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Molecular characterization of *Vibrio* species isolated from dairy and water samples

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Vibrio **species can cause foodborne infections and lead to serious gastrointestinal illnesses. The purpose of this research was to detect the *Vibrio cholerae* and *Vibrio parahaemolyticus* in raw milk, dairy products, and water samples. Also, it investigated the virulence factors, antibiotic resistance and biofilm formation in isolated bacteria. Conventional and molecular approaches were used to identify the isolates in this study. *Vibrio* species were detected in 5% of the samples. *Vibrio cholerae* and *Vibrio parahaemolyticus* were isolated from 1.25 and 1.5%, respectively, of the total samples. Penicillin resistance was detected in all strains of *Vibrio cholerae* and *Vibrio parahaemolyticus*, with a MAR index ranging from 0.16 to 0.5. Four isolates were moderate biofilm producer and three of them were MDR. When *Vibrio cholerae* was screened for virulence genes, *ctx*AB, *hly*A, and *tcp*A were found in 80, 60, and 80% of isolates, respectively. However, *tdh* + /*trh* + associated-virulence genes were found in 33.3% of *Vibrio parahaemolyticus* isolates.**

Vibrio species can cause the bacterial disease Vibriosis, which is a leading cause of mortality[1]. The gram-negative bacteria *Vibrio cholerae* (serogroups O1 and O139) also causes cholera epidemics[2]. Aquatic habitats frequently harbour non-O1/non-O139 strains, which have lately been connected to occasional instances of diarrhoea[3]. Ingestion of *V. parahaemolyticus*-contaminated food can cause nausea, diarrhoea, vomiting, and other food poisoning signs[4]. In extreme situations, it can result in sepsis and death[5]. *V. parahaemolyticus* food poisoning is becoming more common, that is dangerous to the health and safety of the general population. Its risk as a foodborne illness has overtaken that of salmonellosis, seriously compromising people's health and resulting in enormous economic losses[6]. Cholera toxin (CT), which is expressed by the *ctxAB* gene, and the toxin-coregulated pilus (TCP), which is encoded by the *tcpA* gene, are the two main virulence factors of *V. cholerae* O1 and O139[2]. While hemolytic toxin, which includes heat-resistant hemolytic toxin (TDH), hemolytic toxin linked with heat-resistant hemolytic toxin (TRH), and heat-labile hemolytic toxin, is the main factor contributing to *V. parahaemolyticus*'s pathogenicity (TLH)[7].

Antibiotics are the most commonly used treatment for bacterial diseases, and most *Vibrio* spp. are susceptible to them. Antibiotics that are effective against Vibriosis include tetracycline, quinolones, trimethoprim, oxytetracycline potentiated sulfonamides, quinolones, oxolinic acid, and sarafloxacin[8,9]. Due to the widespread and disorderly use of antibiotics, resistant strains of *Vibrio* spp. are developing and becoming more prevalent[10], It could be harmful to human health because it introduces resistant bacteria into the food chain or because mobile genetic elements can spread resistance genes to other human pathogens[11,12].

Vibrio spp. are able to produce adhesion factors, allowing them to adhere to surfaces and cause the development of biofilms[2]. *Vibrio* spp. enter food products through contamination of equipment and instruments by growing biofilms on inert surfaces like stainless steel, polyvinyl chloride, and glass, which leads to cross-infection and considerable antibiotic resistance[6]. This renders disinfectants, antibodies, and antibiotics ineffective against bacteria in the biofilm mass[2]. So this study was directed to detect the *V. cholerae* and *V. parahaemolyticus* in raw milk, dairy products, and water samples. Also, investigation of *Vibrio* isolates for existence of virulence factors, antibiotic resistance and biofilm formation.

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Results

Phenotypic identification of *Vibrio* spp.. Presumptive colonies of *Vibrio* spp. (yellow, blue-green) on TCBS agar plates were biochemically characterized. Out of 60 isolates, 33.3% (20/60) were presumptively selected as *Vibrio* spp. As Gram –ve curved rods exhibits ability for growth in 1% NaCl broth, oxidase positive reaction, has the ability to ferment glucose, nitrate reduction,H_2S production, lysine decarboxylation and gelatin liquefaction inveterate the characteristics of isolates.

Characterization of *V. cholerae* and *V. Parahaemolyticus* using *ctx* and *tox*R genes. These isolates were molecularly confirmed as *Vibrio* spp. using housekeeping *16S rDNA gene primers* and recovered from 2 and 6% of dairy and water samples, respectively, and a total of 5% of the total samples. Eleven of the molecularly confirmed *Vibrio* spp., were later identified on species level by *ctx* and *tox*R amplification, sequencing, and phylogenetic analysis as *V. cholerae* (n = 5, 1.25%) and *V. parahaemolyticus* (n = 6, 1.5%) (Table 1, Fig. 1). The sequences obtained in this investigation have been put in GenBank under the accession Nos. (OP414272, OP414273, OP414274, OP414275, OP414276, OP414277, OP414278, OP414279, OP414280, OP414281 and OP414282).

Antimicrobial resistance profile, virulence and biofilm production of *V. cholerae* and *V. parahaemolyticus*. The antibiotic resistance profiles of *Vibrio* isolate for the twelve antibiotics are stated in Table 2. All the obtained isolates exhibited resistance to Penicillin family followed by erythromycin (81.8%), while no resistance was recorded to phenolics and Quinolones. Four patterns of antimicrobial resistance and four isolates showed multiple antimicrobial resistances to drugs of at least three families. MAR indices were between 0.16 to 0.5; maximum MAR index was assigned to the isolates that exhibited resistance to six antibiotics (Table 3). Four isolates were MDR.

The virulence genes in the *V. cholerae* and *V. parahaemolyticus* isolates are displayed in Table 4. The biomarker genes, *ctxAB*, *hly*A and *tcp*A, codes for the virulence factors of *V. cholerae* were detected in 80, 60 and 80% of the obtained isolates, respectively. Meanwhile, there are two *trh* + and two *tdh* + /*trh* + *V. parahaemolyticus* strains, accounting for 33.3% per each pattern. Four isolates were moderate biofilm producer and three of them were MDR. All characters of the identified isolated are displayed in Fig. 2.

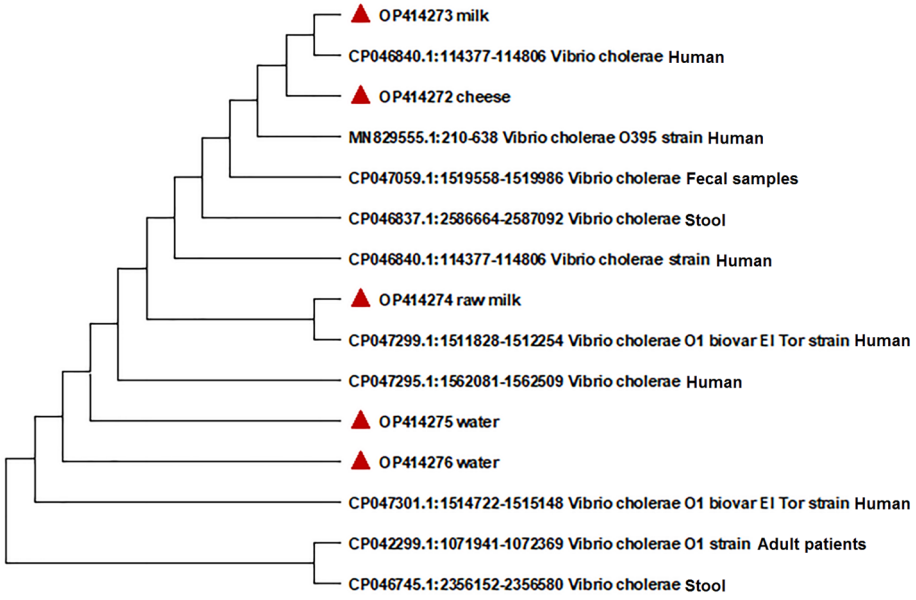
Discussion

Vibrio cholerae is a genus of zoonotic bacteria with global economic and health significance. It is a significant universal public health burden, producing significant morbidity and mortality in the population. Over the years, a number of researchers have focused on the severity of infections caused by *V. cholerae*, ignoring relatively minor *Vibrio* species that are important medically and some of which are emerging pathogens that can cause mild to severe human diseases[13]. A variety of methods were used in this study to isolate and identify the *vibrio* species, including TCBS culturing, the using of *Vibrio* housekeeping *16S rDNA* gene primers, and the amplification and sequencing of the *ctx* and *tox*R genes. Huq et al.[14] reported that molecular detection approaches had enhanced the incidence of finding harmful microbes while conventional culture-based detection methods might have failed. Many authors endorsed the using of *16S rDNA* gene to identify *Vibrio* spp[15–18]. In addition, the genetic information gained from the sequencing of the *ctx* and *tox*R genes distinguishes between species that are closely related, such as *V. cholerae* and *V. parahaemolyticus*[19]. The phylogenetic analysis of the previously sequenced genes revealed a relationship between our strains isolated from milk, milk products, and water and phylogenetically related reference strains on GenBank isolated from water, food, and patients. These findings may provide information about the source of contamination[20–22]. In the current investigation, three of the examined dairy samples included *V. cholerae*, of which two were isolated from raw milk. Tahoun et al.[23], Sharma et al.[24], and Islam et al.[25] also found higher results. This is corroborated by findings from Waturangi et al.[26] who found that *V. cholerae* could survive for at least three months in all items tested, including UHT milk and ice cream that was kept in a freezer.

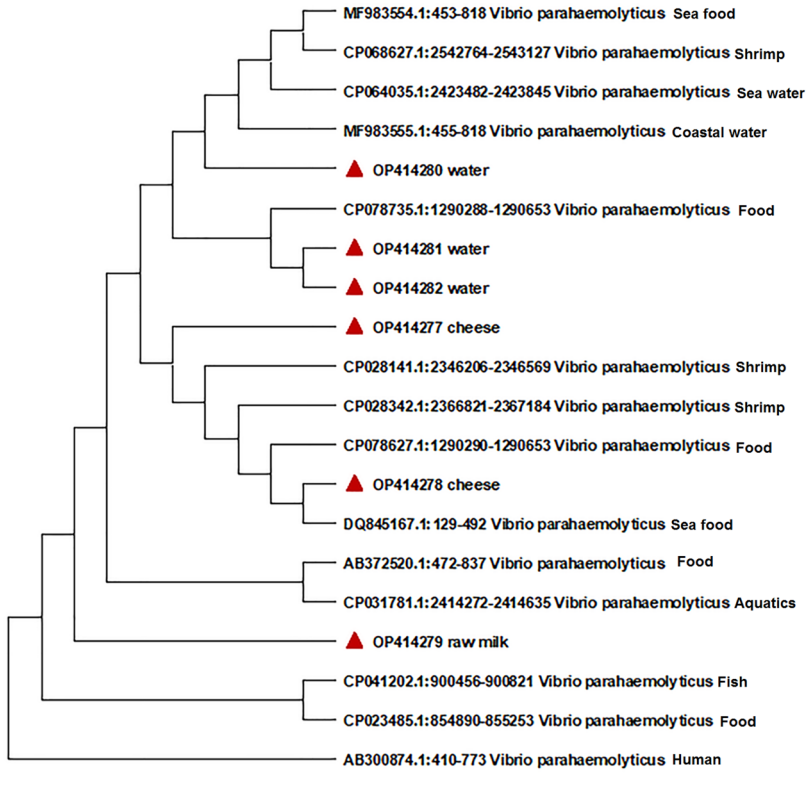
Several processes are involved in the production of Domiati cheese, including natural milk fermentation, salting, renneting, and salted whey solutions. The salt in cheese milk, which can range from 5.46 to 9.50%[27], plays

Type of samples	No. of samples	No. of *Vibrio* species positive samples (%)	No. of species (%)		
			V. cholerae	*V. parahaemolyticus*	Other *Vibrio* spp.
Raw milk	100	3 (3)	2 (2)	1(1)	0
Domiatti cheese	50	1 (2)	0	1 (2)	0
Kareish cheese	50	2 (4)	1 (2)	1 (2)	0
Yoghurt	50	0	0	0	0
Ice cream	50	0	0	0	0
Sub-Total	300	6 (2)	3(1)	3(1)	0
Water	100	14 (14)	2 (2%)	3(3%)	9 (9%)
Total	400	20 (5)	5 (1.25)	6 (1.5)	9 (2.25)

Table 1. Prevalence of *V. cholerae* and *V. parahaemolyticus* isolated from the examined samples.



(a)



(b)

Figure 1. The Maximum Likelihood tree shows the *ctx* and *tox*R genes phylogenetic relationships of (**a**) *V. cholerae* and (**b**) *V. parahaemolyticus* isolated from raw milk, cheese and water and phylogenetically related reference strains on GenBank.

an essential role in the production and processing process by promoting or suppressing bacterial development. *Vibrio* spp. are directly tied to a marine environment and are salt tolerant. According to several investigations *Vibrio* spp. and other bacteria associated with a marine environment were found in the cheese flora[27–30]. The

Antibiotic class	Antibiotic	S	I R	R
Penicillins	Ampicillin	0	0	11 (100%)
	Ampicillin-Sulbactam	0	0	11 (100%)
Cephems	Ceftazidime	7 (63.6%)	0	4 (36.4%)
	Cefotaxime	11 (100%)	0	0
Aminoglycosides	Gentamicin	7 (63.6%)	0	4 (36.4%)
Tetracyclines	Tetracycline	9 (81.8%)	0	2 (18.2%)
Fluoroquinolones	Ciprofloxacin	11 (100%)	0	0
Quinolones	Nalidixic Acid	11 (100%)	0	0
Folate Pathway Inhibitors	Sulfonamides	11 (100)	0	0
Macrolides	Erythromycin	2 (18.2%)	0	9 (81.8%)
Phenolics	Chloramphenicol	9 (81.8%)	2 (18.2%)	0
Carbapenem	Meropenem	11 (100%)	0	0

Table 2. Antimicrobial resistance profile of *V. cholerae* and *V. parahaemolyticus* isolated from the examined samples.

Resistance pattern	Frequency of occurrence	*Vibrio* spp. (source)	(No.)	MAR index
AMP + SAM + CAZ + GEN + TE + E	2	*V. cholerae*(raw milk)	1	0.5
		V. parahaemolyticus (water)	1	
AMP + SAM + CAZ + GEN + E	2	*V. cholerae*(water)	1	0.4
		V. parahaemolyticus (water)	1	
AMP + SAM + E	5	*V. cholerae* (kareish cheese and water)	2	0.25
		V. parahaemolyticus (cheese and water)	3	
AMP + SAM	2	*V. cholerae* (raw milk)	1	0.16
		V. parahaemolyticus (raw milk)	1	

Table 3. Multiple antibiotic resistance (MAR) index of *V. cholerae* and *V. parahaemolyticus* isolates.

Samples	Serogroup of *V. Cholerae* (No.)	*V. cholerae* virulence genes (%)			*V. parahaemolyticus* virulence genes (%)			
		ctxAB	*hlyA*	*tcpA*	No	*tdh*	*trh*	*tdh*/*trh*
Raw milk	O1 (2)	2(100)	1(50%)	2(100)	1	0	0	1(100)
Domiatti cheese	0	–	–	–	1	0	0	0
Kareish cheese	Non-O1/non O139 (1)	0	1(100)	0	1	0	1(100)	0
Sub-total	3	2(66.7)	2(66.7)	2(66.7)	3	0	1(33.3)	1(33.3)
Water	O1 (2)	2(100)	1(50)	2(100)	3	0	1(33.3)	1(33.3)
Total	5	4 (80)	3 (60)	4 (80)	6	0	2(33.3)	2(33.3)

Table 4. Distribution of the virulence-associated genes in *V. cholerae* and *V. parahaemolyticus* isolated from the examined samples.

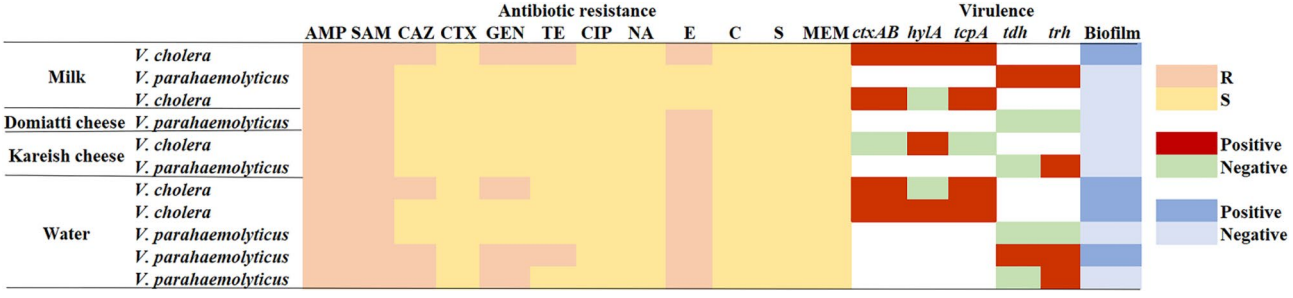


Figure 2. Heat-map summary of antimicrobial resistance, virulence, and biofilm formation by *V. cholerae* and *V. parahaemolyticus* strains isolated from the examined samples.

usage of contaminated raw milk and/or contaminated water may have promoted the occurrence of *V. cholerae* and *V. parahaemolyticus* in Kariesh cheese, a popular type of artisanal raw milk soft cheese in Egypt.

Vibrio spp. were absent from ice cream and yoghurt samples. Similar finding were reported by Kim et al[31]. Contrarily, Islam et al.[32] detected *Vibrio* spp. in street ice cream and Tahoun et al.[23] recovered *V. cholerae* and *V. parahaemolyticus* from yoghurt samples.

Vibrio cholerae was also isolated from 2% of the examined freshwater samples. Higher results obtained by Ismail et al.[33]. Although *V. parahaemolyticus*, is a halophilic bacterium and linked to marine water[34,35], it could be isolated from dairy and freshwater samples. The presence of *V. parahaemolyticus* in dairy samples is supported by the findings of Tahoun et al.[23] who also recommended that further investigation be performed to ensure its ability to survive in milk. It worth mentioning that the salinity of the Nile elevates from Aswan to Cairo because of lowering the flow of the Nile water[36], and the elevation continues in the direction of the Mediterranean Sea Nile outlets, where the maximum salinity values were registered[33] resulting in survival of *V. parahaemolyticus* in certain niches of fresh water and the scenario of subsequent contamination of food through the use of this water in different processing steps is potentially existed. Due to *V. parahaemolyticus* is found in water and the aquatic environment in general[37,38], it is possible for it to get to milk and its products through utensils washed with contaminated water. Also there are other possible means accelerated the bacterial contamination, including; contaminated hands, unsanitary manufacturing unit conditions, and poor materials quality[39].

Furthermore, recent discoveries confirming the presence of *V. cholerae* in river sediments demonstrate that the risk of infection associated with river exposure may increase under conditions of sediment resuspension[40]. Isolation of *V. cholerae* from river sediments improved the knowledge of the potential *V. cholerae* sources that accounted for the disease epidemics and have afflicted different developing and Sub-Saharan African countries[13].

Vibrio cholerae in raw milk was most likely caused by contamination with farm soil during milking and poor dairy farm management[40]. Workers' unclean hands, poor milk quality, unsanitary circumstances in the manufacturing unit, inferior quality of materials utilised, and water given for washing utensils could all be factors in encouraging bacterial contamination of milk products and post-manufacturing contamination[23,33,39,41–43].

To assess the actual risk to human health posed by the presence of *V. cholerae* and *V. parahaemolyticus* in the studied samples, identification of the microbe should be followed by PCR detection of the virulence genes responsible for cholera toxin production (CTX) and toxin co-regulated pilus (TCP) in *V. cholerae* and TDH and TRH toxins in *V. parahaemolyticus*. Pathogenic *V. cholerae* strains have two distinct important genetic elements, the cholera toxin element (CTX) and the Vibrio pathogenicity island (VPI), both of which are involved in the coding of the cholera toxin (CTX) and the toxin co-regulated pilus (TCP), respectively[44]**.** Only the O1 and O139 serogroups of *V. cholerae* are known to cause epidemic or pandemic cholera. Serogroup O1 has been linked to pandemics since 1899, and serogroup O139 has been linked to pandemics since 1992, with both serogroups being found in recent outbreaks of cholera[45,46]. In serotyping of pathogenic isolates (O1 and O139), combined genotypic and phenotypic analyses have largely replaced serotyping[47].

V. cholerae O1 isolates obtained from raw milk samples harbored *ctx*AB and *tcp*A genes, indicating that the genes encoding the virulence and surface organelles needed for intestinal attachment and colonisation were preserved. As a result, both strains maintained the core of the CTX genetic element as well as TCP, both of which are regarded as essential characteristics in pathogenicity. Similar results reported by Tahoun et al.[23] where all *V. cholerae* isolates from milk belonged to O1 serogroup and also, they were positive for the *ctxAB*, *hly*A, and *tcp*A genes. The virulent genes *ctxA* and *toxR* were not found in any of the isolates studied by Meena et al.[48], but the *hly*A gene was identified as the dominant biomarker gene among the isolates.

The non-O1/O139 *V. cholerae* strains rarely possess cholera toxin, which is encoded by the *ctx*AB gene, but they can cause less severe diarrhea-like symptoms[49] and the enterotoxic activity is most likely due to the produced hemolysin[50]**.** Out of the five *V. cholerae* isolates, only one strain was non-O1/O139 and carried only the virulence *hly*A gene. Contradictory findings were obtained by Ahmed et al.[43], Sarkar et al.[44], Bakhshi et al.[51]. Serogroup non O1/O139 have been associated to a large number of cases of diarrhoea in different regions of the world, including Sudan in 1968[44,52,53]. The lack of the *ctx* gene in this serogroup of *V. cholerae* does not preclude the risk posed by its existence[54,55]. Previous studies have shown that antigenic translation of *V. cholerae* non O1/O139 to *V. cholerae* O1 takes place under favourable conditions[56–58].

DH-related hemolysin (*trh*) and thermostable direct hemolysin (*tdh*) of *V. parahaemolyticus* have comparable hemolytic activity on living cells and induce erythrocyte lysis[59]. About 0.2–3% of environmental *V. parahaemolyticus* isolates are potentially pathogenic owing to the occurrence of *tdh* and/or *trh* genes, as per studies carried out in various areas[60]. Of the six examined *V. parahaemolyticus* isolates from dairy and water samples, 33.3% of each were positive for both *tdh* and/or *trh* genes. Similar results reported by Tahoun et al.[23] Also, Ahmed et al.[43] found that 33.3% of the obtained isolates harboured both *tdh* and *trh* genes and Abd-Elghany and Sallam[61] detected *tdh* and/or *trh* genes in 11.1% of the isolates. These findings are supported by the fact that not all of *V. parahaemolyticus* strains are pathogenic, and the pathogenic ones that are associated with the majority of illness and deaths are distinguished by producing TDH and/or TRH hemolysin encoded by *tdh* and *trh* genes, respectively[62].The majority of *V. parahaemolyticus* strains of environmental or from food origin are not pathogenic to humans[63]. Two out of six isolate harboured *trh* gene only. TDH negative strains were found to produce TRH and are also considered pathogenic to man[64,65].

The presence of this bacterium species in the aquatic environment raises human worries about food safety since, depending on environmental conditions, it has the ability to produce disease outbreaks[66]. The Centre for Disease and Control (CDC) recommends using antibiotics combined with fluid replacement to treat *Vibrio* infections[67]. Most antibiotics tested, including ampicillin, chloramphenicol, ciprofloxacin, gentamicin, erythromycin, quinolone and tetracycline are prescribed as first-line antibiotics for *Vibrio* infections treatmen[8,67–69]. The emergence of antibiotic resistance is a challenging problem that repetitively links human, environmental, and pathogen-related characteristics[70].

The ampicillin and ampicillin-sulbactam resistance by of all isolates noted in the current study is consistent with the CLSI standards[71], that notified *Vibrio spp.* as inherently resistant to ampicillin, which also is in line with previous studies[23,24,43,48]. The development of resistant bacteria to the penicillin antibiotic class in past years has restricted its effectiveness as one of the most effective antibiotics in primary care[72]. The isolates in this study were extremely resistant to erythromycin, ceftazidime, and Gentamicin. This was supported by Sharma and Malik[24], Islam[25]. and Ahmed et al.[43] who also recorded high resistance to Ciprofloxacin, Nalidixic acid and Cefotaxime in contrast to this study. Unlike this study, all *Vibrio* isolates obtained by Meena et al.[48] were sensitive to erythromycin but on the other hand similar results are recorded for Ciprofloxacin and Nalidixic acid drugs by them.

MAR indices between 0.16 to 0.5; maximum MAR index was contributed to the isolates displayed resistance to six antibiotics and four out of 11 *Vibrio* isolates were MDR with. This is consistent with Tahoun et al.[23] who found that most isolates showed MDR with MAR index ranging from 0.15 to 0.54, and Islam et al.[25] and Ahmed et al.[43] who found that all the isolates showed MDR with an MAR index ranging from 0.58 to 1. Meena et al.[48] recorded MAR index ranging from 0.11 to 0.22. Elevated levels of MDR may be a result of the increased opportunity for resistance genes located on plasmids to be exchanged among environmental isolates via horizontal gene transfer due to the widespread, unsupervised use of antimicrobials in the infection treatment[73,74].

A MAR higher than 0.2 indicates that high-risk sources, like farmers and farm animals that regularly take antibiotics, are the source of contamination, posing a threat to consumers. The current study found high MAR indices in water isolates, suggesting that these isolates came from high-risk sources; thus, antimicrobial resistance monitoring is critical for ascertaining the efficacy of new antibiotics and ensuring food safety[75]. Municipal and industrial waste water have been outlined as a potential source of resistant isolates in the aquatic ecosystem. A significant amount of the antibiotics that people take for medical reasons are expelled in their faeces and urine in an active biological form[76–78]and between 30 to 90% of the antibiotics that animals consume are also eliminated in faeces and urine[79]. Antibiotic-resistant bacteria and antibiotics were found to pollute the environment through animal excreta[80,81]. This phenomenon was newly confirmed in a study of 20 calf farms in the Netherlands. Antibiotics were found in 75% and 95% of the calf faeces and cattle farms, respectively, and the most common residual antibiotics recovered were oxytetracycline, doxycycline, and sulfadiazine[78]. It is a possible scenario for water contamination and subsequent contamination of milk and dairy products with antibiotic resistant pathogens.

The US National Institutes of Health reported that biofilm is accountable for more than 80% of bacterial diseases[82]. Extracellular polymeric substances produced by biofilm-producing bacteria, such as *Vibrio*, provide an appropriate medium for surface colonialization to produce biofilms. Once the biofilm is formed, the structure of the biofilm permits bacteria to remain alive and thrive in adverse environments such as high salinity and antibiotics[83]. Four isolates are biofilm producers, three recovered from water samples and one from raw milk samples and most of them are *V. cholerae*. *Vibrio* spp. ability to produce biofilm were recorded by various studies[6,84,85]. Three isolates are MDR this is supported by the finding that biofilm is related to appearance of multidrug resistance[86].

V. cholerae's ability to form biofilms is essential to the colonisation of the intestine; however, the biofilm structure generated throughout infection, in addition to their involvement in intestinal colonisation and pathogenicity, currently unclear[2]. It has been reported that *V. cholerae* biofilms are more resistant to acid inactivation[87]. Furthermore, biofilm-derived cells have competitive advantage over planktonic cells when it comes to limited nutrients in the small intestine[88]. Planktonic cells that disengaged from an infecting biofilm should move toward the intestinal mucosa, where they must break through the mucus barrier and proceed to the underlying epithelium. *Vibrio* cells that fail to form biofilm-like aggregates within the mucus gel due to lack of encoding genes (*vps*) or their expression[89]and/or penetrate the protective mucosa are leached inertly as a consequence of persistent mucosal degeneration[90].

Given that getting cholera includes the oral intake of virulent *Vibrio* cells that can express TCP and CT in the pattern of planktonic cells or biofilms and infection is contracted naturally in the latter form which represents a fast pathway for disease spreading during outbreaks[2], also *V. cholerae* cells in a biofilm demonstrate a smaller infective dose and totally dominate their planktonic cells' colonisation[91], and biofilms mostly require greater concentrations of antibiotics to be eliminated than planktonic bacteria[92], as a consequence, there is a potential that the obtained isolates represent a high potential threat.

In this study, a link was revealed between the patterns of drug resistance and virulence phenotype. Two out of four MDR isolates harboured virulence genes and exhibited phenotypic biofilm capacity. The earlier finding was corroborated by Katongole et al.[93] who discovered that biofilm-forming organisms harboured more virulence genes and were more MDR than non-biofilm generating pathogens. This is because biofilms are crucial because they act as hubs for horizontal gene transfer, which promotes the spread of virulence and antibiotic resistance genes[94]. These findings raise significant concerns regarding food safety and public health since raw milk may be a reservoir for bacteria that are resistant to antibiotics and virulent strains that may spread to humans through the food supply, and so this concern becomes more severe with regard to high temperatures in the study area, this is in line with reports of *V. cholerae* outbreaks occurring in a stressful environment of high temperatures during the spring and summer seasons[95,96]. Furthermore, the result presented here are is consistent with the findings of many authors who have shown that the introduction of *V. cholerae* O1 into nonendemic areas of less-than-ideal sanitation frequently causes the disease to spread more quickly. This is done by means of a fast faecal-oral pathway that takes advantage of the transitory hyper infective stage of *V. cholerae* found in fresh cholera stool[97–100].

Conclusions

The use of TCBS agar and PCR in the isolation and identification of *Vibrio* spp. is critical for biotype detection and differentiation. The isolation of *V. cholerae* and *V. parahaemolyticus* from dairy and water samples necessitates application of strict hygienic measures. Most *Vibrio* isolates were virulent, exhibited MDR with a high MAR index and four isolates were biofilm producer, representing public health hazard.

Materials and methods

Sample collection. The present study was designed to determine the prevalence of *Vibrio* spp. in 300 raw milk and dairy products were collected from farmers' houses, dairy farms, local dairy shops and vendors in Qena, Egypt. These samples included 100 raw cow and buffalo milk (50 samples each), and cheese, yoghurt and ice cream (50 samples each). Additionally, 100 water samples were collected from farmer's houses and dairy farms where the raw milk was collected. These samples were collected from May to October 2021.

Isolation and identification of *Vibrio* species. The Food and Drug Administration's Bacteriological Analytical Manual (FDA 2004)[101] was followed for the isolation of *Vibrio* species. Briefly, one ml/g of each milk and water samples was blended with 9 ml of sterile alkaline peptone water (APW) (Oxoid, CM1028, UK) while 10 g for each Yoghurt, cheese, ice-cream were blended in 90 ml APW and incubated at 35 °C ± 2 °C for 24–48 h (ISO-TS-21872–1, 2007)[102]. A loopful of the enriched broth, was streaked onto Cholera Medium TCBS agar (Oxoid, CM0333, UK), then plates were incubated at 37 °C for 24 h. Presumptive *Vibrio* colonies (yellow, blue green) were streaked on tryptone soya agar (Oxoid, CM0131, UK) containing 2% NaCl for purification. Gram staining and biochemical tests including; growth in 0,1% NaCl, oxidase, catalase, motility, glucose oxidation/fermentation, gelatinase production, nitrate reduction, arginine dehydrolase utilization, lysine and ornithine decarboxylase utilization, mannose, arabinose, sucrose and lactose sucrose mannose were used for identification[103].

Molecular characterization of *Vibrio* spp. strains. Uniplex and multiplex PCR reactions used in molecular identification of *Vibrio* spp. The primers and target genes used in this study, as well as the PCR cycling conditions and amplicon sizes, are described in their respective references and are listed in Supplementary Table 1. PCR amplification was carried out in an Applied Biosystem 2720 thermal cycler. To validate the presence of amplified DNA, PCR products were examined using 1.5% (w/v) agarose gel electrophoreses in 0.5 TBE buffer at a constant voltage of 90 V. A gel documentation system (Alpha Innotech, Biometra, Göttingen, Germany) used to photograph the gel.

Preparation of genomic DNA. Genomic DNA was extracted with the DNA extraction QIAamp DNA Mini kit (Qiagen GmbH, Hilden, Germany). DNA was extracted from 1 ml of overnight-grown cultures in tryptic soy broth medium (TSB) (Oxoid, CM0129, UK) and reconstituted in 100 µl of DNA hydration buffer.

Molecular identification of the obtained isolates. Confirmation of the biochemically identified *Vibrio* colonies was done by PCR using housekeeping *16S rDNA* gene primers set for *Vibrio* species[104]. Specific primers targeting the *toxR* and *ctx* genes were used to confirm *V. parahaemolyticus* and *V. cholerae*[105,106]. Serotyping of *V. cholerae* isolates was molecularly performed using O1-rfb and O139-rfb genes[107].

Sequencing of the *ctx* and *toxR* genes was done to confirm the molecular identification of *V. cholerae* and *V. parahaemolyticus* isolates. PCR products were purified using a QIAquick PCR Purification Kit as directed by the manufacturer (Qiagen, Valencia, CA). The sequence reaction was conducted with a Bigdye Terminator V3.1 cycle sequencing kit (Thermo Fisher Scientific in the United States), and it was purified with a Centrisep spin column. The genetic analyzer Applied Biosystems 3130 (HITACHI, Tokyo, Japan) was used to obtain DNA sequences. All the obtained sequences of *ctx and tox*R genes were analysed using Lasergene (version 7.2; DNASTAR, Madison, WI) and submitted to the GenBank (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome). The strains of *V. cholerae* MN829555.1, CP042299.1, CP047059.1, CP046837.1, CP047295.1, CP046840.1, CP046840.1, CP047299.1, CP047301.1 and CP046745.1 and *V. parahaemolyticus* CP028342.1, CP028141.1, CP078627.1, DQ845167.1, DQ845167.1, CP041202.1, MF983554.1, AB372520.1, CP023485.1, CP031781.1, MF983555.1, CP064035.1, CP078735.1, CP068627.1 and AB300874.1 from the NCBI database were included in the phylogenetic analysis. The sequence alignment and phylogenetic tree were carried out using multiple alignment algorithms in MegAlign (version 10.2.4; DNASTAR, Wisconsin, USA).

Molecular detection of virulence determinants. *V. cholerae* isolates were characterized for their virulence using specific primers targeting the *ctx*AB[49] the *hly*A[108] and the *tcp*A[108]virulence determinants. While *V. parahaemolyticus* isolates were tested for *tdh* and *trh* virulence genes[109].

Antimicrobial susceptibility test. Antimicrobial resistance of *V. cholerae* and *V. parahaemolyticus* isolates (n = 11) was determined using disk diffusion method on Mueller Hinton Agar (Oxoid, UK) according to the guidelines by the Clinical and Laboratory Standards Institute (CLSI,2015)[110]. Twelve antibiotic disks (Oxoid, UK) used in this study including ampicillin (AMP) (20 µg), Ampicillin-sulbactam (SAM) (10 µg), Ceftazidime (CAZ) (30 µg), Cefotaxime (CTX) (30 µg), Tetracycline (TE) (30 µg), Ciprofloxacin (CIP) (5 µg), Gentamicin (GEN) (10 µg), Nalidixic Acid (NA) (30 µg), Erythromycin (E) (15 µg), Sulfonamides (S) (300 µg), Chloramphenicol (C) (30 µg), and Meropenem (MEM) (10 µg). The zones of inhibition were measured and compared

to the world standards CLSI, isolates were recorded as Resistant (R), and Sensitive (S) accordingly. For nalidixic acid (NA), the *Enterobacteriaceae* interpretation criteria was used.

Determination of MAR index. The method described by Osundiya et al.[111] was used to generate the MAR index[107], in which the number of antibiotics to which an isolate is resistant (a) is divided by the total number of antibiotics used in the study (b). The formula for calculating is illustrated below:

$$\text{MAR Index} = a / b.$$

Biofilm formation. The biofilm production of bacterial strains was investigated using a microtiter plate assay as per[112]. Briefly, bacteria were cultured for 24 h in Tryptone Soy Broth (TSB) (Oxoid, CM0129B, UK) supplemented with 3% NaCl at 37 °C. In fresh TSB (3% NaCl), 200 µL of a 1:100 dilution of overnight cultures was adjusted to 0.5 McFarland turbidity. The diluted solutions were then distributed into the wells, and the plats were incubated at 37 °C for 24 h. Three well of uninoculated TSB with 3% NaCl were set as the control. The wells' contents were discarded, and the wells were rinsed twice with phosphate buffer saline (PBS). Each well received 200 µL of crystal violet dye (1%) before being incubated at room temperature (25 °C) for 1 h. The staining dye was removed, and the wells were rinsed three times with PBS before being allowed to air-dry at 25 °C. Then 200 µL of acetic acid (33%) for 30 min were added to each well to resolubilize the adherent cells and the optical density at 570 nm was measured. Three separate tests were carried out. All isolates' biofilm-producing ability was classified according Stepanović et al.[113] as follows: No biofilm formation if OD test < OD control; weak biofilm formation if OD control < OD test < 2OD control; moderate biofilm formation if 2ODcontrol < OD test < 4OD control; and strong biofilm formation if OD test > 4OD.

Statistical analysis. The mean and standard deviation of the data were displayed. The Graph Prism 8 one-way ANOVA test was employed. The criterion for significance was $P < 0.05$.

Ethical approval. The study was approved by the Animal Ethics Committee for Veterinary Research (75/02. 10.2022), Faculty of Veterinary Medicine, South Valley University, Qena, Egypt.

Data availability

Dr. Mon A. El-Zamkan and Hams M. A. Mohamed have data available upon request.

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Author contributions

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Competing interests

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