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Report of the Scientific Committee of the Spanish Agency for Food Safety and Nutrition (AESAN) on the microbiological criteria for *Vibrio cholerae*, as additional control measures at border control posts, applicable to imported frozen prawns and other fishery products

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Working group

Antonio Valero Díaz (Coordinator), Rosa María Capita González, Baltasar Mayo Pérez, Azucena del Carmen Mora Gutiérrez and María Dolores Rodrigo Aliaga

Scientific Committee

Concepción María Aguilera García
Universidad de Granada

María Pilar Guallar Castillón
Universidad Autónoma de Madrid

Azucena del Carmen Mora Gutiérrez
Universidad de Santiago de Compostela

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Hospital Gregorio Marañón de Madrid

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María del Carmen Recio Iglesias
Universitat de València

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Araceli Díaz Perales
Universidad Politécnica de Madrid

Baltasar Mayo Pérez
Consejo Superior de Investigaciones Científicas

Ana María Rivas Velasco
Universidad de Granada

María Roser Vila Casanovas
Universitat de Barcelona

Technical Secretary
Vicente Calderón Pascual

Technical management of the report AESAN: Paula Arrabal Durán

Abstract

In recent years, an increase in the presence of *Vibrio* spp. in fishery products has been detected as a result of phenomena associated with climate change, international trade and the development of new detection and diagnosis methods. Specifically, it is the serogroups of *V. cholerae* O1 and O139, as well as the strains carrying the gene that codes for cholera toxin (*ctx* positive strains), that pose a risk to the consumer through the intake of contaminated fishery products. The lack of a harmonised criterion on border controls, together with the increasing presence of non-toxigenic non-O1/

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non-O139 strains, highlights the need to more accurately assess the risk to the consumer of the presence of *V. cholerae* in frozen prawns and other fishery products, both raw and cooked ready-to-eat, and establish microbiological criteria, within the framework of controlling these products.

Based on the literature analysed, the prevalence of *V. cholerae* O1 and O139, as well as *ctx* positive strains in imported frozen prawns and other fishery products is low, so the risk to the consumer is mainly associated with poor handling and storage practices. On the other hand, the pathogenicity of non-toxigenic *V. cholerae* serogroups is not yet well defined, and there is no solid evidence of foodborne infection.

Therefore, based on the evidence found, it is recommended to maintain the microbiological criterion of absence in 25 g of product, whether frozen, raw or cooked ready-to-eat prawns or other fishery products, in the case of *V. cholerae* O1 and O139, as well as other *ctx* positive strains, given the inherent risk associated with disease. For those strains of non-toxigenic *V. cholerae*, there is not enough evidence about their pathogenicity, so no intervention measures are suggested beyond the monitoring of Good Hygiene Practices (GHP), applicable to any fishery product.

In relation to the risk associated with the presence of *V. cholerae* in raw, frozen prawn and other fishery products, it follows that cooking treatments at 70 ºC for 2 minutes in the centre of the product guarantee the elimination of the pathogen. In the case of frozen, cooked ready-to-eat prawns and other fishery products, microbiological risk is associated with contamination after cooking treatment. In order to mitigate the risk to the consumer, it is necessary to apply GHP and the principles of Hazard Analysis and Critical Control Points (HACCP) throughout the production, distribution and consumption chain.

Keywords

Vibrio cholerae, foodborne outbreak, microbiological criteria, frozen prawns, fishery products.

Suggested citation

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1. Introduction

The genus *Vibrio* is widely distributed in nature in fresh or salt water environments, in coastal areas and estuaries of tropical regions. It is formed by, at least, 12 species pathogenic to humans; 10 of these could cause diseases that are transmitted through food. Most are caused by *Vibrio cholerae*, *V. parahaemolyticus* and *V. vulnificus*.

Except in particular situations, consumer exposure to these pathogens is very low, due to their low prevalence in fishery products, the inhibition of growth at refrigeration temperatures, sensitivity to heat treatment and the limited consumption of raw fishery products (fish, molluscs and crustaceans) in Spain.

Hence, the risk of suffering from a disease due to the consumption of fishery products from third countries contaminated by *Vibrio* spp. in Spain has so far been classified, in general terms, as low or very low, based on the available data on the identification, prevalence and concentration of *V. cholerae, V. parahaemolyticus* and *V vulnificus*. However, various studies suggest that climate change and increased sea temperature are contributing to an increase in the prevalence and concentration of *Vibrio* in water, associated with an increase in outbreaks reported worldwide and its geographical spread to regions where the disease did not previously exist. Furthermore, factors such as the increase in population density on the coast and the improvement of diagnostic methods could be an explanation for these observations.

An inadequate storage temperature can produce a considerable increase in *Vibrio* counts, which can reach levels of health risk, even starting from very low concentrations of the pathogen in the capture of the product or upon arrival at border checkpoints. Cross-contamination during handling, as well as through contact with contaminated food, may increase the risk associated with the presence of *Vibrio* spp.

Regulation (EC) No. 2073/2005 on microbiological criteria for foodstuffs (EU, 2005) does not lay down criteria at a European Union level for any *Vibrio* spp., so there is no harmonised criterion on border controls, and each country takes its own decision regarding its actions to control these pathogens.

According to the report of the meeting of the Joint FAO/WHO Expert Meetings on Microbiological Risk Assessment (JEMRA, 2021), various approaches are already available for the isolation, detection, enumeration and characterisation of *Vibrio* spp. from matrices such as water and seafood. In particular, the international method for the detection of *V. parahaemolyticus*, *V. cholerae* and *V. vulnificus* (UNE-EN ISO 21872-1:2017 (UNE-EN ISO, 2017)) includes molecular detection options using conventional and real-time polymerase chain reaction (PCR) (qPCR).

Vibrio spp., mainly the species *V. parahaemolyticus* and *V. vulnificus*, have been identified by the European Food Safety Authority (EFSA) as biological hazards with a high probability of becoming emergent in the near future in Europe. EFSA is preparing an opinion on *Vibrio* spp. associated with the consumption of seafood, scheduled for publication in June 2024 (EFSA, 2022). Likewise, the *Codex Alimentarius* Committee on Food Hygiene (CCFH) is reviewing the 2010 document, Guidelines on the application of general principles of food hygiene for the control of pathogenic *Vibrio* spp. in seafood (Codex Alimentarius, 2010), following the most current scientific evidence collected by the Food and Agriculture Organization/World Health Organization (FAO/WHO).

As a result of the unfavourable result of *V. cholerae* controls in frozen crustaceans from third countries at border control posts during the years 2022 and 2023, official controls were intensified throughout the European Union for frozen prawns from some supplier establishments. In these controls, the established criterion was the absence of *V. cholerae*, without distinction of serogroups.

With these premises, the Scientific Committee of the Spanish Agency for Food Safety and Nutrition (AESAN) is requested to prepare a report determining:

i. The existing differences regarding the risk to the consumer between the presence in frozen prawns of the *V. cholerae* strains belonging to serogroups O1 or O139; the non-O1/non-O139 serogroups carrying the *ctx* gene that encodes cholera toxin (CTX); the non-O1/non-O139 serogroups not carrying the *ctx* gene that encodes CTX.
ii. The existing differences in terms of risk to the consumer of the presence of *V. cholerae* in frozen prawns, among raw products, and those ready-to-eat cooked products.

2. Background

2.1 Taxonomic aspects

The genus *Vibrio* belongs to the *Vibronaceae* family and is formed by highly mobile, Gram-negative halophilic bacteria with curved-road shape, with a single polar flagellum. The microorganism is between 1 and 3 µm long and between 0.5 and 0.8 µm wide, is oxidase-positive facultative anaerobic, non-spore forming and is capable of fermenting sugars (glucose, sucrose and mannitol) (Kraus et al., 2003) (Ryan and Ray, 2004). *Vibrio* spp. are typically found in aquatic environments and some pose a serious danger to human health as causative agents of foodborne infections (Haque et al., 2023). *Vibrio* spp. can cause cholera, vomiting, sepsis, bloody diarrhoea, abdominal pain, fever and nausea, processes that are often associated with the consumption of contaminated seafood (Haque et al., 2023). 12 of the 30 species of the genus are considered human pathogens; among these, *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* are the most frequently reported (Haque et al., 2023).

V. cholerae is divided into more than 200 serogroups determined by the structure of the lipopolysaccharide O-antigen (LPS). Among them, a subset of strains belonging to serogroups O1 and O139 can cause cholera due to their ability to produce CTX, responsible for profuse watery diarrhoea, and have been mainly associated with epidemics. CTX has two subunits, A and B, the latter with five components. The *V. cholerae* strains that cause cholera harbour a filamentous bacteriophage (CTXΦ) that encodes CTX, and a virulence factor, the *Toxin-Coregulated Pilus* (TCP), a type IV *pilus* that is essential for colonisation (it allows adjacent bacterial cells to bind to each other, facilitating the formation of microcolonies within the intestines of infected humans and animals, and facilitates adhesion to enterocytes) and also serves as a receptor for the CTXΦ bacteriophage (Waldor and Mekalanos, 1996). In addition to TCP, many other cellular structures and activities, including the O-LPS antigen, cell curvature, motility, and certain metabolic processes, have been implicated in intestinal colonisation by *V. cholerae* (Baker-Austin et al., 2018). The production of these virulence factors (CTX and TCP) is strongly influenced by environmental conditions (Bhandari et al., 2023). Other virulence factors, such as zonula occludens toxin (Zot), accessory cholera enterotoxin (Ace), hemolysin (HlyA), heat-stable enterotoxin, type III and VI secretion systems (T3SS and T6SS), and

the ability to form biofilms are involved in the pathogenicity of *V. cholerae*. Therefore, even isolates of *V. cholerae* negative for CTX genes could be a cause for concern due to the potential presence of other virulence genes (Jantapaso et al., 2024), although this is an aspect that has been the subject of little research so far.

The vast majority of cholera cases are caused by strains of serogroup O1, which are divided into three serotypes, called Ogawa, Inaba and Hikojima, according to the methylation status of the terminal perosamine of the LPS. Ogawa strains are methylated, Inaba strains are unmethylated, and Hikojima strains express methylated and unmethylated O antigens. While the Ogawa and Inaba serotypes can circulate simultaneously during epidemics and are capable of interconversion (Stroeher et al., 1992), the Hikojima serotype is rare and evidence indicates that it is an unstable transitional form that occurs when a strain has a serotype change from Ogawa to Inaba (Karlsson et al., 2014). In turn, the *V. cholerae* O1 serogroup is classified into two biotypes, classical and El Tor, which can be distinguished based on a set of phenotypic and genetic markers (Barzelighi et al., 2016) (Clemens et al., 2017). Thus, in principle, these two biotypes differ in their haemolytic capacity, agglutination reaction with erythrocytes and resistance to polymyxin B, characteristics present in the El Tor strains (Sharma et al., 1997) (Sharifnia et al., 2012). A gene that helps encode the development and regulation of TCP, *tcpA*, is also used to distinguish biotypes (El Tor or classical), based on sequence deletions in the classical allele (Iredell and Manning, 1994). Interestingly, there are some differences between the two biotypes in terms of infection patterns. El Tor strains are more efficient in host-to-host transmission, survive better in the environment and in the human intestine, and have a higher incidence of asymptomatic than symptomatic carriers, compared to classical strains (Nair et al., 2006).

In general, strains that do not belong to these serogroups (commonly referred to as "non-O1/non-O139 *V. cholerae*") lack the *ctx* gene and are not pathogenic, or cause mild and sporadic diseases in healthy people, such as gastroenteritis, otitis or wound infections (Farina et al., 2010). In immunocompromised people or those who have an underlying disease, non-O1/non-O139 *V. cholerae* strains are capable of causing necrotising fasciitis and septicaemia, with associated lethality rates of up to 47 % (Trubiano et al., 2014), as well as gastroenteritis potentially as severe as cholera (Calduch et al., 2003) (Dutta et al., 2013) (Octavia et al., 2013) (Fernández-Ruiz et al., 2017) (Zhang et al., 2020). Infections caused by non-O1/non-O139 strains are often related to environmental exposure, particularly to the consumption of raw or undercooked seafood, and, mainly, affect immunocompromised patients (Morris, 1990) (Patel et al., 2009). During the summer of 2014, there was a considerable increase in the number of reported non-O1/non-O139 *V. cholerae* infections in the Baltic Sea area, which corresponded both temporally and spatially with a significant heatwave. Most cases consisted of self-limited ear and soft tissue infections associated with swimming or exposure to water (Baker-Austin et al., 2018).

Although the clinical importance and the impact on public health of non-toxigenic serogroups of *V. cholerae* have been questioned for a long time, their involvement in outbreaks of diarrhoea after the consumption of contaminated seafood has been documented in several European countries (Le Roux et al., 2015). Some non-O1/non-O139 *V. cholerae* strains produce a heat-stable enterotoxin

(called NAG-ST), which closely resembles the heat-stable enterotoxin of enterotoxigenic *Escherichia coli*. These strains, called "enterotoxigenic *V. cholerae*", can cause diarrhoeal diseases in humans, according to observations in volunteers and epidemiological studies of outbreaks and sporadic cases (Bagchi et al., 1993). The pathogenicity of non-toxigenic *V. cholerae* can be increased due to the presence of a wide spectrum of virulence factors, including extracellular enzymes, enterotoxins and hemolysins (Restrepo et al., 2006) (Ottaviani et al., 2009). Hence, it is possible that non-toxigenic serogroups of *V. cholerae* will be considered in the future as an emerging cause of gastrointestinal infection in our environment, especially in immunocompromised patients, where the infection could be associated with significant mortality (Fernández-Ruiz et al., 2017).

Non-O1/non-O139 strains that produce CTX have sometimes been isolated (O141 and O75 in the United States; O5, O6, O10, O12, O14 and O37 in other parts of the world) (Crump et al., 2003) (Tobbin-D'Angelo et al., 2008) (Aidanian et al., 2015). These serogroups may cause sporadic cases of cholera or small outbreaks, but do not appear to be capable of causing epidemic cholera. In this regard, it has been indicated that some non-O1/non-O139 serogroups could also serve as a reservoir of the cholera toxin phage genome (Udden et al., 2008). In phylogenetic studies these non-O1/non-O139 serogroups are not grouped with the *V. cholerae* strains responsible for epidemic diseases (which tend to be positioned in close proximity) and, in general, lack multiple genes/gene complexes that have been associated with "typical" cholera (Li et al., 2002). This fact coincides with the concept that there is an "epidemic genotype" that includes multiple genes necessary for epidemic disease (Aidanian et al., 2015). Specifically in Europe, there is a lack of mandatory reporting systems for *Vibrio*-associated diseases other than those caused by *V. cholerae* O1/O139, which prevents an accurate estimate of the number of infections. However, the prevalence of outbreaks caused by non-O1/non-O139 *V. cholerae* is increasing, probably as a result of the progressive increase in sea surface temperature (Castello et al., 2022).

2.2 Cholera

V. cholerae infection may be asymptomatic or present with severe clinical forms (cholera *gravis*). Cholera can occur as a sporadic, epidemic or endemic disease, known to cause severe diarrhoea and dehydration that can cause death in the absence of medical treatment (Bhandari et al., 2021). In endemic areas, 75 % of cases are asymptomatic, 20 % are mild to moderate and between 2 % and 5 % are severe forms. Symptoms include the abrupt onset of watery "rice grain" diarrhoea, occasional vomiting, and abdominal cramps (Ryan and Ray, 2004). Dehydration occurs with symptoms and signs such as thirst, dry mucous membranes, decreased skin turgor, sunken eyes, hypotension, weak or absent radial pulse, tachycardia, tachypnoea, hoarse voice, oliguria, cramps, renal impairment, convulsions, drowsiness, coma and, occasionally, death (Kraus et al., 2003). Death from dehydration can occur in a few hours or days, especially in children without medical treatment, with the disease being especially dangerous for pregnant women during the last stage of pregnancy, since miscarriages, premature births and foetal death can occur. In cases of cholera *gravis*, which involve severe dehydration, up to 60 % of those affected may die; however, less than 1 % of infections are fatal with rehydration treatment. The incubation period of cholera can range from 12 hours

to 5 days, and the disease usually lasts between 4 and 6 days (Azman et al., 2013). Several studies demonstrate that clinically apparent *V. cholerae* infection induces protective immunity against subsequent infections in humans. Symptomatic patients can eliminate *Vibrio* spp. before clinical signs of the disease and up to 2 weeks later. In addition, a carrier state may exist (where the patient has the infectious agent without any clinical manifestation), in this case the *Vibrio* spp. being eliminated in small and intermittent amounts for several weeks (Gangarosa et al., 1966).

V. cholerae is transmitted from person to person by the faecal-oral route or indirectly through contaminated food or water (Montero et al., 2023), with epidemics caused by the consumption of seafood having been reported (Krauss et al., 2003) (Ryan and Ray, 2004) (Baker-Austin et al., 2018). In developed countries, cases of *Vibrio* gastroenteritis are almost always related to the consumption of seafood, particularly raw or undercooked (Hlady and Klontz, 1996). However, in environments with limited resources, the pattern is usually less clear, due in part to the increased risk of faecal contamination of food and water and/or cross-contamination of other food with contaminated fishery products. Temperature and salinity are the two most important factors affecting the distribution of *Vibrio* worldwide. Therefore, infections usually arise during the summer and autumn, when surface waters are comparatively warmer (Deeb et al., 2018). The rapid warming of the marine environment, together with the increase in extreme weather events, such as heat waves, are favouring the spread of *Vibrio* spp. around the world, and outbreaks of *Vibrio* spp. infection have recently been reported in temperate regions such as Spain (*V. parahaemolyticus* (Martínez-Urtaza et al., 2018)), Sweden and Finland (*V. cholerae* and other species (Baker-Austin et al., 2016)).

After ingestion, the pathogen proliferates until reaching a high concentration throughout the mucosal surface of the small intestine, but does not alter the integrity of the epithelial barrier or cause substantial damage to epithelial cells (it is an extracellular pathogen). Instead, the bacteria elicit an intense secretory response, resulting in profuse watery diarrhoea. Studies in various animal models and in volunteers have shown that cholera diarrhoea is, primarily, a response to CTX secreted by the pathogen. Thus, it has been observed that the elimination of the *ctxA* and *ctxB* genes (which encode the A and B subunits of the CTX, respectively) of *V. cholerae* removes the ability of the bacterium to induce diarrhoea in animal models, while the administration of purified CTX is sufficient to cause diarrhoea in human volunteers (Baker-Austin et al., 2018).

Thanks to advances in sanitation through education and improved infrastructure (proper wastewater management), developed countries have successfully eradicated cholera, except for a few occasional outbreaks or as a result of a few imported cases (WHO, 2018). In contrast, cholera is endemic in almost 70 countries worldwide, with the disease being more frequent in tropical and subtropical areas. Most cases are found in the Indian subcontinent and in Africa (in 2002, the WHO estimated that 97 % of cholera cases occur in Africa) (Ali et al., 2015), where socio-economic issues such as population growth and consequent overcrowding, unplanned urban development and poor health infrastructure, extreme poverty, forced migration and prolonged warfare contribute to the transmission of the disease (WHO, 2018). In endemic areas, cholera cases tend to be more common in children under the age of 5, which can be explained by a deficient immune system. In addition to endemic patterns of transmission, cholera often occurs within the context of large and

devastating epidemics. Epidemics in endemic areas tend to occur during the hot season and seem to be increasing as a result of climate change (Kraus et al., 2003). In 2022, there were about half a million cases of cholera in the world, and 2549 deaths from this cause (WHO, 2023). However, some researchers estimate that 3 million cases of human disease occur annually, associated with more than 100 000 deaths (Ali et al., 2012).

In the last 200 years there have been seven major cholera pandemics. The classical biotype of *V. cholerae* O1 was responsible for the first six cholera pandemics, which occurred between 1817 and 1925. From that year, leaving aside some local outbreaks, no more cholera pandemics were reported until the 1960s (Wachsmuth et al., 1994) (Dziejman et al., 2002). The seventh cholera pandemic, caused by the El Tor biotype, began in 1961 in Sulawesi (Indonesia), reached Africa in 1970 and Latin America in 1991 (Rivera et al., 2003). In August 2000, South Africa experienced one of the worst cholera epidemics in its history, with over 114 000 recorded cases.

The strains of the seventh O1 El Tor pandemic, which continues today, have been the most persistent probably as a result of their continued adaptability to environmental and other changes (selection pressure, evolutionary events, gene transfer, etc.). These El Tor strains replaced the classical strains shortly after their appearance in 1961, except in Bangladesh, where both strains were reported simultaneously for a decade (1982-1993) (Ansaruzzaman et al., 2007). Until the early 2000s, there were still some sporadic cases worldwide due to the classical biotype. Since 1990, *V. cholerae* O1 has switched to hybrid and altered El Tor variant strains. These variant strains of El Tor have unique or mixed phenotypes compared to classical and El Tor strains, and at the same time produce the classical-type CTX. These emerging variants of *V. cholerae* have been classified as strains of "atypical El Tor variant" (Safa et al., 2010) (Klinzing et al., 2015).

El Tor was temporarily replaced, in 1992, by a strain from a serogroup other than O1, which caused a massive outbreak of a cholera-like disease in coastal villages in India and Bangladesh. This strain is phenotypically and genetically very close to El Tor, with some characteristics of classical strains, and is classified as a new serogroup (O139 Bengal). In August 1996, *V. cholerae* re-emerged as the causative agent of cholera in Calcutta, being named the strain responsible O139 Calcutta. These O139 Calcutta strains are genetically similar to O139 Bengal and have an additional *ctx* element of the El Tor type (Sharma et al., 1997). The rapid spread of this strain of serogroup O139 Bengal in most of Asia was thought to herald the beginning of the eighth pandemic, but this strain did not spread outside of Asia and was consequently not classified as a pandemic. Cholera associated with the O139 Bengal strain declined in 1996, but increased in 2002, and again in 2005. Since then, O139 Bengal has not been associated with any major outbreaks and is only sporadically isolated. Currently, cholera outbreaks coexist by strains of the O139, O1 El Tor and O1 El Tor hybrid/atypical biotypes (Bhandari et al., 2023). The El Tor biotype continues to be the main causative agent of cholera worldwide (Baker-Austin et al., 2018).

Notable are the population changes (almost complete replacement of the prevalent strains by new strains) and the continuous evolution of *V. cholerae* O1 from the eighteenth to the twenty-first century. Horizontal gene transfer and acquisition of exogenous DNA, including mobile genetic elements such as plasmids, bacteriophages, transposons, integrative and conjugative elements, and

genomic islands, help bacteria to increase their fitness in different environmental conditions. The appearance of new pathogenic clones such as, for example, *V. cholerae* O1 El Tor, *V. cholerae* O139 and atypical clones of *V. cholerae* O1 El Tor seems to be due to the extensive genetic recombination produced by horizontal gene transfer, mainly genomic islands (Bhandari et al., 2021). In fact, the analysis of the *V. cholerae* genome suggests that recent outbreaks of cholera in various parts of the world have occurred due to the rapid evolution of multiple descendants of an ancestor of *V. cholerae* O1 El Tor, mainly due to horizontal gene transfer events through transduction, conjugation and transformation (Chun et al., 2009).

2.3 Cholera and food. Prevalence of *V. cholerae* in fishery products

Among different foods, fishery products are the main source of infection by *V. cholerae* globally. Thus, this bacterium is one of the most important pathogens associated with its consumption in several countries (Yaashikaa et al., 2016) (Castello et al., 2022). Contamination of these foods with *Vibrio* spp. can occur at various points in the production chain, including the life cycle of seafood, as well as its processing, conservation and storage (for example, from plankton, pond water, water used for cleaning, ice and by cross-contamination with different surfaces) (Jantapaso et al., 2024), especially when these operations are performed in inadequate hygienic conditions. As a result, fishery products may be contaminated with different species of *Vibrio* that not only contribute to their deterioration, but also to the spread of cholera and other foodborne diseases (Haque et al., 2023).

Physicochemical characteristics of foods that favour the survival and growth of *V. cholerae* include high water content, neutral or alkaline pH, and absence of competing bacteria. In addition, *V. cholerae* has the ability to form biofilms, which can play an important role in the persistence and transmission of the pathogen (Fernández-Delgado et al., 2016). Thus, as with many other microorganisms, the resistance of *V. cholerae* biofilms to chlorine and other disinfectants has been documented (Carrascosa et al., 2021), which makes it difficult to control this bacterium in the food industry.

Humans are a reservoir of *V. cholerae*, as are animals found in aquatic environments (Krauss et al., 2003). Climate change, increasing water pollution and the anthropisation of coasts are factors that favour the global spread of *Vibrio* spp., as well as the appearance of antibiotic-resistant isolates (Castello et al., 2022). Since *Vibrio* spp. requires a warm saline aquatic environment for optimal growth (>15 ºC), it has been suggested that the increase in average sea temperatures as a result of global warming could explain the increase in the incidence of *V. cholerae* infection, even in northern latitudes (Ottaviani et al., 2009) (Le Roux et al., 2015).

The presence of *Vibrio* spp. in seafood has been described in different countries of Asia, Europe and Latin America (Ripabelli et al., 1999) (Lhafi and Kuhne, 2007) (Raghunath et al., 2008) (Lopatek et al., 2015) (Sperling et al., 2015) (Tra et al., 2016) (Neetoo et al., 2022) although, in the majority of the research, CTX-producing strains are not detected (Zhang et al., 2014). In Thailand, some authors have observed very high prevalences of *Vibrio* spp., as is the case with Woodring et al. (2012), which found the microorganism in 92 % of raw seafood samples from markets in Bangkok analysed in 2008. For their part, Preeprem et al. (2014) found that 55 of 125 seafood samples analysed in Thailand were

contaminated with *V. cholerae*, with 99 of the 100 isolates identified as non-O1/non-O139. These researchers did not detect CTX-producing strains. Preeprem et al. (2023) recently isolated *Vibrio* spp. from seafood from retail establishments in the province of Yala (Thailand) and found that 34 % of the isolates corresponded to *V. cholerae*. Also in that country, Dalsgaard et al. (1995a) detected the presence of *V. cholerae* O1 in farmed prawns, but subsequent molecular studies (Dalsgaard et al., 1995b) showed that the strains were negative for the *ctx* gene, so these authors suggest the importance of using molecular techniques, such as PCR, to characterise the environmental isolates of *V. cholerae* and thus be able to detect the pathogenic strains of this microorganism. Chitov et al. (2009) have indicated that the level of *V. cholerae* in raw seafood in Thailand could range from 10^2 to 10^4 CFU/g, and the serogroups detected are generally non-O1/non-O139.

Haque et al. (2023) detected *V. cholerae* in 24.7 % of the fish farm samples tested in Bangladesh, with a higher prevalence in prawn samples (38 %) than in mud (20 %) and water (16 %) samples. Gopal et al. (2005) studied the presence of various *Vibrio* spp. in water, sediment and prawn samples from multiple farm environments on the east and west coast of India. These researchers detected *V. cholerae* in some cases, although all isolates were negative for the gene for CTX. For their part, Elhadi et al. (2004) examined 768 samples of seafood from Malaysia including prawns, squid, crabs, cockles and mussels. 97 isolations of *V. cholerae* were performed, of which 1 belonged to serogroup O1, and 14 to serogroup O139. In this study, all *V. cholerae* isolates were negative for the gene for CTX (*ctx*), studied by PCR.

With respect to Europe, Castello et al. (2022) studied, in 603 samples of seafood in Sicily (Italy), the presence of *Vibrio* spp., as well as the pathogenicity and antimicrobial resistance of the strains. 165 isolates were made, of which 12 (7.3 %) corresponded to *V. cholerae*, all of them different from serogroups O1 and O139, not carrying any virulence genes. Low prevalences of *V. cholerae* have also been observed in seafood from other regions of Italy. In particular, a study performed by Passalacqua et al. (2016) revealed a prevalence of *V. cholerae* in clams (*Ruditapes philippinarum*) of 0 % and 3 % in Emilia Romagna and Sardinia, respectively. For their part, Normanno et al. (2006) obtained a prevalence of 0.3 % for *V. cholerae* in mussels (*Mytilus galloprovincialis*) sold in Puglia. Another research study showed a higher prevalence than indicated regarding the presence of non-toxigenic *V. cholerae* in prawns (17 %) and mussels (9 %) collected from the Italian coast (Ottaviani et al., 2009).

In a recent study, the prevalence of *Vibrio* spp. in the seafood retail trade in Berlin (Germany) was determined (Vu et al., 2018). The presence of the microorganism was investigated in a total of 160 samples of raw seafood from different retail establishments, consisting of prawns (n= 80) and bivalve molluscs (n= 80). Using the ISO/TS 21872 method and a multiplex PCR, the overall prevalence of *Vibrio* spp. in retail seafood was 55 %, and the prevalence of *V. cholerae* was 6.3 %. Isolates of *V. cholerae* (n= 27) lacked the *ctxA* gene. This prevalence of *Vibrio* spp. is similar to that observed in other studies carried out in France (34.7 %) (Robert-Pillot et al., 2014) and Mexico in 2012 and 2013 (44.3 %) (Franco-Monsreal et al., 2015). According to data reported in the latest European zoonosis report One Health in 2022, the Netherlands tested 185 individual samples and 327 batches of raw fish and crustaceans, such as shrimp, collected at border control posts to verify the presence of *Vibrio*

spp. Non-toxigenic *V. cholerae* was detected in 11 batches, and *V. parahaemolyticus* was detected in 18 individual samples and in 1 batch, for a total of 30 (5.9 %) positive results (EFSA/ECDC, 2023).

In addition to fishery products, *V. cholerae* has been detected in other foods. In these cases, contamination occurs, mainly, when they are processed in unhygienic environments or by contact with infected handlers. Despite their low prevalence, some foods (rice, millet porridge and contaminated vegetables) have also been implicated in epidemic outbreaks (Budiman et al., 2022). Meat and dairy products also have a certain potential to transmit cholera (Clemens et al., 2017).

3. Alerts and outbreaks of foodborne toxi-infections associated with *V. cholerae* in fishery products in the European Union and worldwide

In recent years, there has been a significant increase in the number of outbreaks and food alerts by pathogenic species of *Vibrio* due to the consumption of fishery products, so it is necessary to implement effective guidelines and management strategies for their control.

Previous studies indicated that the intake of a high number of viable cells was necessary for the pathogen to survive the pH of the gastrointestinal tract and cause infection in the host. With the increase in pathogenic serogroups and strains, it is possible that the infective doses may be lower depending on their virulence and the host's immune system.

Outbreaks caused by cholera mainly affect developing countries, and are due to the intake of water and food contaminated with faecal matter. Epidemics caused by cholera are recurrent in various countries in Africa (McAteer et al., 2018) (Sinyange et al., 2018). Previously, in 2010, more than 23 cases of cholera were also reported in the United States caused by consumption of imported fish (Newton et al., 2011). All of these outbreaks have been associated with *V. cholerae* belonging to serogroup O1. The *ctxB* allelic variants of *V. cholerae* O1 Ogawa have been related to various outbreaks in India (Jain et al., 2011) (Bhusan Pal et al., 2021). In 2018, an outbreak of *V. cholerae* O1 (Ogawa serotype) was reported with 74 cases due to contaminated water intake (EFSA/ECDC, 2019). Finally, outbreaks have been reported in recent years, such as in Haiti, where in 2023 more than 20 000 cases were reported with a hospitalisation rate of 79 % and a mortality rate of 3 % (Ocasio et al., 2023).

On the other hand, *V. cholerae* strains that do not belong to serogroups O1 and O139 (known as non-O1/non-O139) can cause infection producing sporadic cases of diarrhoea, or bacteraemia, although the symptomatology is generally milder than that caused by *ctx* positive strains. However, outbreaks of gastroenteritis caused by non-O1/non-O139 strains have been reported due to the acquisition of virulence factors (T3SS/T6SS) and antibiotic resistance genes (Arteaga et al., 2020). Other outbreaks reported by non-O1/non-O139 *V. cholerae* species occurred in the United States, in 2011, (*V. cholerae* O75) with 10 confirmed cases related to the consumption of contaminated oysters (Onifade et al., 2011). Other recorded cases associated with non-toxigenic species of *V. cholerae* occurred between 2014 and 2018 in Norway, Finland, Poland, Sweden, Denmark and Estonia, and were attributed to an increase in temperatures in that period. In particular, 100 cases were reported, although most of them (67 %) were not serious (Amato et al., 2022). However, in other European countries such as Austria, despite having reported cases associated with non-toxigenic *V. cholerae*

due to contact with contaminated water, there does not seem to be a clear correlation between this and global warming (Rehm et al., 2023).

According to the data present in the RASFF portal (Rapid Alert System for Food and Feed), 43 alerts have been notified, between the years 2020-2024, due to the presence of *V. cholerae* strains in imported fishery products (RASFF, 2024), being notified mainly in Spain and, to a lesser extent, in other countries such as Denmark, Sweden, Norway or Romania. Detection in frozen product shows that the pathogen can survive this treatment and produce toxin if environmental conditions allow its subsequent growth.

However, outbreaks associated with *V. cholerae* in the European Union are highly sporadic. According to the information reported in the latest zoonosis report (EFSA/ECDC, 2023), in 2022 an outbreak was reported in the European Union associated with a non-toxigenic strain of *V. cholerae*, with 4 cases in total and one person hospitalised. The source of contamination was attributed to the intake of ready meals.

Finally, the cases reported in Europe in the 2018-2022 period (EFSA/ECDC, 2023) show an increase in cases reported in 2022 (26), in line with those reported in 2018 and 2019. Likewise, in 2022, about 20 % of them needed hospitalisation (5) while 82.6 % were associated with travel-related cases. In relation to the distribution of cases by age groups, most of them took place among people between 25 and 64 years of age, with more than 50 % of the total notifications.

4. Microbiological criteria applied in the European Union for *V. cholerae* in fishery products

At the date of preparation of this report, European legislation lacks harmonised microbiological criteria for the monitoring of *Vibrio* spp. contamination in fishery products.

In some countries, a distinction is made between ready-to-eat cooked foods and those that are not, mainly due to the greater risk associated with the consumption of cooked prawns in relation to raw products, since the latter are subjected to heat treatment. In some cases, the presence of *ctx* positive strains of *V. cholerae* is taken into account but, in other cases, this criterion is not taken into account. Other criteria that are applied are based on considering the most important *Vibrio* spp. related to raw and processed fishery products, and do not take into account the pathogenicity factors when carrying out the tests. If no pathogenicity factors are considered and a batch is positive, Article 14 on food safety requirements of Regulation (EC) No. 178/2002 (EU, 2002) applies. Through this Regulation, the competent authorities are empowered to take appropriate measures to impose restrictions on its marketing or demand its withdrawal from the market when there are indications that the food is not safe. Finally, it should be noted that there are countries that do not consider the rejection of shipments appropriate only on the basis of Article 14 of Regulation (EC) No. 178/2002 (EU, 2002).

In 2010, the *Codex Alimentarius* published general guidelines for the control of pathogenic *Vibrio* spp. in seafood (CAC/GL 73-2010) (Codex Alimentarius, 2010). However, they did not provide definitive microbiological criteria, although it did indicate the need to improve microbiological approaches in this section. As indicated in the introduction to this report, Regulation (EC) No. 2073/2005

(EU, 2005) establishes microbiological criteria for food products produced and marketed in Europe, but does not include specific microbiological criteria for *Vibrio* spp.

In the case of Spain, a zero tolerance criterion (absence in 25 g) is applied at border control posts for *V. cholerae* without distinction between serogroups, following the recommendation of AESAN in its Report of the Scientific Committee on the applicable microbiological criteria for pathogenic species of the genus *Vibrio* in imported fishery products, as additional control measures at border inspection points (AESAN, 2010). In the case of non-O1/non-O139 serogroups, it is recommended to identify the products that may present a greater risk to the consumer and adopt effective surveillance measures over those that could be isolated from environmental, clinical or food samples. In addition, this report assesses the importance that species of this genus can have in the contamination of fishery products and how, despite the low risk that exists in our country, it is necessary for the health authorities to maintain a strict control.

For their part, the Italian guidelines related to Regulations (EC) No. 882/2004 (EU, 2004a) and 854/2004 (EU, 2004b) specify *V. cholerae* (O1, O139, non-O1/non-O139) and *V. parahaemolyticus* as hazards to be controlled in fishery products during official controls. Furthermore, their guidelines mention suitable methods for the detection of potentially enteropathogenic *Vibrio* spp. (Rahman et al., 2023). Although Regulation (EU) 2017/625 (EU, 2017) repeals the previous regulations (Regulations (EC) No. 882/2004 (EU, 2004a) and 854/2004 (EU, 2004b)), the guidelines remain valid in Italy according to note No. 0069887/2019 (Ministero della Salute, 2019).

In accordance with Opinion No. 011/2022 of the *Bundesinstitut für Risikobewertung* (BfR, 2022), and given that, at present, there is no European Union regulation that establishes microbiological limit values for *Vibrio* spp. in fishery products, it establishes applying the following recommendations to foods, both raw and ready-to-eat cooked:

- Absence of pandemic strains of *V. cholerae* O1, O139 with CTX (carriers of the *ctx* gene), and positive *ctx* strains from other serogroups.
- Absence of *V. vulnificus.*
- Absence of toxin-forming strains of *V. parahaemolyticus* (*tdh+*, *trh+*).

The Technical Instruction DGAL/SDSSA/2024-73 of the *Ministère de L´Agriculture et de la Souveraineté Alimentaire* of France (MASA, 2024) aims to define the criteria to judge the conformity of a batch of fishery products or live bivalve molluscs that are contaminated with *Vibrio* spp. in official controls. This update of the information contained in the previous Technical Instruction DGAL/SDSSA/2023-117 (MASA, 2023), regulates, in particular, the possibility of performing heat treatments for certain batches of contaminated fishery products. Among others, the Instruction applies to imports, in the particular case of reinforced controls (IOC, *contrôles renforcés a l'import*) related to the search for *V. cholerae* in frozen prawns. These reinforced controls are validated by the European Commission and batches must be sent to customs pending the result of the analysis. Table 1 indicates the *Vibrio* spp. species considered pathogenic that may give rise to a non-compliant result, within the framework of an official control, i.e. carried out within the framework of a surveillance plan, reinforced control or after a food outbreak.

Table 1. *Vibrio* spp. considered pathogenic that may give rise to a non-compliant result, within the framework of an official control

Pathogenic *Vibrio* spp.	Food matrix	Sampling plan	Limit	Method
Vibrio cholerae O1 or O139 *Vibrio cholerae* non-O1/non-O139 but carrying the *ctx* gene coding for cholera toxin	Fishery products and live bivalve molluscs	n= 5 (*) c= 0	Absence in 25 g of meat and intravalvular liquid for bivalve molluscs [a] * or Absence in 25 g of meat for fishery products [b] *	Standard NF EN ISO 21872-1 in force
Vibrio parahaemolyticus carrying, at least, one of the hemolysin genes (*tdh*+ or *trh*+ or *tdh*+/*trh*+)				
Vibrio vulnificus				

*In the event of collective food toxi-infection, the analysis shall be adapted to the number and size of the remaining samples.
[a]A minimum of 12 oysters; 30-40 mussels and shells; 20-30 clams.
[b]Shelling fishery products such as whole crustaceans and whole fish.
Source: (MASA, 2024).

In order for the sample to meet the requirements, none of the five sampling units, each analysed separately, must show the presence of pathogenic *Vibrio* spp. (n= 5 and c= 0).

In the particular case of reinforced import controls, the investigation only concerns *V. cholerae*, where any detection leads to declaring "non-compliant", regardless of the serogroup and whether the *ctx* gene is present or not.

There are other *Vibrio* spp. that are considered non-pathogenic. Consequently, batches contaminated by *V. alginolyticus, V. fluvialis*, *V. mimicus*, *V. carchariae*, *V. metschnikovii*, *V. furnissii*, *V. cincinnatiensis* or by other *Vibrio* spp. whose species are not identified may be sold.

Table 2 summarises the criteria for judging the conformity of a batch of fishery products or live bivalve molluscs contaminated by *Vibrio* spp. following official controls, in accordance with Technical Instruction DGAL/SDSSA/2024-73 (MASA, 2024).

Table 2. Criteria for judging the conformity of a batch of fishery products or live bivalve molluscs contaminated by *Vibrio* spp. in official controls

***Vibrio* spp.**		**Management measures**
Vibrio cholerae (PSCP or subsequent poisoning)	Belonging to serogroups O1 or O139	Non-compliant batch, recall Heat treatment possible, with conditions
	Non-O1/non-O139 but carrier of the *ctx* gene encoding cholera toxin	
	Non-O1/non-O139 and does not possess the *ctx* gene encoding cholera toxin	No intervention measures
Vibrio cholerae (particular case of reinforced control)	All	Non-compliant batch, recall Heat treatment possible, with conditions
Vibrio parahaemolyticus	Possess at least one of the hemolysin genes (*tdh*+ or *trh*+ or *tdh*+/*trh*+)	Non-compliant batch, recall Heat treatment possible, with conditions
	Not having the hemolysin genes (*tdh* or *trh*)	No intervention measures
Vibrio vulnificus		Non-compliant batch, recall Heat treatment possible, with conditions
Other *Vibrio* spp.		No intervention measures

PSCP: Monitoring Plans and Control Plans.
Source: (MASA, 2024).

Non-conforming batches shall be withdrawn from the market, identified as animal by-products not intended for human consumption of category 2 as soon as possible and treated as such in accordance with Article 13 of Regulation (EC) No. 1069/2009 (EU, 2009).

However, in accordance with Regulation (EC) No. 2073/2005 (Article 7, paragraph 2) (EU, 2005), a heat treatment may be accepted to use the products for human consumption, which aims to destroy the bacteria. To this end, the Technical Instruction establishes a series of conditions:

1. The products are initially raw, intended for the end consumer, and have not reached the retail establishment.
2. The operator justifies a minimum pasteurisation value (VP) of 3 in their cooking/heat treatment process.
3. The intervention file of these batches that are known to be contaminated (detection within the framework of the reinforced control) will be updated.

As part of the reinforced control, the DDPP (*Direction Départementale de la Protection des Populations*) of the destination department must accept the processing in advance, before the border inspection post delivers the batch to the kitchen establishment. The DDPP must then complete the TRACES software.

The lack of standards for *Vibrio* spp. in fishery products has been due, for a long time, to the absence of adequate and validated discriminatory methods. The basis of microbiological procedures for the detection of *Vibrio* spp. in food is UNE-EN ISO 21872-1:2017 Microbiology of the food chain. Horizontal method for the determination of *Vibrio* spp. Part 1: Detection of potentially enteropathogenic *Vibrio parahaemolyticus*, *Vibrio cholerae* and *Vibrio vulnificus* (UNE-EN ISO, 2017). The European Commission entrusted the European Committee for Standardisation (CEN) with the task of providing validation data for 15 microbiological methods that could support the development of Community legislation. The results of this validation have allowed the integration of the two existing technical specifications intended for the detection of the main *Vibrio* spp. transmitted by food, the simplification of the set of recommended biochemical identification tests and the introduction of molecular procedures that provide both species-level identification and discrimination of pathogenic strains of *V. parahaemolyticus*. The results of these validations were reflected in the revised standard UNE-EN ISO 21872-1:2017, published in July 2017 (UNE-EN ISO, 2017).

5. Risk assessment associated with *V. cholerae* serogroups in raw and ready-to-eat cooked frozen prawns

In order to understand the magnitude of the potential risk associated with the presence of *V. cholerae* in raw and ready-to-eat cooked frozen prawns, it is necessary to consider the consumption trends of these products in Spain. According to the data provided in the 2022 Report on Food Consumption in Spain (MAPA, 2023), the annual per capita consumption of shrimp and prawns was 1.95 kg, 12.5 % less than 1 year previously. However, if the distribution by type of seafood species at the end of 2022 is taken into account, it can be seen that shrimp/prawns represent more than a quarter of the market with 28.3 % of the volume, gaining relevance with respect to the previous year, when their weight was 27.9 %. Within the consumption of prawns, in proportion, the consumption of frozen product amounts to 58 %, while fresh and cooked prawns represent a consumption share of 26 % each (Mercasa, 2022).

According to the main sources of transmission, the *V. cholerae* serogroups associated with cholera come from the intake of water or seafood contaminated, mainly due to its wide dissemination in marine environments.

Previous studies have shown that there is an association between zooplankton levels in the water and the adhesion of *V. cholerae* to the exoskeleton of crustaceans (Magny et al., 2011). However, the prevalence of disease-causing *V. cholerae* species is highly variable, depending on environmental factors and handling conditions during capture. In countries with a higher incidence of cholera, there are asymptomatic carriers who can transmit the pathogen to food during handling.

In order to assess the impact of contamination by *V. cholerae*, as well as by other *Vibrio* spp., studies have been carried out for some years on Microbiological Risk Assessments (MRA) associated with fishery products and waters. In the case of contamination by pathogenic *Vibrio* spp. in seawater, the associated microbiological risk is low ($<5 \times 10^{-4}$), as demonstrated in some MRA carried out for this purpose (Dickinson et al., 2013). Infection usually occurs by the entry of the pathogen through wounds and contact with the mucous membranes.

In relation to contamination through food, since 2001, organisations such as FAO/WHO have developed MRA aimed at estimating the risk associated with species of *V. parahaemolyticus*, *V. vulnificus* and *V. cholerae* in seafood (FAO/WHO, 2005a, b, 2011, 2016, 2020). With respect to *V. cholerae* O1 and O139, MRA stands out in shrimp imported from temperate waters (FAO/WHO, 2005b). In the development of the MRA, information was collected about the conditions of capture, processing, preparation and consumption, developing qualitative and quantitative MRA. According to the results provided, in general, the risk attributed to the disease due to the intake of contaminated shrimp was considered low, with values between 0.009 and 0.9 cases/country/year. The average risk was between 2 and 9 cases of disease/10^9 servings of product. However, the lack of information prevented the distinction between raw and ready-to-eat cooked frozen products and, in addition, the emerging risk posed by the increase of other non-O1/non-O139 serogroups was not considered at that time.

Although the risk attributable to the presence of *V. cholerae* O1 and O139 in contaminated food is classified as low, in recent years there has been an increase in the detection of non-O1/non-O139 *V. cholerae* species in consignments of contaminated fishery products that have been studied in various MRA. López-Hernández et al. (2022) developed an MRA associated with the consumption of raw oyster contaminated with *V. cholerae* and *V. parahaemolyticus* in Mexico. The results obtained showed that the average risk from consuming unrefrigerated raw oyster for 10 hours and contaminated with non-O1/non-O139 *V. cholerae chxA*+ was estimated to be 99 cases/100 000 servings in summer, 1.5-6.6 times higher than that calculated for the other seasons. However, the average risk is classified as low. In contrast, the risk in oysters with 24 hours without refrigeration during the summer was 42.4 times greater than that calculated with 10 hours without refrigeration. Oh et al. (2021) developed a non-O1/non-O139 *V. cholerae* MRA associated with uncooked octopus consumption in South Korea. According to the data provided, like the previous study, the probability of contracting disease was very low, with an average value of 7.08×10^{-13}. Therefore, despite the increase in the incidence of non-O1/non-O139 serogroups, according to the available information, the risk associated with infection by *V. cholerae* due to the consumption of contaminated seafood is classified as low or very low.

Recently, a review of the advances and risk assessment tools for *V. parahaemolyticus* and *V. vulnificus* species in seafood was published (FAO/WHO, 2021). This document shows that there is growing concern about the effects caused by climate change on the spread of foodborne pathogens present in marine environments, such as *Vibrio* spp. Globally, ocean warming has significantly increased in areas suitable for the proliferation of pathogenic *Vibrio* spp. and causing diseases in humans (Watts et al., 2019). In fact, there are studies that point to a correlation between the increase in water temperature and the risk associated with emerging *Vibrio* spp. (Baker-Austin, 2013). Furthermore, *Vibrio* spp. has a relatively low doubling time compared to other bacterial species, which makes them highly sensitive to favourable environmental stimuli, such as temperatures above 15 ºC (Baker-Austin et al., 2016) (FAO/WHO, 2021). Hence, infections caused by these pathogens are now being reported in areas with little or no prior incidence, with clear implications for future risk.

In relation to the infective doses, there is great variability in the data used by the different MRA. In any case, the number of data associated with dose-response ratios of *V. cholerae* is limited. In the event that the intake occurs through contaminated water, it is estimated that a higher dose is needed to cause disease (10^3-10^6 CFU), while when it is associated with contaminated food, the infective doses are usually lower (10^2-10^4 CFU) due to the neutralising effect of stomach acid on food (Seas and Gotuzzo, 2010). Other studies suggest that the intake of a high dose (10^8 CFU) is required to cause severe cholera in healthy volunteers, while a lower dose (10^5 CFU) is sufficient when administered together with antacids to neutralize the pH of the stomach (Sack et al., 2004). These values are consistent with other dose-response models developed for *V. cholerae*, which show an increase in disease from doses higher than 10^2 CFU (QMRA Wiki, 2024) with dose estimates equivalent (N_{50}) to 6.82 x 10^3 CFU, under which 50 % of individuals would contract disease, which are similar to those reported by Watson et al. (2018). Other studies show that doses can be very variable depending on the capacity of acid production in the stomach, ranging from 10^3 to 10^8 CFU.

In the last decade, a variety of MRA approaches have been developed using satellite-based remote sensing tools to study marine systems, which have been useful for estimating the human health risk associated with *Vibrio* spp. (Grimes et al., 2014). These methods of global application have been used mainly to analyse strains of *Vibrio* non-O1/non-O139, from recreational waters and from outbreaks associated with the consumption of seafood (Semenza et al., 2017). These approaches have been successfully used to analyse environmental conditions such as temperature and salinity, which are well-established variables that can modulate the risk of *Vibrio* spp. Numerous studies, such as those focusing on bathing water and infections associated with seafood, have shown the usefulness of these methods to attribute an increased risk before and during outbreak episodes (Baker-Austin et al., 2016). It also highlights the development of the Vibrio Suitability Tool that predicts the risk associated with the presence of *Vibrio* spp. in water from the Baltic Sea according to temperature and salinity variables (ECDC, 2024). The model is based on data from non-O1/non-O139 *V. cholerae* strains and is able to estimate the geographical area where favourable environmental conditions can develop for the presence of pathogenic *Vibrio* spp.

A key risk factor for ready-to-eat cooked shrimp and prawns is microbiological contamination after the cooking process. Poor post-cooking management practices, such as using contaminated seawater or drinking water to cool cooked shrimp and prawns, can lead to *V. cholerae* contamination. Likewise, cross-contamination with contaminated raw products, followed by an abuse of storage temperature can increase the risk to the consumer due to the presence of *V. cholerae* (FSANZ, 2005) (ICMSF, 2005). As for the possibility of transmission of the pathogen through thawing water, there is to date no verifiable evidence in this regard. However, it should be noted that due to the effect of freezing on the reduction of *V. cholerae* viability and the low levels of contamination present in fishery products, the probability of its dissemination through the thawing water to other products is considered very low. Cross-contamination between foods should be avoided. In particular, in the display of fishery products on ice at sales counters in the retail trade, it should be organised in such a way that ice and iced water are not mixed or reused between separate products. Consequently, the storage and display of fishery products must be organised by using separate containers or

arranged with sufficient physical separation so that there is no contamination. The use of drinking water, as well as the monitoring of Good Hygiene Practices (GHP) and the principles of Hazard Analysis and Critical Control Points (HACCP) seem vital to minimise the risk of product contamination.

6. Mitigation of the risk associated with *V. cholerae* in food

To control cholera, reduce its incidence and reduce the number of deaths caused by this disease, it is necessary to adopt multidisciplinary criteria that involve various aspects such as (i) surveillance and knowledge of the pathogen, (ii) treatment and sanitation of drinking water and wastewater, (iii) correct processing, transport and storage of food, (iv) personal hygiene, (v) adequate treatment of the disease, and (vi) its prevention through vaccines. Some of these measures can be addressed jointly through the application of GHP and a HACCP system, which would allow the identification, throughout the entire value chain, of the practices and processes that present the greatest risks.

In the case of fishery products, the foods with the greatest capacity to transmit *V. cholerae*, the decisive points for control include the treatment of breeding water, purification treatments, adequate storage and transport at low temperature (in refrigeration or freezing) of raw and processed products, and careful hygiene of work utensils and handlers (FAO/WHO, 2005b). In this regard, some official statistics show that hand washing with soap reduces the risk of diarrhoeal diseases by half (Osei-Asare et al., 2020).

Below, the factors that influence survival are detailed, as well as the measures of prevention and control of *V. cholerae* in food.

6.1 Factors influencing the survival of *V. cholerae* in food

The optimal growth temperature of *V. cholerae* is 37 ºC. However, this pathogen can grow in the range of 10 to 43 ºC. The optimal pH for growth is 7.6, but it can develop in the range of 5.0 to 9.6. The optimal water activity (a_w) of *V. cholerae* is 0.984, although it can develop between 0.998 and 0.970. Additionally, *V. cholerae* can grow with a sodium chloride range between 0.1 and 4.0 %, with an optimal value of 0.5 % sodium chloride (Singleton et al., 1982) (Huq et al., 1984) (ICMSF, 1996).

6.2 Measures for the prevention and control of *V. cholerae*

6.2.1 Water disinfection

Chlorination is a fundamental hygiene measure for the elimination of pathogens from water. Generally, chlorine is very effective at eliminating *V. cholerae*. However, there is little data on its action in different types of water (fresh *versus* salt) or under different dosage regimens. For this reason, it is recommended to carry out more research to establish adequate chlorine doses and determine concentrations that do not affect sensory properties such as taste and smell (String et al., 2022). In aquaculture, chlorination is usually carried out in fish farming or fattening tanks, but the measure cannot be implemented in open water.

In addition to chlorination, the development of new technologies that are as or more effective and at the same time more respectful of health and the environment remains a scientific and technical challenge. In this regard, because it requires a short contact time, ultraviolet (UV) radiation is

increasingly used for water disinfection. UV radiation treatment is sufficient to inactivate the *Vibrio* spp. that are released into the water during purification (Chen et al., 2018).

6.2.2 Purification of molluscs

Purification is a common pathogen removal process that is carried out on molluscs before they are placed on the market. It consists of keeping them in germ-free water so that they "purge" the pathogenic bacteria. Key process parameters include time, temperature, salinity and current intensity (Campbell et al., 2022). Purification of *V. cholerae* has a limited effect when performed at room temperature (Eyles and Davey, 1984). At about 20 ºC, the purification of oysters contaminated with *V. vulnificus* (a species close to *V. cholerae*) requires, at least, 16 days (Kelly and Dinuzzo, 1985). The decrease in water temperature to 15 ºC increases the purification efficiency, but lower temperatures (between 5 and 10 ºC) seem to reduce it (Chae et al., 2009).

6.2.3 Storage temperature

The multiplication of *V. cholerae* in food is influenced by temperature, and both refrigeration and freezing delay its development and reduce its concentration. The bacterium, however, can remain viable with both treatments for a time that depends on the microbial load and storage conditions, as has been shown in various experiments. In shrimp homogenate inoculated with a concentration of 7.8 log CFU/g, stored at 7 ºC, a *V. cholerae* O1 survival of up to 21 days has been observed (Reilly and Hackney, 1985). In raw shrimp with an initial count of 5 log CFU/g, a survival of between 4 and 9 days was observed at 5 and 10 ºC, respectively (Pesigan et al., 1967). In freezing, a *V. cholerae* survival greater than 3 months has been verified according to temperature (ICMSF, 1996) (Waturangi et al., 2015). In fresh foods, including freshwater fish, *V. cholerae* O1 remains viable for up to 90 days at -5 ºC and up to 30 days at -25 ºC. Storage at temperatures between -12 and -20 ºC for 15 and 60 days reduced the concentration of the pathogen in cooked frozen prawns between 2 and 6 log CFU/g (Nascumento et al., 1998). The previous steps of washing and placing the product in ice can further reduce the *V. cholerae* load by 3 log CFU/g (Sumner, 2011).

6.2.4 Heat treatment

V. cholerae is a thermolabile microorganism, so heat treatment is an effective measure for its inactivation in food. It has a D value (time in minutes to destroy 90 % of the population) of 2.65 at 60 ºC (ICMSF, 1996). A treatment of 1-2 minutes at 80 ºC reduced the counts by just over 7 logarithmic cycles in peeled prawns (Nascumento et al., 1998). Torres-Vitela et al. (2000) concluded that a boiling treatment for 3 minutes was able to reduce the *V. cholerae* O1 load by 8 log CFU/g without significantly altering the colour, smell and aroma of ceviche prepared with treated fish. Pathogen reduction is important even with lower temperature treatments. Thus, heating rice, fish and meat for 20 minutes at temperatures between 50 and 60 ºC (cold pasteurisation) seems to be sufficient to eliminate *V. cholerae* O1 (Nascumento et al., 1998). The food safety agencies consider that reaching a temperature of 70 ºC for 2 minutes inside seafood is sufficient for the inactivation of *Vibrio* spp. (Wright and Schneider, 2010) (BfR, 2022).

6.2.5 Additives

Sodium metabisulphite (E 223) is authorised as a food additive in molluscs and crustaceans according to Regulation (EC) No. 1333/2008 (EU, 2008). According to Januário and Dicks (2008), the addition of metabisulfite (at 1 %) could reduce the growth of *V. cholerae* under commercial refrigeration conditions.

6.2.6 High Hydrostatic Pressures

High Hydrostatic Pressures (HHP) are a non-thermal food processing technology capable of inactivating pathogens and reducing the microbial load. Treatment of fishery products with HHP reduces microbiological risks, increases shelf life and has few harmful effects on sensory quality. *Vibrio* spp. is sensitive to HHP treatment. The effectiveness of such treatment increases combined with moderate temperatures, both heating (Ye et al., 2012) and cooling. Treatment of strains of various *Vibrio* spp. and serogroups at 250-300 MPa for 10 minutes at 25 ºC reduced the concentration of the tested strains below the detection limit (Berlin et al., 1999). On the other hand, a treatment of 150 MPa for 4 minutes combined with freezing temperatures (-2 ºC) was able to reduce the microbial load of *Vibrio* in fresh oysters by 4.7 log CFU/g (Kural and Chen, 2008). The combination of mild APH treatments (250-300 MPa for 2 minutes at 21 ºC) followed by storage under refrigeration (on ice, for 5-10 days) or frozen (-18 ºC, for 7 days), was found to be sufficient for the complete inactivation (reduction >7 log CFU/g) of *V. parahaemolyticus* (Ye et al., 2013).

6.3 Vaccines

Vaccines are useful and effective tools to prevent microbial infections. In countries where *V. cholerae* is endemic and there is a high chance of contracting the disease, mass vaccination is a good way to control cholera. Infection with *V. cholerae* also confers strong immunity, which underlines the viability of preventive vaccination (Mathebula et al., 2023). Authorised by the WHO, three different commercial brands of oral cholera vaccine are currently available (WHO, 2017). All three require two doses for full protection.

6.4 Unauthorised methods

At an early stage of development and without authorisation for use in food, we can mention experimental treatments such as the use of bacteriophages against *V. cholerae* or predatory bacteria of the genus *Bdellovibrio*. Bacterial viruses are the most abundant biological entities on the planet and the natural enemies of bacteria. Administration of active phage cocktails against *V. cholerae* could be used in fish farming tanks or ponds (Mittal et al., 2023). *Bdellovibrio* spp., on the other hand, could be used for the elimination of *V. cholerae* from water (Cao et al., 2015).

In water disinfection, photocatalysis is also investigated. This technology is based on the interaction of light and solid semiconductor nanoparticles with UV light, which generates reactive oxygen species (Wennberg et al., 2013). TiO_2 and ZnO catalysts are economical, structurally stable and non-toxic (Das et al., 2015). Due to its simplicity and cost-effectiveness, sunlight-assisted photocatalysis is also gathering attention (Chatterjee et al., 2021).

In 2005, the FDA (Food and Drug Administration) approved the use of gamma and X-rays to reduce contamination in shellfish (FDA, 2005). The application of low doses of ionising radiation (up to 10 kGy) is authorised in Spain to eliminate non-sporulated pathogenic bacteria from some foods, but not in fishery products (BOE, 2001).

Conclusions of the Scientific Committee

This report describes the main factors associated with the risk of the presence of *V. cholerae* in fishery products, including frozen prawns. The most recent data on the prevalence and outbreaks of food infection caused by pathogenic species indicate a low risk, mainly associated with sporadic cases of food intake with a high degree of contamination or poor processing and storage practices. Nevertheless, this assessment may change in the future depending on the evolution of climate conditions in the coming years, as well as improvements in data collection.

In the case of strains belonging to O1 and O139 serogroups of *V. cholerae*, as well as non-O1/non-O139 strains that carry the *ctx* gene that codes for cholera toxin, their prevalence in imported frozen prawns and other fishery products is low based on the available evidence in this regard. Likewise, it is considered that the transmission of the pathogen through contaminated food or processing water poses a low risk to the consumer, according to the estimates published in the different Microbiological Risk Assessments. However, due to the symptomatology of the disease (especially in vulnerable population) and its rapid transmission, it is recommended to continue applying an absence criterion in 25 g in these products.

On the other hand, in light of the available information, an increase in the presence of non-O1/non-O139 non-toxigenic *V. cholerae* strains has been observed, possibly due to different environmental factors associated with climate change. The pathogenicity of these emerging serogroups through the intake of contaminated food is not yet well defined. However, in the published Microbiological Risk Assessments it has been seen that the probability of contracting disease is very low. Therefore, given the low level of risk of these serogroups for the general population, it does not seem necessary, at the present time, to implement measures related to the application of microbiological criteria beyond compliance with Good Hygiene Practices throughout the production-consumption chain.

With regard to the risk to the consumer derived from the presence of *V. cholerae* in raw frozen prawns and other fishery products, the previous phases of washing and placing in ice, together with the application of a freezing process, can reduce the concentration of the pathogen. Cooking treatments at 70 ºC for 2 minutes in the centre of the product guarantee the elimination of *V. cholerae*. The risk associated with the intake of ready-to-eat cooked frozen prawns and other fishery products is related to post-cooking contamination. Since the product does not undergo any inactivation treatment after cooking and prior to consumption, it is necessary to follow Good Hygiene Practices and the principles of Hazard Analysis and Critical Control Points to reduce the risk of contamination of the product.

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