Food Research 8 (1) : 190 - 209 (February 2024)
Journal homepage: https://www.myfoodresearch.com

FOOD RESEARCH

REVIEW

Insights into foodborne *Vibrio parahaemolyticus* – a review

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Article history:
Received: 22 May 2022
Received in revised form: 29 June 2022
Accepted: 16 September 2023
Available Online: 30 January 2024

Keywords:
Vibrio parahaemolyticus,
Virulence factors,
Antimicrobial resistance

DOI:
https://doi.org/10.26656/fr.2017.8(1).267

Abstract

Vibrio parahaemolyticus is a bacterium which normally inhabits the aquatic environment. In the Asian region, as well as in the United States, it is one of the main causative agents of foodborne illnesses in humans after consumption of raw or partially processed marine hydrobionts. The pathogenetic mechanism of *V. parahaemolyticus* involves a number of factors such as hemolysin, two types of secretory systems, and adhesion agents. Foodborne outbreaks caused by *V. parahaemolyticus* usually occur in summer when the water temperature is above 15°C. In view of the rising global temperatures, *V. parahaemolyticus* is becoming more widespread and risks becoming a serious threat to countries that lack control systems for this pathogen. At the same time, attention should be paid to the reports about the presence of many field strains of *V. parahaemolyticus* resistant to a number of antibiotics such as ampicillin, streptomycin, and cefazolin. This article aimed to support the understanding of *V. parahaemolyticus* as a threat to public health by summarizing the main methods of isolation and identification, incidence, antimicrobial resistance and factors that affect the survival rate of the pathogen in seafood.

1. Introduction

Vibrio parahaemolyticus is one of the most common causes of foodborne illness in humans (Letchumanan *et al.,* 2014). It occurs as a result of consumption of raw or undercooked contaminated seafood and most commonly is clinically manifested by symptoms of acute gastroenteritis, nausea, vomiting, stomachaches, and slight fever (McLaughlin *et al.,* 2005). The disease is self -limited, moderately severe, lasting an average of about 3 days in immunocompetent patients (Yeung and Boor, 2004). In rare cases, *V. parahaemolyticus* can cause wound and ear infections or septicemia in people with comorbidities or immunocompromised patients (Zhang and Orth, 2013). *Vibrio parahaemolyticus* is the cause of foodborne outbreaks in Japan (Su and Liu, 2007; Hara-Kudo *et al.*, 2012), Taiwan (Yu *et al.*, 2013), China (Li *et al.*, 2014), Bangladesh (Bhuiyan *et al.*, 2002), Laos (Matsumoto *et al.*, 2000), Hong Kong and Indonesia (Matsumoto *et al.*, 2000). In the United States, *V. parahaemolyticus* is the leading pathogen causing acute gastroenteritis associated with the consumption of marine hydrobionts (Newton *et al.*, 2012). Data published by the Centers for Disease Control and Prevention (CDC) in the Foodborne Diseases Active Surveillance Network (FoodNet) and Morbidity and Mortality Weekly Report (MMWR) show that in the United States in 2016, *V. parahaemolyticus* was detected in approximately 34664 cases of foodborne infection and is reported as a major pathogen compared to other *Vibrio* spp. (Huang *et al.*, 2016). Most infections with *Vibrio* spp. with the exception of cases associated with toxigenic *V. cholerae* (O1/O139), are not subject to notification in Europe and as such are likely to be rarely reported (Baker-Austin *et al.*, 2010). However, there is a growing concern that due to global warming, pathogenic vibrios could become an unexpected public health risk in non-endemic areas such as Europe (Baker-Austin *et al.*, 2017). This article aimed to help understand *V. parahaemolyticus* as a threat to public health by summarizing the main methods of isolation and identification, incidence, antimicrobial resistance and factors that affect the survival rate of the pathogen in seafood.

2. Characteristics of *Vibrio parahaemolyticus*

Vibrio parahaemolyticus belongs to the family Vibrionaceae, genus *Vibrio*, comprising 30 types of Gram-negative, straight and slightly curved non-spore-forming rods with a width of 0.5-0.8 μm and a length of 1.4-2.6 μm. Thirteen of them are pathogenic to humans

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eISSN: 2550-2166

(Drake *et al.*, 2007). *Vibrio parahaemolyticus* is a bacterium that is normally present in marine, estuarine and coastal areas around the world. It is to be found in warm, slightly salty seawater, usually colonizing various marine hosts such as mollusks, shrimp, fish or sediment (DePaola *et al.*, 1990). *Vibrio parahaemolyticus* is found mainly in coastal waters and not so much in the open sea. It cannot be isolated when the water temperature is below 15°C and cannot survive the pressure in deeper waters. During the winter months, when water temperatures fall below 15°C, its resistance in the sediment ensures its survival, from where it can be restored, even when the water temperature is below 10°C (Adams and Moss, 2007). For survival and reproduction, *V. parahaemolyticus* requires the presence of salt in optimum concentrations of 1 to 3% (Yeung and Boor, 2004). *Vibrio parahaemolyticus* is a multiserotypic bacterium. All strains share a common H antigen, but to date, 12 O (somatic) types and over 70 K (capsule) antigens have been described, although many other strains are untyped (Miwatani and Takeda, 1976). This is the reason why serotyping is used mainly to detect *V. parahaemolyticus* and to study its pathogenesis (Xu *et al.*, 2014). Three serotypes (O3:K6, O4:K68 and O1:K) are highly virulent and pathogenic to humans and are considered to be the main causes of foodborne infections (Jones *et al.*, 2012). *Vibrio parahaemolyticus* is a halophilic bacterium (1-7% NaCl), grows in an alkaline environment (pH 8.6-9.4) and is resistant to various agents such as detergents, some antibiotics (colistin and polymyxin B) and chemotherapeutics, dyes such as bile salts and metachrome yellow II RED (Donovan and Netten, 1995).

3. Methods of isolating and identifying *Vibrio parahaemolyticus*

3.1 Enrichment broths

Proving *V. parahaemolyticus* to be the causative agent of gastroenteritis after consumption of contaminated seafood is extremely important and for this reason, many different types of enrichment broths and solid selective media have been developed and improved over the years. The development of each of them uses the natural characteristics of the bacterium, determined by the normal living environment.

Alkaline peptone water (APW) is one of the most commonly used enrichment broths in methods for isolating *V. parahaemolyticus*. The high concentration of sodium chloride combined with pH in the range of 8.5-9.0 provides optimal conditions for bacterial growth and at the same time inhibits the growth of other bacteria (DePaola *et al.*, 2004).

Raghunath *et al.* (2009) compared the efficacy of alkaline peptone water and enrichment broth containing bile salts and sodium taurocholate (ST broth). Their study showed a higher percentage of isolated and identified pathogenic *V. parahaemolyticus* upon enrichment with ST broth. This is probably related to the hypothesis of Pace *et al.* (1997) about the increase of the virulence of *V. parahaemolyticus* strains in the presence of bile salts. This contributes to the high percentage of pathogenic *V. parahaemolyticus* in clinical isolates despite their low prevalence in the environment. Bisha *et al.* (2012) used Salt polymyxin B broth (SPB), glucose salt teepol broth, salt colistin broth, and alternative protein source (APS) as enrichment media.

Hara-Kudo *et al.* (2001) developed a double-enrichment method using the non-selective Salt Trypticase soy broth first, followed by a second enrichment in SPB selective medium. SPB contains polymyxin B sulfate, which inhibits the growth of Gram-positive microorganisms. This two-step enrichment technique proved to be much more effective in isolating *V. parahaemolyticus* compared to a single enrichment in SPB broth alone.

3.2 Solid selective media

Various solid selective media have been developed to isolate and identify *V. parahaemolyticus*. Thiosulfate-citrate-bile salts-sucrose agar (TCBS) is most often used as a selective medium, which is a highly differentiating medium used not only for *V. cholerae* but also for all other pathogenic *Vibrio* spp. with the exception of *V. hollisae* (Kobayashi *et al.*, 1963). TCBS is a selective medium containing bile (0.8%) and NaCl (1%), alkaline pH 8.6, which inhibits the growth of other Gram-positive microorganisms. The main advantage of TCBS agar is the presence of a sucrose/bromothymol blue system that differentiates sucrose-positive vibrios such as *V. cholerae* from colonies of other *Vibrio* species. *V. cholerae* produces yellow 2-3 mm colonies on TCBS agar (Letchumanan *et al.*, 2014). *V. parahaemolyticus* does not ferment sucrose and therefore produces green colonies on TCBS agar (Sangadkit *et al.*, 2020). Despite its widespread use, TCBS agar also has its drawbacks, such as difficulties in isolating *V. parahaemolyticus* from seafood. This is due to the huge amount of yellow colonies produced by sucrose-fermenting bacteria on agar, which makes it difficult to effectively isolate, differentiate, and enumerate *V. parahaemolyticus* (Bisha *et al*., 2012). To this must be added that in addition to *V. parahaemolyticus*, green colonies on TCBS agar are also formed by *V. hollisae*, *V. mimicus* and *V. vulnificus* (Hara-Kudo *et al.*, 2001). To compensate for this problem, Hara-Kudo *et al.* (2001) developed an

https://doi.org/10.26656/fr.2017.8(1).267

enrichment methodology and a new selective medium for the detection of *V. parahaemolyticus* in hydrobionts. Samples are enriched in selective SPB broth and plated on CHROMagar Vibrio (CV) chromogenic agar. CHROMagar contains colorimetric substrates for β-galactosidase and has been developed specifically to differentiate ortho-nitrophenyl-β-galactoside-positive *V. parahaemolyticus* from other closely related vibrio species (Bisha *et al.*, 2012). In this chromogenic medium, the purple-colored colonies of *V. parahaemolyticus* are easily distinguished and differentiated from other *Vibrio* species. Many researchers have compared the two nutrient media (CV and TCBS) and have reported a higher percentage of isolated *V. parahaemolyticus* in CV compared to TCBS, indicating that CV is more specific and accurate than TCBS in detecting *V. parahaemolyticus* (Su and Liu, 2007; Letchumanan *et al.*, 2014).

Lee *et al.* (2020) developed a new ChromoVP agar that can be used to specifically distinguish *V. parahaemolyticus* from other closely related vibrio species. They then compared it to the two commercially available CHROMagar™ Vibrio and TCBS agar. ChromoVP agar was developed as a selective medium allowing direct identification of *V. parahaemolyticus* based on specific color development of the colonies using a substrate system based on β-glucosidase. *V. parahaemolyticus* forms smooth, flat, purple colonies with a diameter of 2-4 mm. In contrast, *V. alginolyticus*, *V. vulnificus*, *V. cholerae* and *V. mimicus* form colorless or milky white colonies 0.5-2 mm in diameter. When the ChromoVP agar plates are left at room temperature for additional 24 hours, the purple color of *V. parahaemolyticus* colonies intensifies and the colony size increases, allowing even better recognition of these bacteria. Better specificity and accuracy have been found in the new selective medium, which has been identified as an alternative method for isolating and enumerating *V. parahaemolyticus* from seafood.

Wagatsuma agar has been developed to detect pathogenic *V. parahaemolyticus*. It contains human or rabbit blood with NaCl, mannitol, crystal violet and K_2HPO_4. It differentiates *tdh*-positive strains that cause β -hemolysis on this agar (Nishibuchi and Kaper, 1995). The main drawback is that it is impossible to distinguish *trh*-strains of *V. parahaemolyticus* from non-pathogenic strains, as they do not cause hemolysis on the Wagatsuma agar (Letchumanan *et al.*, 2014).

3.3 Biochemical tests

The biochemical characteristics of pathogenic *V. parahaemolyticus* isolated from seafood show that the bacterium is positive for oxidase, ornithine and lysine decarboxylase, negative for arginine dihydrolase, grows at 42°C and 3, 6 and 8% NaCl, and forms acid from arabinose, mannose and mannitol (Elliot *et al.*, 1995).

3.3.1 Urease test

All *V. parahaemolyticus* isolates have to be tested for the presence of urease. Urease broth enriched with 2% NaCl, Christensen urea agar enriched with 2% NaCl or via API 20E can be used. Clinical strains from the West Coast of the United States, as well as those from Asian countries, are predominantly positive for urease. Urease production is associated with the presence of the *tdh* and/or *trh* genes (Suthienkul *et al.*, 1995; Osawa *et al.*, 1996). The urease reaction is a valuable screening test for potentially pathogenic strains (Kaysner *et al.*, 1994).

3.3.2 Salt tolerance test

Most *Vibrio* species have physiological requirements for NaCl concentration, and salt is an important ingredient in selective enrichment broths and solid nutrient media for *Vibrio* spp. It is typical for *V. parahaemolyticus* that it cannot grow in salt-free media, but develops optimally in 3, 6 and 8% saline broths or agar media (Food and Drug Administration (FDA), 2021).

Sangadkit *et al.* (2020) propose a new integrated platform including colorimetric detection with an enrichment step to provide early information on the presence or absence of *V. parahaemolyticus* in seafood. Identification is performed within 24 hours with a detection limit of 0.1-1.0 log CFU/mL by carbohydrate fermentation and amino acid decarboxylation. NaCl was added and the pH was adjusted to suppress competing microorganisms that could interfere with screening. This method gives 100% sensitivity and improves specificity to 95-100% compared to 88% specificity of the conventional method.

3.4 Molecular methods

Polymerase chain reaction (PCR) is the most common method for identification of pathogenic bacteria. Multiplex PCR protocols targeting *toxR*, *tlh*, *tdh*, *trh* and *fla* genes have been developed to detect pathogenic and non-pathogenic strains of *V. parahaemolyticus* from clinical and environmental samples (Rosec *et al.*, 2009; Hossain *et al.*, 2013; Paydar, 2013).

Tarr *et al.* (2007) used multiplex PCR to amplify the *rpoB* gene of *V. cholerae*, *V. parahaemolyticus*, *V. vulnificus* and *V. mimicus* followed by DNA sequencing. Multiplex PCR analysis using collagenase-targeted

https://doi.org/10.26656/fr.2017.8(1).267

primer pairs to detect *V. alginolyticus*, *V. cholerae* and *V. parahaemolyticus* has been developed by Di Pinto *et al.* (2005). In the multiplex PCR technique developed by Neogi *et al.* (2010) species-specific PCR primers have been designed based on the *toxR* gene for *V. cholerae* and *V. parahaemolyticus* and the *vvhA* gene for *V. vulnificus*. The *toxR* gene is present in all pathogenic or non-pathogenic *V. parahaemolyticus* (Dileep *et al.*, 2003; Sujeewa *et al.*, 2009) and is used in its identification (Kim *et al.*, 1999). The presence of *toxR* in a *V. parahaemolyticus* isolate does not prove whether it is pathogenic (Paydar, 2013).

Chen *et al.* (2012) used O-specific serogroup PCR analysis to identify pathogenic *V. parahaemolyticus* in clinical and environmental samples. A quantitative PCR method combined with propidium monoazide was used to quantify viable *V. parahaemolyticus* cells in seafood (Zhu *et al.*, 2012).

Loop-mediated isothermal amplification (LAMP) is a specific and highly sensitive technique for DNA amplification under isothermal conditions with specific primers and is widely used to detect pathogenic bacteria in food (Qi *et al.*, 2012). Targets in LAMP are *tlh*, *tdh* or *toxR* genes for rapid and highly sensitive detection of *V. parahaemolyticus* (Yamazaki *et al.*, 2008; Chen and Ge, 2010). LAMP *in situ* is a new method for rapid detection of *V. parahaemolyticus* strains in food, which has a higher specificity and is performed in a shorter time compared to conventional LAMP techniques and other methods based on PCR (Wang *et al.*, 2013).

4. Pathogenic factors of *Vibrio parahaemolyticus*

A number of factors such as hemolysins, adhesins, and enzymes determine the pathogenicity of *V. parahaemolyticus*. The pathogenicity of *V. parahaemolyticus* is associated with the Kanagawa (KP) phenomenon being β-hemolysis on Wagatsuma agar. Almost all clinical isolates of *V. parahaemolyticus* are KP-positive, while only 1-2% of environmental strains are KP-positive (Nishibuchi and Kaper, 1995). It is now known that the Kanagawa phenomenon is the result of the action of thermostable direct hemolysin (*tdh*). It is a hemolytic protein that is not inactivated at 100°C for 10 mins, hence its name (Nishibuchi and Kaper, 1995). Its hemolytic activity is not increased by the addition of lecithin, suggesting direct activity on erythrocytes (Nishibuchi and Kaper, 1995). Kaper *et al.* (1984) were the first to clone the gene encoding the *tdh* protein (designated *tdh* 1) from *V. parahaemolyticus* strain WP1, which is of clinical origin. Later, Hida and Yamamoto (1990) found that the *V. parahaemolyticus* strain WP1 actually contained a second different *tdh* gene, which was designated *tdh* 2.

Regardless of the importance of the Kanagawa factor and the *tdh* protein, KP-negative strains of *V. parahaemolyticus* are sometimes associated with gastroenteritis. Honda *et al.* (1987, 1988) reported that some KP-negative strains of *V. parahaemolyticus* causing diseases in humans produce a *tdh*-like hemolysin designated *trh*. The *trh* protein was first discovered in O3:K6 strains. This new hemolysin, found mainly in environmental isolates of *V. parahaemolyticus*, causes high lethality in mice (Sarkar *et al.*, 1987). Some clinical isolates contain both *tdh* and *trh* genes, while most environmental isolates do not have either (Xu *et al.*, 1994).

Thermostable direct hemolysin exhibits various biological activities such as hemolytic activity, cytotoxicity, cardiotoxicity and enterotoxicity. *tdh* is a toxin that forms pores with a diameter of ~ 22 nm in the erythrocyte membrane (Matsuda *et al.*, 2010). The large pore size allows water and ions to cross the cell membrane (Honda *et al.*, 1992). The diarrhoea observed during infection with *V. parahaemolyticus* is a result of these changes in the water-ion flow in the intestines.

Trh is a thermolabile toxin and immunologically similar to *tdh* (Honda *et al.,* 1988) with both genes being 70% homologous (Kishishita *et al.*, 1992). Like *tdh*, *trh* also activates chlorine channels in the cell membrane, which alters ion flux (Takahashi *et al.*, 2000). Although *tdh* and *trh* are present in pathogenic strains, they do not fully determine the pathogenicity of *V. parahaemolyticus* (Lynch *et al.,* 2005). There are studies that reported that some of the clinical strains do not contain *tdh* and/or *trh* genes (Jones *et al.,* 2012; Pazhani *et al.*, 2014). Even in the absence of these hemolysins, *V. parahaemolyticus* remains pathogenic, proving that other pathogenic factors exist (Jones *et al.*, 2012). The study by Mahoney *et al.* (2010) reported that isolates of *V. parahaemolyticus* from the environment with missing *tdh* and/or *trh*, have other pathogenic factors such as extracellular proteases, biofilm formation, and siderophores. These results clearly show that the cytotoxicity and enterotoxicity of pathogenic *V. parahaemolyticus* cannot be explained only by the presence of *tdh* and *trh*, and other factors are also responsible for the pathogenicity.

4.1 Type III secretion system T3SS

The type III secretion system T3SS of *V. parahaemolyticus* is thought to be one of the main factors determining the pathogenicity of the bacterium (Broberg *et al.*, 2011). In many studies, T3SS1 has been associated with cytotoxicity, lethality in mice, and possibly induction of autophagy (Park *et al*., 2004). It is likely that the T3SS2 system determines enterotoxicity

https://doi.org/10.26656/fr.2017.8(1).267

and ensures the survival of strains in the environment (Park *et al.*, 2004; Matz *et al.*, 2011). All isolates of *V. parahaemolyticus* possess T3SS1, while T3SS2 is usually associated with *V. parahaemolyticus* carrying a *tdh* and/or *trh* gene (Park *et al.*, 2004). Two separate T3SS2 lines have been described showing a correlation of *tdh* with T3SS2α and *trh* with T3SS2β (Park *et al.*, 2004). Recently, however, T3SS2β has also been found in *tdh*- and *trh*-negative strains of *V. parahaemolyticus* from the environment (Paranjpye *et al.*, 2012). Jones *et al.* (2012) studied 77 clinical isolates of *V. parahaemolyticus* from wound infections or foodborne infections. It was found that 21 of 77 (27%) clinical strains of *V. parahaemolyticus* were negative for *tdh*, *trh* and T3SS2. The results of these studies raise some doubts about the idea that genes encoding *tdh* and *trh* proteins and T3SS2 determine the pathogenicity of *V. parahaemolyticus* (Raghunath, 2015).

4.2 Type VI secretion system T6SS1 and T6SS2

Comparison between pandemic and non-pandemic strains of *V. parahaemolyticus* led to the identification of type VI secretory systems - T6SS1 (VP1386-VP1420) and T6SS2 (VPA1030-VPA1043) located on chromosomes 1 and 2 of *V. parahaemolyticus* RIMD 2210633, respectively (Boyd *et al.*, 2008; Izutsu *et al.*, 2008). T6SS2 is unlikely to be involved in cytotoxicity, but promotes adhesion to host cells (Yu *et al.*, 2012). Because the T6SS2 and T3SS2 systems coexist, they are thought to interact in the process of infecting the host. T6SS2 initiates the first step of infection by adhering to host cells, while T3SS2 exports effector molecules by inducing enterocytotoxicity (Yu *et al.*, 2012). The role of T6SS1 has not yet been conclusively proven, but Salomon *et al.* (2013) reported that T6SS1 is most active in warm marine conditions, while T6SS2 is in low salt content. T6SS is used as a marker of virulence in the differentiation of *V. parahaemolyticus* strains. Chao *et al.* (2010) reported that most pandemic strains isolated in China contain the full set of T6SS genes, while most non-pathogenic strains have only a partial set of T6SS genes.

Various enzymes also form the pathogenicity of *V. parahaemolyticus*. Studies of several factors such as enzymatic (lipase, gelatinase and hemolysin), biological (adhesion, cytotoxicity and enterotoxicity) and enteropathogenic strains of *V. parahaemolyticus* isolated from seawater have shown that virtually all tested strains have lipase and gelatinase activity, while only 10% have hemolytic activity. 80% and 90% of the tested *V. parahaemolyticus* isolates have adhesive and cytotoxic abilities, respectively (Baffone *et al.*, 2001).

A detailed description of the pathogenic factors of *V. parahaemolyticus* and their function are presented in Table 1.

5. Incidence of pathogenic *Vibrio parahaemolyticus* in seafood

The incidence of *V. parahaemolyticus* in marine environments depends on water temperature. Studies show that the bacterium is rarely found in seawater until temperature rises to 15°C and above. A study of *V. parahaemolyticus* in the Chesapeake Bay, Maryland, found that it survived in sediments in winter and was released into the water when its temperature rose to 14°C in late spring or early summer (Kaneko and Colwell, 1973). *V. parahaemolyticus* can live freely in water, attached to the surface of marine hydrobionts or colonize the gastrointestinal tract of fish. Crustaceans and molluscs have often been found to be associated with the incidence of *V. parahaemolyticus* (Mala *et al.*, 2016; Yu *et al.*, 2016). Thus, shellfish and other aquatic organisms are a means of transmitting this microorganism. Although *V. parahaemolyticus* is a well-known halophile, some studies have shown that it can also be found in freshwater organisms (Nair *et al.*, 2007; Otomo *et al.*, 2013). As marine hydrobionts account for a large proportion of a healthy diet and their consumption is associated with various health benefits (Iwamoto *et al.*, 2010), there is also a potential risk associated with the consumption of contaminated products.

In Malaysia, Tan *et al.* (2020) tested 140 samples of blood clams (*Anadara granosa*), shrimps (*Penaeus* spp.), Surf clams (*Paphia undulata*), and squids (*Loligo* spp.) for the presence of *V. parahaemolyticus*. The pathogen was isolated from 85.71% (120/140) of the samples. The highest number of positive samples showed blood clam (91.43%; 32/35), followed by shrimps (88.57%; 31/35), surf clams (82.86%; 29/35) and squids (80%); 28/35).

Abd-Elghany and Sallam (2013) investigated the presence of potentially pathogenic *V. parahaemolyticus* in 120 shellfish samples (shrimp, crab, and cockle) purchased from fish stores in Mansoura, Egypt. A total of 27 isolates were confirmed as *V. parahaemolyticus*, and *tdh* and/or *trh* genes were detected in 3 (11%). One shrimp isolate is positive for *tdh* and *trh* genes, while another one from cockle is positive for the *tdh* gene only. The third shrimp isolate is a carrier of the *trh* gene only. Both isolates carrying the *tdh* gene also gave a positive reaction to Kanagawa.

Another large-scale study in China found that 41.1% (143/348) samples of freshwater products (chub, mud eel, freshwater shrimp, freshwater fish from Lake Gaoyou) and 56.6% (128/226) samples of seafood (scallops, short-necked clam, and marine fish) were positive for *V. parahaemolyticus*. Nineteen isolates of *V.*

https://doi.org/10.26656/fr.2017.8(1).267

Table 1. Pathogenic factors of *V. parahaemolyticus.*

Pathogenic factors of *V. parahaemolyticus*	Functions	References
Single flagellum	Polarly located single flagellum with a shell that lengthens the bacterial membrane. Provides the ability for the bacterium to move freely in the aquatic environment. It is composed of six different proteins, which facilitate attachment. The motility of the single flagellum is provided by the driving force of sodium, which is an advantage in salty waters with pH 8.0.	McCarter, (1999); Broberg *et al*. (2011)
Lateral flagella	The lateral flagella are uncoated and allow the bacterium to slide on hard and semi-hard media and surfaces. It consists of a protein called flagellin.	McCarter, (1999); Broberg *et al*. (2011)
MAM7	Multivalent adhesive protein is required for the initial binding of *V. parahaemolyticus* to the host cell.	Broberg *et al*. (2011)
Siderophores	Facilitating the purification of iron from the environment - vibrioferrin, ferrichrome, aerobactin.	Broberg *et al*. (2011)
T3SSs	Comprising needle-like bacterial mechanisms that inject protein effectors directly into the membrane and cytoplasm of eukaryotic cells without entering the extracellular environment.	Cornelis (2006)
T3SS1	Present in all clinical and field strains of *V. parahaemolyticus*. During tissue cell infection, T3SS1 initiates a series of changes leading to cell lysis.	Paranjpye *et al*. (2012); Sreelatha *et al*. (2013)
T3SS2	Pathogenicity factor associated with pandemic *V. parahaemolyticus* strains. T3SS2 effectors translocate into the host cell and exhibit cytotoxicity and enterotoxicity.	Paranjpye *et al*. (2012)
TDH	Hemolysin is present in almost all clinical and less than 5% field strains. One of the main indicators of pathogenicity. Causes β-haemolysis on Wagatsuma blood agar (KP-positive) and gastroenteritis. Exhibits many different biological activities - haemolysis, enterotoxicity, cytotoxicity and cardiotoxicity.	Hiyoshi *et al*. (2010)
TRH	Immunologically and biologically similar to TDH. Thermolabile, decomposes at 60°C for 10 min. Its action is expressed by an increase in Cl-secretion with a subsequent increase of intracellular calcium.	Takahashi *et al*. (2000)
Urea hydrolysis	Additional markers of virulence in some pathogenic strains. Correlation has been demonstrated between the presence of the *trh* gene and urease production.	Suthienkul *et al*. (1995); Osawa *et al*. (1996);

parahaemolyticus were detected in 35.9% of a total of 53 food samples from food poisoning outbreaks. 8.5% of freshwater and marine product isolates are positive for the *tdh* gene, while 1.5% are positive for the *trh* gene (Chao *et al.*, 2009).

Lei *et al.* (2020) investigated the incidence of *V. parahaemolyticus* in various seafood and ready-to-eat foods including 324 shrimp (*Metapenaeus ensis*), 200 fish (*Megalobrama amblycephala*, *Parabramis pekinensis*, *Carassius auratus*, and *Larimichthys crocea*), 260 RTE food samples 180 deli meat samples, 57 cold vegetable dishes or noodles in sauce, and 23 fried rice or noodle). The pathogen was isolated from 163 (20.8%) of a total of 784 samples. Shrimp (32.6%; 106/324) and fish (22%; 44/200) showed a higher number of contaminated samples compared to ready-to-eat food (4.96%; 13/260).

Xu *et al.* (2014) studied 111 fresh shrimp samples, 73 frozen shrimp samples, and 89 dried shrimp samples. Of these 273 samples, a total of 103 samples (37.7%) were positive for *V. parahaemolyticus* with 78 (70.3%) being fresh shrimp, 16 (21.9%) being frozen shrimp and 9 (10.1%) being dried shrimp. No *tdh*-positive *V. parahaemolyticus* was detected. However, 5 (2%) *trh*-positive isolates were identified in fresh shrimp samples, while no *trh*-positive isolate was detected in frozen and dried shrimp samples.

Martinez-Urtaza *et al.* (2020) isolated *V. parahaemolyticus* from 194 (12.5%) of a total of 1551 mussel samples from four areas of the west coast of Spain on the Atlantic Ocean. The highest number of positive samples was registered in the autumn (61; 18%) with their number amounting to 1234 MPN/100 g.

Bauer *et al.* (2006) examined 885 samples of Norwegian blue mussels (*Mytilus edulis*) from 102 production sites approved by the Norwegian Food Safety

https://doi.org/10.26656/fr.2017.8(1).267

Authorities and five sites with wild mussels. *V. parahaemolyticus* was isolated from 91 (10.3%) of the samples. A number of 1800 CFU/g and 200 CFU/g was found in two samples, while the others contained <100 CFU/g. No *V. parahaemolyticus* isolate contained the *tdh* gene, but four were *trh*-positive.

6. *Vibrio parahaemolyticus*-associated foodborne outbreaks

Vibrio parahaemolyticus does not cause disease due to pre-formed toxins (Chai *et al.*, 2019). Foodborne infection due to *V. parahaemolyticus* occurs after an infectious dose of 10^4 CFU (FDA, 2005). The incubation period is usually within 24 hours but can range from 4 to 96 hours after ingestion of bacteria (CDC, 2006). The average duration of clinical manifestations is 6 days, and the classic symptoms include watery, sometimes bloody diarrhoea, abdominal cramps, nausea, vomiting, and fever.

Vibrio parahaemolyticus was first associated with disease after consuming seafood in the East Asian region. This bacterium was isolated in 1951 from contaminated food in Osaka, Japan, after consuming shirasu, which resulted in 272 infected patients and 20 deaths (Fujino *et al.*, 1953). Since then, many cases of gastrointestinal disease due to the habit of eating raw or unprocessed seafood such as sushi, sashimi, crustaceans, crab meat, fish, squid, and sea urchin have been reported in Japan (Wu *et al.*, 2014). The trend of registered cases also increased from 1993 (837 cases) to 1998 (12,318 cases). A drastic decline was recorded in 1999 (14 cases), but in 2009 it rose again to 280 cases (Wu *et al.*, 2014).

In China, *V. parahaemolyticus* has been a major cause of food poisoning since the early 1990s. Crustaceans are the main means of spreading the pathogen (Liu *et al.*, 2004). The number of outbreaks decreased to 322 cases between 2003 and 2008 (Liu *et al.*, 2004). *V. parahaemolyticus* was found to be the most common cause of acute diarrhoea in 2007-2012 in the southern coastal region of China with the most common serotype O3:K6, followed by O4:K8 and O3:K29 (Yu *et al.*, 2013). A large number of cases of gastroenteritis due to *V. parahaemolyticus* have also been reported in Taiwan (Matsumoto *et al.*, 2000; Su and Liu, 2007; Yu *et al.*, 2013).

Vandy *et al.* (2012) studied a food explosion among guests at a wedding party in Cambodia, which was attended by 256 guests. Part of the menu was a vegetable salad with raw octopus. Fifty-two of the guests had clinical symptoms including watery diarrhoea, mucous stools, bloody diarrhoea, abdominal pain, vomiting, nausea, and fever. The incubation period varied from 7 to 51 hours. *V. parahaemolyticus* was isolated in 3 rectal samples. Although no other enteropathogenic bacteria were detected in the other samples, it was considered to be enteritis caused by *V. parahaemolyticus*.

In Cambodia, 49 cases of acute diarrhoea caused by *V. parahaemolyticus* have been reported (Thongjun *et al.*, 2013), while in Thailand the pandemic O3:K6 serotype of *V. parahaemolyticus* is the cause of most cases of foodborne infections between 2006 and 2010 (Yano *et al.*, 2014). Bilung *et al.* (2005) isolated *V. parahaemolyticus* from 62 of a total of 100 samples of cockles (*Anadara granosa*). The gene encoding thermostable direct hemolysin (*tdh*) was identified in two of the isolates, while the gene for *tdh*-related hemolysin (*trh*) was detected in 11 isolates.

A study by Noorlis *et al.* (2011) in Malaysia demonstrates the presence of *V. parahaemolyticus* in freshwater fish catfish and red tilapia. Of the 150 samples taken, 25% of the catfish samples and 22.6% of the red tilapia samples with an established number of *V. parahaemolyticus* of $1.1x10^7$ MPN/g were positive.

In India, *V. parahaemolyticus* has been isolated from clinical and environmental samples. Serotype O3:K6 of *V. parahaemolyticus* was found in Calcutta (Ceccarelli *et al.,* 2013; Pazhani *et al.*, 2014). Subsequently, it became widespread in Asia. A clinical study identified 178 isolates of *V. parahaemolyticus* from 13607 diarrhoea patients admitted to a hospital in Calcutta from 2001 to 2012 (Kanungo *et al.*, 2012). There are also reports of diarrhoea caused by *V. parahaemolyticus* in Calcutta, India (Reyhanath and Kutty, 2014).

Vibrio parahaemolyticus was first identified as the cause of three outbreaks with 425 cases of gastroenteritis associated with the consumption of improperly prepared crabs in Maryland, USA in 1971 (Daniels *et al.*, 2000). Since then, periodic outbreaks of *V. parahemolyticus* have been reported in coastal areas of the United States due to the consumption of raw shellfish or uncooked seafood (Letchumanan, Loo, Law *et al.*, 2019).

CDC reported 40 outbreaks of *V. parahaemolyticus* infection between 1973 and 1998 (CDC, 1999). Four of them included more than 700 cases of diseases associated with the consumption of raw oysters on the shores of the Persian Gulf, the West Pacific coast of North America and the northeastern Atlantic between 1997 and 1998. In the summer of 1997, 209 people were affected by *V. parahaemolyticus* after consumption of raw oysters in Oregon, Washington, California and British Columbia of Canada, with one death reported (McLaughlin *et al.,* 2005; Letchumanan, Ab Mutalib,

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Wong *et al.*, 2019). In 1998, two separate reports of *V. parahaemolyticus* infection were made in Washington (43 cases) and Texas (416 cases) (Letchumanan, Loo, Law *et al.*, 2019). Between July and September 1998, there were eight cases of *V. parahaemolyticus* infection reported around Connecticut, New Jersey and New York after consuming oysters and mussels collected from New York (McLaughlin *et al*., 2005). In the summer of 2006, another outbreak broke out, affecting 177 people with symptoms of gastroenteritis following consumption of *V. parahaemolyticus*-contaminated oysters in Washington and British Columbia (Alanis *et al.*, 2005). The presence of *V. parahaemolyticus* in both clinical and environmental samples poses a serious food safety risk in the United States (Letchumanan, Loo, Law *et al.*, 2019).

Infections with *V. parahaemolyticus* have been reported rarely in European countries, in contrast to Asia and the United States, where it is common (Miwatani and Takeda, 1976). Over the years, cases of gastroenteritis caused by *V. parahaemolyticus* have been proven in Spain, Greece, Great Britain, Turkey, Denmark, Yugoslavia, the Scandinavian regions and Italy (Molero *et al.*, 1989; Serracca *et al.*, 2011). *Vibrio parahaemolyticus* was detected in 8 cases of acute gastroenteritis after consumption of fish and mussels in Spain in 1989 (Robert-Pillot *et al.*, 2004). In 1997, a large outbreak of *V. parahaemolyticus* broke out in France involving 44 patients after consuming shrimp imported from Asia (Lozano-Leon *et al.*, 2003). In 1999, the first major outbreak of *V. parahaemolyticus* broke out in Galicia, Spain, affecting 64 people and involving the consumption of raw oysters (Martinez-Urtaza *et al.,* 2005). Another outbreak in Spain in 2004 affected 80 people who attended a restaurant wedding. The investigation revealed that the outbreak was caused by the consumption of cooked crabs prepared under unhygienic conditions (Martinez-Urtaza *et al.*, 2005). Between 2004 and 2005, 57 cases of *V. parahaemolyticus* infections were reported in the United Kingdom, most of which occurred when travelling to endemic areas (Wagley *et al.*, 2003). The pandemic serotype O3:K6 *V. parahaemolyticus* was isolated from patients in Spain and patients with gastrointestinal infections in Italy (Wagley *et al.*, 2003; Ottaviani *et al.*, 2010).

7. Antimicrobial resistance

Vibrio spp. are usually susceptible to most antimicrobial agents used in veterinary and human medicine. However, many studies have reported that *V. parahaemolyticus* has demonstrated multidrug resistance due to antibiotic abuse as a means of controlling infections in aquaculture production. In addition, both field and clinical isolates show similar profiles of antibiotic resistance. The most commonly observed profiles of antibiotic resistance include ampicillin, penicillin and tetracycline, regardless of the geographical location of the countries. The presence of many antibiotic-resistant bacteria in seafood and in the aquatic environment is a major problem in aquaculture and human health (Elmahdi *et al.*, 2016).

Clinical and Laboratory Standards Institute (Clinical and Laboratory Standards Institute (CLSI), 2015) determines the limits of sensitivity and resistance of *V. parahaemolyticus* to antimicrobials.

A more worrying fact is that both environmental isolates and clinical isolates have similar profiles of antibiotic resistance. Most commonly, antibiotic resistance is observed to ampicillin, penicillin, and tetracycline (Elmahdi *et al.*, 2016). A study conducted in the United States in 2005 and 2006 tested the antibiotic resistance of 168 isolates of *V. parahaemolyticus* from Louisiana Bay oysters. A total of 95 isolates (57%) are resistant to ampicillin (Han *et al.*, 2007).

Shaw *et al.* (2014) investigated the antimicrobial resistance of *V. parahaemolyticus* isolated from the surface waters of the Chesapeake Bay and Maryland. Most of the isolates are sensitive to the antibiotics of choice for vibrio infections, but 96% of *V. parahaemolyticus* are resistant to chloramphenicol and 68% are resistant to penicillin.

Lopatek *et al.* (2018) tested the antibiotic resistance of 104 isolates of *V. parahaemolyticus* from 595 samples of raw shellfish and marine fish sold commercially in Poland. Most isolates were resistant to ampicillin (75%) and streptomycin (68.3%), but all were sensitive to chloramphenicol and tetracyclines. 46.2% of the isolates showed resistance to ampicillin and streptomycin, 9.6% were resistant to ampicillin, streptomycin and gentamicin and 1% were resistant to ampicillin, streptomycin and ciprofloxacin.

Ottaviani *et al.* (2013) investigated the antimicrobial resistance of 107 isolates of *V. parahaemolyticus* from shellfish and clinical samples in Italy. 62% of all isolates were resistant to four classes of antibiotics. All isolates were resistant to ampicillin and amoxicillin, but sensitive to chloramphenicol and doxycycline. Resistance was found to tetracycline (11.2%), oxytetracycline (8.4%) and trimethoprim/sulfamethoxazole (3.7%).

From a total of 218 *V. parahaemolyticus* isolates from olive flounder, black rockfish, red sea bream, and sea bass, Oh *et al.* (2011) found that 65.1% were resistant to more than one antimicrobial agent. The isolate resistance to ampicillin was the highest (57.8%),

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followed by rifampin (11.9%), streptomycin (8.7%) and trimethoprim (6.4%). Multidrug resistance to four or more antimicrobial agents occurred in 5% of *V. parahaemolyticus* isolates.

Zhao *et al.* (2018) tested 114 *V. parahaemolyticus* isolates from water taken from 26 shrimp farms. Resistance to streptomycin (78.9%), ampicillin (64.9%) and gentamicin (53.5%) was found. In addition, multidrug resistance was demonstrated in 61.4% of the isolates, as well as the presence of genes for resistance to doxycycline, florfenicol and trimethoprim/ sulfamethoxazole.

Li *et al.* (2020) examined 905 food samples, from which 202 *V. parahaemolyticus* isolates were isolated. 79.2% were found to be resistant to ampicillin. In addition, they showed high levels of resistance to cephalothin (74.7%), streptomycin (65.8%), cefazolin (58.9%) and kanamycin (44.5%). The isolates were sensitive to nalidixic acid (97.5%), ciprofloxacin (96%), chloramphenicol (90.5%), tetracycline (78.7%), trimethoprim/sulfamethoxazole (78.2%) and azithromycin (77.2%).

The emergence of antimicrobial resistance in *Vibrio* spp. is increasingly common worldwide and the declining effectiveness of publicly available antibiotics poses a global threat to public health (Dutta *et al.*, 2021). Continuous monitoring and more extensive and in-depth studies on the incidence and level of resistance of *V. parahaemolyticus* are needed to ensure the protection of public health. Summary data on the antimicrobial resistance of *V. parahaemolyticus* isolated from different hydrobiont species are presented in Table 2.

8. Impact of various factors on the survival rate of *Vibrio parahaemolyticus* in seafood

Various preservation methods such as low-temperature storage, drying, smoking or salting are used in food processing and transportation to control the growth and survival rate of proteolytic and pathogenic microorganisms (Yang *et al.*, 2008). Although most strains of *V. parahaemolytcus* are sensitive to low temperatures (Lin *et al.*, 2004), some survive at least 3 weeks at 4°C with little or no reduction in cell number, but proliferation may occur at favorable temperatures (DePaola, Nordstrom, Bowers *et al.*, 2003, DePaola, Ulaszek, Kaysner *et al*., 2003).

Shen *et al.* (2010) investigated the survival of *V. parahaemolyticus* during low-temperature storage and high-temperature treatment. *V. parahaemolyticus* culture enriched in sterile APW with 1.5% NaCl (APW-saline broth) remained at 37°C for 12-16 h. It was then stored at various temperatures from -30, -18, 0, 5, 10, 15 to 20° C. The high-temperature treatment was performed at 50, 55, 60, 70, 80 and 90°C. The results showed that *V. parahaemolyticus* in APW-saline broth multiplied rapidly at temperatures above 15°C and gradually decreased at 0 and 5°C.

Shen *et al.* (2010) demonstrated that storing a bacterial suspension of *V. parahaemolyticus* with a density of 8.59 log CFU/mL at -18 and -30°C for 15 days significantly reduced the number of bacteria to 2.04 (at -18°C) and 3.84 (-30°C) log CFU/mL. This is probably due to the damage of bacterial cells by the larger ice crystals that form at -18°C compared to -30°C. Heating at 60°C for 5 mins, 70°C for 2 mins, and 80°C for 1 min also reduced *V. parahaemolyticus* from 10000 MPN/g to undetectable levels.

Mathur and Shaffner (2013) tested the effect of lime juice on the growth and survival rate of *V. parahaemolyticus*. Tilapia fillets (*Oreochromis niloticus*) were inoculated with *V. parahaemolyticus* (>7 log CFU/ g) and incubated at 25 and 4°C for 30 or 120 mins in the presence of fresh lime juice in concentrations typical of the preparation of the popular ceviche dish. Similar amounts of cells were also inoculated in fresh lime juice. The number of *V. parahaemolyticus* was reduced by ~5 log under all tested conditions. The number of *V. parahaemolyticus* inoculated in lime juice was also reduced by ~5 log.

Salem and Amin (2012) studied the effect of citric and acetic acid on the growth and survival rate of *V. parahaemolyticus* inoculated in fresh shrimp. After contamination of the shrimp with *V. parahaemolyticus* at a density of 10.91 log cfu/g, they were immersed in 5 and 10% citric acid and 4 and 8% acetic acid for 5, 15, 30, 60 mins and 24 hours. The initial number of *V. parahaemolyticus* decreased after immersion in 5 and 10% citric acid for 5 mins with 5.68 log CFU/g and 7.91 log CFU/g, respectively, and in 4% acetic acid for 5 mins with 6.61 log CFU/g. The growth of *V. parahaemolyticus* was completely inhibited after immersion in 8% acetic acid for 5 mins, 4% acetic acid for 15 mins and 10% citric acid for 30 mins.

Cho *et al.* (2016) studied the survival rate of *V. parahaemolyticus* in raw ready-to-eat crab (*Portunus trituberculatis*) marinated in soy sauce. The crabs were inoculated with a bacterial suspension with a density of 4.1-4.4 log CFU/g, immersed in a soy sauce with a salt content of 15.6% and pH 4.6 and stored in a refrigerator at 5 or 22°C for 28 days. Regardless of the storage temperature, *V. parahaemolyticus* was not isolated from the samples during the storage period. This proves the high sensitivity of *V. parahaemolyticus* to low pH.

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Table 2. Antimicrobial resistance of *V. parahaemolyticus*.

Source	Number of isolates tested	Antibiotics tested	R	I	S	References
Shrimps	168	Ampicillin	57%	24%	-	Han *et al.* (2007)
Water	77	Chloramphenicol		96%	-	Shaw *et al.* (2014)
		Penicillin	68%			
Fish, mussels, shrimps	104	Ampicillin	75%	-		Lopatec *et al.* (2018)
		Streptomycin	68.30%			
		Chloramphenicol			100%	
		Tetracyclines			100%	
Fish	218	Ampicillin	57.80%	-	-	Oh *et al.* (2011)
		Rifampin	11.90%			
		Streptomycin	8.70%			
		Trimethoprim	6.40%			
Ready-to-eat food, shrimps, fish	163	Ampicillin	82.21%	-	-	Lei *et al.* (2020)
		Gentamycin	19.63%			
		Tetracyclines	14.11%			
		Ciprofloxacin	4.91%			
		Levofloxacin	4.91%			
Shrimps	10	Ampicillin	90%	20%		Melo *et al.* (2012)
		Amikacin	60%			
		Chloramphenicol			100%	
Shrimps	114	Streptomycin	78.90%	-	-	Zhao *et al.* (2018)
		Ampicillin	64.90%			
		Gentamycin	53.50%			
Shrimps, mussels	65	Ampicillin	63.10%	-		Al-Othrubi *et al.* (2014)
		Cefalexin	35.40%			
		Tetracycline			97%	
		Ciprofloxacin			49.30%	
Shrimps	59	Ampicillin	98.30%	-		Kang *et al.* (2018)
		Cephalothin	62.70%			
		Streptomycin	76.30%			
		Tetracycline			64.40%	
		Nalidixic acid			86.40%	
Bivalve mollusks	70	Ampicillin	87.50%	-		Lopatek *et al.* (2015)
		Streptomycin	70.30%			
		Tetracycline			100%	
		Chloramphenicol			100%	
Shrimps	71	Cefpodoxine	100%	-	-	Parthasarathy *et al.* (2021)
		Cefotaxime	90%			
		Ampicillin	90%			
		Ceftizoxime	60%			
		Tetracycline	50%			
		Cetriaxone	40%			
Marine water, fish	49	Ampicillin	97.90%	-	-	Son *et al.* (2005)
		Oxolinic acid	24.10%			
		Amicacin	17.20%			
Ready-to-eat food	213	Ampicillin	96.90%	-	-	Kim *et al.* (2005)
		Amikacin	29.20%			
		Tetracycline	27.70%			

R: Resistant, I: Intermediate, S: Susceptible

https://doi.org/10.26656/fr.2017.8(1).267

Table 2 (Cont.). Antimicrobial resistance of *V. parahaemolyticus*.

Source	Number of isolates tested	Antibiotics tested	R	I	S	References
Marine water, fish, bivalve mollusks	716	Vancomycin	97.30%	-	92%	Han *et al.* (2012)
		Ampicillin	87.30%			
		Cephalothyn	48.80%			
		Rifampin	46.10%			
		Sulfamethoxozole-trimethoprim				
		Chloramphenicol			92%	
		Gentamicin			82.30%	
		Tobramycin			74.80%	
		Tetracycline			69.40%	
Marine water	30	Ampicillin	100%	-	100%	Yang *et al.* (2017)
		Cefazolin	66.70%			
		Tetracycline				
		Amikacin			60%	
		Gentamicin			80%	
Shrimps, mussels	385	Ampicillin	85%	-	-	Letchumanan, Ab Mutalib, Wong *et al.* (2019)
		Amikacin	66.80%			
		Kanamycin	50%			
		Cefataxime	55.80%			
		Ceftazidime	34%			
Crustaceans, mussels	208	Ampicillin	94.20%	-	-	Hu and Chen (2016)
		Rifampin	93.30%			
		Streptomycin	77.90%			
Marine water	41	Carbenicillin	98%	-	-	Ghenem and Elhadi (2008)
		Ampicillin	88%			
		Cephalothin	76%			

R: Resistant, I: Intermediate, S: Susceptible

Filipović *et al.* (2016) tested the antibacterial activity of 16 spices at a concentration of 2.5% against *V. parahaemolyticus* at 5 and 37°C. At 5°C all spices except anise and coriander seeds showed antibacterial activity against *V. parahaemolyticus* as the number decreased by at least 1 log. Strong antibacterial properties have been found at the same temperature for oregano, garlic, thyme, cloves, cinnamon, curry, rosemary, ginger and turmeric. Oregano, garlic, thyme, cloves and cinnamon showed strong antibacterial activity at 37°C. This result showed that some spices have the potential to reduce the number of *V. parahaemolyticus* in seafood in combination with low temperatures.

9. Conclusion

Vibrio parahaemolyticus is one of the basic pathogens resulting in foodborne illness and comprising a serious risk to human safety in the consumption of marine hydrobionts. Despite reports from a number of authors for establishing *V. parahaemolyticus* in various hydrobionts, there are still no systematic data about its incidence in a number of European countries. Literature data report a complex set of pathogenic factors. However, there are no systematic studies to clarify the environmental conditions (other than temperature and salt content) that regulate the expression of these factors. Existing conflicting data on the application of different methods for the isolation and identification of *V. parahaemolyticus* indicate that further research is needed in this regard. The presence of genes and multiple serovariants of pandemic branches is evidence of the need for larger studies related to the incidence of *V. parahaemolyticus*.

Conflict of interest

The authors declare no conflict of interest.

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