Muller Hinton agar plates (Pronadisa Laboratories CONDA, Spain) supplemented with 2% NaCl. The standard commercial antibiotic disks used (Bio-Rad Marnes-la-Coquette France) were as follows: colistin (COL) (50 μg), amikacin (AKN) (30 μg), nalidixic acid (NAL) (30 μg), nitrofurantoin (NFE) (300 μg), cefotaxime (30 μg) (CTX), chloramphenicol (CHL) (30 μg), gentamicin (GMI) (15 μg), tetracycline (TET) (30 μg), trimethoprim/sulphamethoxazole (SXT) (1.25/23.75 μg) and Penicillin (P) (6 μg).

After incubation at 37 °C for 18–24 h, the diameter of the inhibition zone was measured using a calliper, and the obtained values were interpreted according to the standard of Clinical and Laboratory Standards Institute (CLSI 2018). Antibiotics' susceptibilities were carried out in triplicate, and the results are expressed as the mean of the three experiments with standard deviation.

Molecular identification and detection of virulence genes by polymerase chain reaction

The preparation of template DNA for the polymerase chain reaction (PCR) was performed by a boiling technique. Briefly, 1 ml of overnight pure culture in tryptic soy broth supplemented with 2% NaCl of each presumptive isolate was centrifuged at 7,000 RPM, at 4 °C for 8 min. The pellet was suspended in 300 μL of Tris–EDTA (TE) buffer (10 mM Tris, 1 mM EDTA, pH 8.2) and boiled at 100 °C for 8 min. Cell suspension was centrifuged for 8 min at 9,000 RPM at 4 °C, and the obtained supernatant containing the DNA was carefully transferred to a new tube and stored at −20 °C for PCR analysis (Alexopoulou *et al.* 2006).

The PCR was performed both on the DNA extract obtained from sample enrichment and from the pure culture of presumptive isolates. The species-specific *toxR* gene (368 bp) was conducted according to the method developed by Kim *et al.* (1999). DNA extracted from *V. parahaemolyticus* isolates was further examined to detect the presence of virulence genes *tdh* (251 bp) and *trh* (250 bp) (Tada *et al.* 1992; Kim *et al.* 1999). Primers used in this study are listed in Table 2.

DNA from *V. parahaemolyticus* AQ 4037 was used as a positive control for *trh* gene and *V. parahaemolyticus* ATCC 43996 was used as a positive control for *toxR* and *tdh* genes. In all PCRs, DNA extracted from *Salmonella typhimurium* ATCC 14028s was used as a negative control. Amplification was performed in a thermal cycler 'TECHNE 3000'. PCR products were electrophoresed on a 1.5% agarose gel, stained with ethidium bromide, visualized and photographed under a UV transilluminator (Bio-Rad ChemiDoc XRS System).

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Table 2 | Primers used in this study

Gene	Primers	Product size (bp)	
toxR	5′-GTCTTCTGACGCAATCGTTG-3′ 5′-ATACGAGTGGTTGCTGTCATG-3′	368	Kim *et al.* (1999)
trh	5′-GGCTCAAAATGGTTAAGCG-3′ 5′-CATTTCCGCTCTCATATGC-3′	250	Tada *et al.* (1992)
tdh	5′-CCACTACCACTCTCATATGC-3′ 5′-GGTACTAAATGGCTGACATC-3′	251	Tada *et al.* (1992)

RESULTS

Isolation and biochemical identification

All 66 seawater samples analysed by cultural methods using CHROMagar culture media showed the absence of typical blue-turquoise colonies relative to *V. cholerae* and green colonies relative to *V. vulnificus* as well. Our analysis showed the presence of yellow colonies in CHROMagar relative to *V. alginolyticus* in all seawater samples. These entire presumptive colonies specific to *V. alginolyticus* in CHROMagar indicated a positive amplification by PCR using collagenase-targeted gene amplification (Di Pinto *et al.* 2005) (data are not shown in this paper).

Only eight samples revealed the presence of purple colonies in CHROMagar (Table 1) characterizing a typical *V. parahaemolyticus*. In total, nine presumptive colonies were selected from CHROMagar, grown on TCBS agar for further differentiation and then isolated in pure culture on TSA agar supplemented with NaCl at 2%.

Hence, the analysis showed that all nine purified isolates are motile, Gram-negative, cytochrome oxidase-positive, catalase-negative and sensitive to vibriostatic compound O_{129} (150 µg/disk). Furthermore, the API 20^{E} biochemical identification confirmed, with a level of acceptance up to 99%, that all presumptive colonies are *V. parahaemolyticus* (Table 3).

Biochemical characterization performed in API 20E revealed that all these isolates were β-galactosidase-negative and positive for glucose fermentation and indole. Some variations were registered on enzyme activity, such as tryptophan desaminase, melobiose and citrate metabolism. All the isolates (Isol) were urease-negative except those from station nos 11 and 12 (Isol 1 and Isol 2), indicating a capacity to metabolize urea.

Molecular characterization

All the colonies identified as *V. parahaemolyticus* by the API 20^{E} test were found to be positive for the *toxR* gene amplification (Figure 2). Simultaneously, perfect agreement has been found for the *toxR* amplification that was performed on DNA extracted from the enriched samples and presumptive *V. parahaemolyticus* isolates obtained by a cultural method. Only the two isolates, Isol 1 and Isol 2, obtained from stations 11 and 12, were found positive for *trh* gene (Figure 3). However, all isolates were negative for the *tdh* gene amplification (Figure 4).

The obtained results showed a limited presence of *V. parahaemolyticus* in the Tunisian coastal seawater, which was found only in seven stations (Table 1). All *V. parahaemolyticus* were isolated at temperatures ranging from 13.8 to 29.3 °C and at salinity between 37.8 and 41.1 psu (Table 1). The two positive *trh V. parahaemolyticus* were isolated from stations 11 and 12 during the month of February (16.7 °C temperature, 38 psu salinity and 0.722 NTU turbidity) (Figure 1; Table 1). *V. parahaemolyticus* was not found in stations 1, 3, 4, 9, 10, 13, 14 and 15 by both cultural and molecular methods.

Antibiotic susceptibility

Antibiotic susceptibility (Table 4) of different *V. parahaemolyticus* isolates shows that all isolates exhibited resistance to colistin, amikacin, penicillin and cefotaxime. However, all strains were sensitive to ceftazidime, tetracycline, ciprofloxacin, chloramphenicol, nitrofurantoin and nalidixic acid, and were registered. Different resistance patterns were recorded for tetracycline (66% of isolates), gentamicin (88% of isolates), nitrofurantoin (33% of isolates) and sulfamethoxazole-trimethoprim (11% of isolates). The multiple antibiotic resistance (MAR) index values show that eight of nine *V. parahaemolyticus* isolates have a value of 0.4, while only one has a 0.5 value (Isol 9).

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Table 3 | Biochemical characterization of *V. parahaemolyticus* isolates (Isol) by the API 20E test

Isolates		Isol 1	Isol 2	Isol 3	Isol 4	Isol 5	Isol 6	Isol 7	Isol 8	Isol 9
Biochemical test	**Abbreviation**									
β-galactosidase	ONPG	−	−	−	−	−	−	−	−	−
Arginine dihydrolase	ADH	−	−	−	−	−	−	−	−	−
Lysine décarboxylase	LDC	+	+	+	+	+	+	+	+	+
Ornithine décarboxylase	ODC	+	+	+	+	+	+	+	+	+
Citrate utilization	CIT	+	+	+	−	+	+	−	+	+
H_2S production	H_2S	−	−	−	−	−	−	−	−	−
Urease production	URE	+	+	−	−	−	−	−	−	−
Tryptophane désaminase	TDA	−	−	+	+	+	−	+	+	−
Indole production	IND	+	+	+	+	+	+	+	+	+
Acétoïne production (Vogues–Proskawer reaction)	VP	+	+	+	+	+	−	+	+	+
Gelatinase production	GEL	+	+	+	+	+	+	+	+	+
Glucose production	GLU	+	+	+	+	+	+	+	+	+
Manitol acidification	MAN	+	+	+	+	+	+	+	+	+
Inositol acidification	INO	−	−	−	−	−	−	−	−	−
Sorbitol acidification	SOR	−	−	−	−	−	−	−	−	−
Rhamose acidification	RHA	−	−	−	−	−	−	−	−	−
Saccharose acidification	SAC	−	−	−	−	−	−	−	−	−
Melobiose acidification du	MEL	+	+	−	−	−	−	−	−	−
Amygdalin acidification	AMY	−	−	−	−	−	−	−	−	−
Arabinose acidification	ARA	+	+	+	+	+	+	+	+	+
Nitrite metabolism	NO_2	+	+	+	+	+	+	+	+	+
Oxydase test	OXY	+	+	+	+	+	+	+	+	+
Probability of identification (%)		99.8	99.8	99.7	99.8	99.7	99.9	99.8	99.7	99.7
Gram staining	Gram	−	−	−	−	−	−	−	−	−
Vibriostatic compound sensibility	O_{129}	S	S	S	S	S	S	S	S	S
Motilité	M	+	+	+	+	+	+	+	+	+

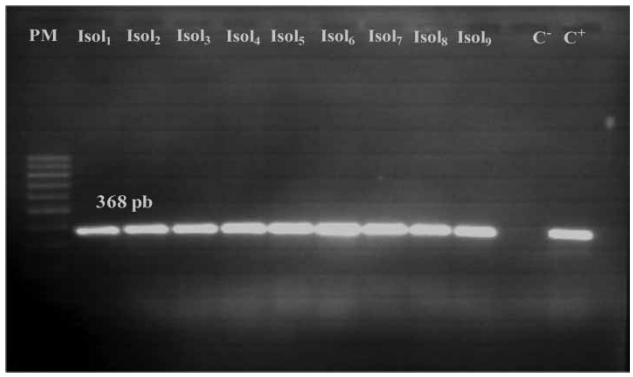

Figure 2 | Detection of *toxR* gene in presumptive *V. parahaemolyticus* isolates on 1.5% agarose gel. Column 1: kb ladder; Columns 2–10: Isol 1–Isol 9; Column 11: negative control (*Salmonella typhimurium* ATCC 14028s), C^-; Column 12: positive control (*V. parahaemolyticus* ATCC 43996), C^+.

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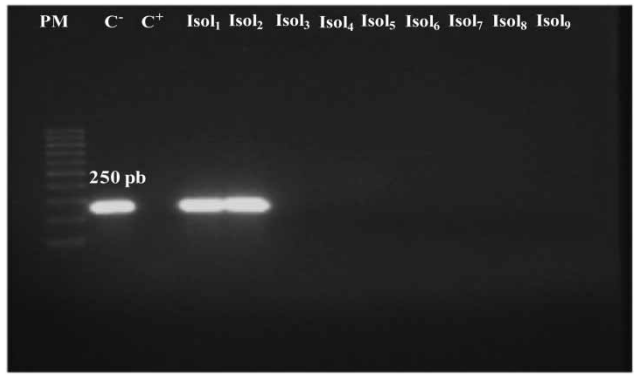

Figure 3 | Detection of *trh* gene in *V. parahaemolyticus* isolates on 1.5% agarose gel. Column 1: kb ladder; Column 2: positive control (*V. parahaemolyticus* AQ 4037), C^{+}; Column 3: negative control (*Salmonella typhimurium* ATCC 14028s), C^{-}; Columns 4–11: Isol 1–Isol 9; only Isol 1 and Isol 2 show positive results.

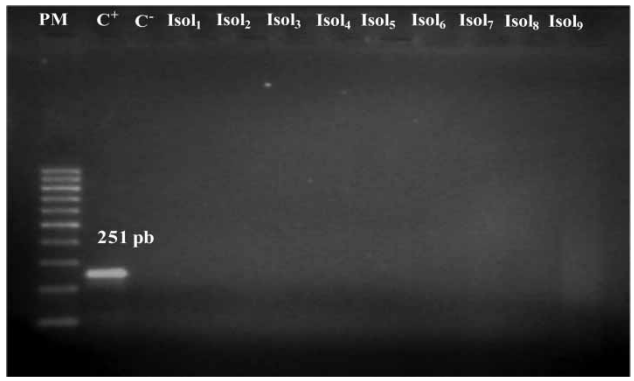

Figure 4 | Detection of *tdh* gene in *V. parahaemolyticus* isolated on 1.5% agarose gel. Column 1: kb ladder; Column 2: positive control (*V. parahaemolyticus* ATCC 43996), C^{+}; Column 3: negative control (*Salmonella typhimurium* ATCC 14028s), C^{-}; Columns 4–12: Isol 1–Isol 9.

DISCUSSION

To our knowledge, this study constitutes the first extended monitoring of the presence of *V. parahaemolyticus* in Tunisian marine waters. This reveals the low occurrence of *V. parahaemolyticus* in Tunisian coasts compared to investigation results found in other Mediterranean countries such as Italy, France and Spain (Lopez-Joven *et al.* 2015; Caburlotto *et al.* 2016). Our results are in agreement with those reported by Snoussi *et al.* (2008), Khouadja *et al.* (2013a), Zrelli *et al.* (2015) and Gdoura *et al.* (2016), who also mentioned the rare presence of *V. parahaemolyticus* in Tunisian seawaters.

V. parahaemolyticus has received extensive attention in numerous countries for its recovery in seafood (Letchumanan *et al.* 2014; Tran *et al.* 2018). However, few resources have been allocated in Tunisia to *V. parahaemolyticus* due to the absence of epidemiological cases (annual report of Institut Pasteur 2017, 2018). A rare infection caused by *V. parahaemolyticus* is probably due to the absence of consumption of raw or undercooked seafood in Tunisian traditional food. The low presence of *V. parahaemolyticus* in the Tunisian coastal seawater compared to data in Asian and European countries (Zulkifli *et al.* 2009; Esteves *et al.* 2015; Odeyemi 2016) suggests that this species adapts poorly to southern Mediterranean conditions. In fact, the viability of *V. parahaemolyticus* in the south Mediterranean is likely influenced by the synergistic effect of high temperature, important salinities and sunlight intensity (Givens *et al.* 2014; Larsen *et al.* 2015).

However, the high sunning and good water transparency in the sampling sites (lighting superior to 25 Klux and turbidity ranging between 0.141 and 2.75 NTU) contribute to the bacterial die-off by the production of reactive oxygen species (Zaafrane *et al.* 2004; Schmitz-Valckenberg *et al.* 2016).

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Table 4 | Mean and standard deviations of diameters of the antibiotic inhibition zone (mm)

Strains	COL	NAL	CTX	SXT	CHL	GMI	NFE	AKN	TET	P	MAR index
Isol 1	0.0 ± 0.0^{a}	22.1 ± 0.2^{b}	0.0 ± 0.0^{a}	21.1 ± 0.0^{b}	31.0 ± 0.0^{b}	13.6 ± 0.2^{c}	22.2 ± 0.1^{b}	0.0 ± 0.0^{a}	21.2 ± 0.1^{b}	0.0 ± 0.0^{a}	0.40
Isol 2	0.0 ± 0.0^{a}	22.0 ± 0.1^{b}	0.0 ± 0.0^{a}	21.0 ± 0.1^{b}	31.1 ± 0.2^{b}	13.4 ± 0.1^{c}	22.1 ± 0.1^{b}	0.0 ± 0.0^{a}	21.3 ± 0.2^{b}	0.0 ± 0.0^{a}	0.40
Isol 3	2.2 ± 0.3^{a}	24.0 ± 0.2^{b}	0.0 ± 0.0^{a}	18.8 ± 0.2^{b}	22.7 ± 0.3^{b}	14.0 ± 0.1^{c}	20.0 ± 0.1^{b}	0.0 ± 0.0^{a}	16.6 ± 0.0^{c}	0.0 ± 0.0^{a}	0.40
Isol 4	4.5 ± 0.2^{a}	21.1 ± 0.1^{b}	0.0 ± 0.0^{a}	19.7 ± 0.0^{b}	23.8 ± 0.0^{b}	15.5 ± 0.1^{c}	22.9 ± 0.3^{b}	0.0 ± 0.0^{a}	17.5 ± 0.1^{c}	0.0 ± 0.0^{a}	0.40
Isol 5	2.3 ± 0.1^{a}	23.0 ± 0.2^{b}	0.0 ± 0.0^{a}	17.7 ± 0.0^{b}	22.8 ± 0.1^{b}	14.5 ± 0.1^{c}	21.1 ± 0.2^{b}	0.0 ± 0.0^{a}	16.5 ± 0.0^{c}	0.0 ± 0.0^{a}	0.40
Isol 6	8.5 ± 0.2^{a}	21.0 ± 0.1^{b}	0.0 ± 0.0^{a}	18.0 ± 0.0^{b}	22.9 ± 0.2^{b}	12.9 ± 0.2^{c}	14.7 ± 0.1^{c}	0.0 ± 0.0^{a}	16.5 ± 0.0^{c}	0.0 ± 0.0^{a}	0.40
Isol 7	6.5 ± 0.3^{a}	16.4 ± 0.3^{b}	0.0 ± 0.0^{a}	16.9 ± 0.1^{b}	22.4 ± 0.1^{b}	14.7 ± 0.1^{c}	16.8 ± 0.2^{c}	0.0 ± 0.0^{a}	16.5 ± 0.0^{c}	0.0 ± 0.0^{a}	0.40
Isol 8	10.0 ± 0.2^{a}	22.1 ± 0.1^{b}	0.0 ± 0.0^{a}	19.2 ± 0.1^{b}	25.5 ± 0.0^{b}	17.0 ± 0.0^{c}	22.9 ± 0.2^{b}	0.0 ± 0.0^{a}	22.2 ± 0.3^{b}	0.0 ± 0.0^{a}	0.40
Isol 9	0.0 ± 0.0^{a}	18.0 ± 0.2^{b}	0.0 ± 0.0^{a}	14.0 ± 0.0^{c}	22.3 ± 0.0^{b}	10.0 ± 0.2^{a}	15.0 ± 0.0^{c}	0.0 ± 0.0^{a}	16.5 ± 0.0^{c}	0.0 ± 0.0^{a}	0.50

MAR, multiple antibiotic resistance; COL, colistin; AKN, amikacin; NAL, nalidixic acid; NFE, nitrofurantoin; CTX, cefotaxime; CHL, chloramphenicol; GMI, gentamicin; TET, tetracycline; SXT, trimethoprim/sulphamethoxazole; Penicillin, P.
The non-observation of the inhibition zone around the disk was considered as 0 mm of diameter of inhibition.
[a]Resistant.
[b]Sensitive.
[c]Intermediate.

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This phototoxicity action induces the rapid passage in viable, but non-culturable (VBNC) cell states in many bacterial species, which partly explains the low presence of *V. parahaemolyticus* and other pathogenic *Vibrio* in Tunisian seawater (Zaafrane *et al.* 2004; Chandran & Hatha 2005; Idil *et al.* 2013).

In addition to the direct effect of sunlight, the scarcity of rivers, discharging into the sea on the Tunisian context contributes to the elevated salinities, and the limitation of nutrient inputs in the coastal zone, which constitute a selective condition for several germs (Millot & Taupier-Letage 2005; Slimani *et al.* 2012).

However, it is important to note the limitation of some current methods in quantifying and identifying bacteria in the marine environment due to the presence of the VBNC state (Li *et al.* 2014; Ding *et al.* 2017).

Underestimated or undetected in environmental and clinical samples by conventional methods of analysis, this physiological state (VBNC) poses a risk to public health. It also requires the use and the development of efficient techniques to quantify and evaluate pathogenic bacteria in marine environments, especially the ones associated with epidemic and pandemic cases. In this context, the use of techniques such as a High-Performance Flow Cytometry and RT-PCR seems to be required for monitoring different states of pathogenic bacteria. However, these rigorous techniques are actually limited for rapid large-scale use (Zhong & Zhao 2018; Robben *et al.* 2019; Wagley *et al.* 2019).

In this study, all *V. parahaemolyticus* isolates were isolated at temperatures ranging from 15.8 to 22.5 °C and salinities ranging from 37.8 to 39 psu, constituting favourable environmental conditions for this bacterium (De Paola *et al.* 2003; Zulkifli *et al.* 2009). Other authors (Martinez-Urtaza *et al.* 2008; Caburlotto *et al.* 2016) showed that the presence of *V. parahaemolyticus* is correlated essentially with low salinity (30.9–36.2 psu).

The absence of *V. parahaemolyticus* during the summer season (Table 1) characterized by an important photoperiod also explains the stressing synergistic effect of sunlight, high temperature and salinity on the presence of this bacterium (De Paola *et al.* 2003; Martinez-Urtaza *et al.* 2008; Liu *et al.* 2015).

Considering the limited number of *V. parahaemolyticus* isolates, and the few salinity fluctuations, it would be difficult to correlate the presence of this bacterium with this parameter (Table 1). However, some monitoring performed in the Tunisian coastal seawater showed that the distribution of *V. alginolyticus* and other pathogenic bacteria is correlated essentially with the water temperature (Cherif *et al.* 2011).

The important presence of *V. alginolyticus* in all samples is due not only to its capacity to tolerate hostile environmental conditions, but also its ability to use a zooplankton chitin as a nutrient (Rao *et al.* 2013). Moreover, several authors demonstrate that *V. alginolyticus* exhibited a better survival and a larger distribution in seawater than *V. parahaemolyticus* and *V. vulnificus* (Munro *et al.* 1994; Eiler *et al.* 2007).

The amplification of the *toxR* gene by the PCR from suspected *V. parahaemolyticus* isolates is in perfect concordance with the results of PCR performed directly from the enriched samples.

The amplification of virulence genes shows only the presence of *trh* in two isolates (stations 11 and 12) (Figure 1). These virulent isolates are found at the same time and in the same area (situated near to the anchorage area of the commercial Sfax harbour) and seem to be derived from ballast water.

The presence of positive *trh* strain is rarely observed in environmental isolates; however, some authors connect this presence with the season (Caburlotto *et al.* 2016; Di *et al.* 2019). Several studies demonstrate that the percentage of presence of *tdh* and *trh* strains in a marine environment remained between 2 and 9% for *tdh* and *trh*, respectively, and this proportion depends on environmental factors and geographical context (Caburlotto *et al.* 2016; Di *et al.* 2019). The two isolates possessing a *trh* gene also exhibit a positive urease activity in the API 20E test.

Normally, most urease-positive strains isolated from the environment are mainly positive for *trh* gene (Wang *et al.* 2017); yet, some research reported the presence of non-virulent strains (trh^- and tdh^-) but having a capacity to hydrolyze urea (Ottaviani *et al.* 2012; Zrelli *et al.* 2015).

Although several authors criticize the use of API 20E systems for the identification of presumptive environmental isolate (Croci *et al.* 2007; Copin *et al.* 2012; Amalina & Ina-Salwany 2016), our results show a high degree of identification by phenotypic methods. In our investigation, cultural methods using CHROMagar and non-fermentation of the sucrose in TCBS seem to be an efficacious procedure for rapid control and differentiation of the three major pathogenic *Vibrio* species in environmental samples (Larsen *et al.* 2015; Nigro & Steward 2015).

Antibiotic susceptibility showed that most isolates are sensitive to sulphamide and chloramphenicol, yet resistant to amikacin, cefotaxime and penicillin. They presented intermediate susceptibility to gentamicin and tetracycline. These results are in agreement with those found by Letchumanan *et al.* (2015), Yang *et al.* (2017) and Elmahdi *et al.* (2018), which recorded an

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increase in multidrug resistance in environmental *Vibrio* and *Aeromonas* isolates. We note that all isolates are sensitive to the antibiotics used in the therapeutic treatment of *V. parahaemolyticus* such as amikacin sulphamide and nalidixic acid.

The tetracycline resistance observed in 66% of *V. parahaemolyticus* isolates is probably attributed to the large use of this antibiotic in aquaculture farms (Yang *et al.* 2017; Fri *et al.* 2018; Park *et al.* 2018). In fact, antibiotics and their residues have been reported frequently at sea and coastal areas (Smaldone *et al.* 2014; Patrolecco *et al.* 2018). In the same context, it is common that the isolation of *V. parahaemolyticus* strains is resistant to β lactams through the penicillinase production (Zago *et al.* 2020; Siddique *et al.* 2021). The recorded sensitivity to chloramphenicol in our case contrary to *V. parahaemolyticus* strains resistance observed in other countries (Han *et al.* 2017) is probably due to the limited use of this family of antibiotics in food product (Angulo *et al.* 2004; Chantziaras *et al.* 2014).

The MAR index ranges from 0.4 to 0.5 are greater than 0.2, which indicate that *V. parahaemolyticus* isolates have been exposed to antibiotics at least once in their cycles and probably have acquired a genetic resistance (Krumperman 1983; Letchumanan *et al.* 2015).

It has been largely demonstrated that antibiotic resistance acquired in bacteria is generally mediated by extrachromosomal plasmids and transmitted to the next generation or exchanged among the different bacterial population (von Wintersdorff *et al.* 2016). In the same framework, Letchumanan *et al.* (2015) demonstrated that the resistance to amikacin, ceftazidime, cefotaxime, kanamycin and levofloxacin depended on a plasmid carry in *V. parahaemolyticus*. Recent research has demonstrated the existence of two cellular signals inducted by bacteria for controlling antibiotic responses: a quorum-sensing signal responsible for inducing antibiotic resistance between bacteria and a quorum-quenching signal that altered this communication (Munita & Arias 2016). The transfer of mobile genetic elements of antibiotic resistance between marine bacteria (Partridge *et al.* 2018) and the large use of antibiotics is mostly responsible for the increase of multidrug resistance. This requires the development of environmental monitoring programmes in order to explore this emergence in marine bacteria.

This study conducted in 2018 showed the rare presence of *V. parahaemolyticus* in Tunisian coastal seawaters. This is probably due to poor adaptation of this bacterium for the south Mediterranean context (high sunning and salinity). However, the presence of virulent *trh* strains suggests the establishment of monitoring programmes for the detection and characterization of antibiotic resistance of *V. parahaemolyticus* and other pathogenic *Vibrio* species in Tunisian seawater and seafood product. Nevertheless, these results do not fail to record the expanding presence of *V. parahaemolyticus* and other pathogenic *Vibrio* species in the Tunisian context as a consequence of climate change as well as the disturbance in water quality.

CONFLICT OF INTEREST

The authors declare no conflicts of interest to disclose.

DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

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