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Vibrio Cholerae as it Relates to Food and Health

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Abstract: Nutrition is a fundamental human need; therefore, food, because it provides vital nutrients, must be safe guarded. Food borne diseases are an important worldwide public health issue because of their high incidence and mortality. Cholera is a Food borne disease caused by the bacterium *Vibrio cholerae* that is contracted by ingesting raw or poorly processed foodstuff, in particular seafood. Currently, to aid in reducing cholera incidences and isolating and detecting *V. cholerae* in foodstuff, protocols and analytical methodologies have been developed for use by food processors and regulatory offices to maintain food monitoring and safety controls. The principal objective of these measures is to maintain food safety for the consumers. In the present study, Food borne diseases are described in a general manner, with a focus on cholera and the characteristics of its causal agent, *Vibrio cholerae*. Analytical methods for the phenotypic and molecular detection of *V. cholerae* in contaminated foods are also discussed.

Key words: Foodstuff, cholera, *Vibrio cholerae*, food borne diseases

Foodstuff and food borne diseases: Nutrition is considered one of man's most critical necessities. Several years ago, it was thought that food was essential to obtain the nutrients and energy required to maintain vital processes. Currently, however, the field of nutrition increasingly emphasizes an equilibrated, safe and healthy diet (Urango Marchena *et al*., 2009). According to the Codex Alimentarius, food is "any substance, whether processed, semi-processed or raw, which is intended for human consumption and includes drink, chewing gum and any substance which has been used in the manufacture, preparation or treatment of "food" but does not include cosmetics or tobacco or substances used only as drugs" (CODEX, 1999).

The evolution of the food industry and food commerce has encouraged worldwide food distribution. Therefore, biological, chemical and physical Food borne hazards can reach populations with increased susceptibility to specific Food borne diseases due to their consumer habits. Of these risks, biological hazards are a serious and growing public health problem in Mexico and in the rest of the world (Carrillo *et al*., 2011; Lopez *et al*., 2014). In the present study, we describe Food borne diseases generally, with an emphasis on cholera and the characteristics of its causative agent, *Vibrio cholerae*. In addition, we discuss the general methodologies for the phenotypic and molecular detection of *V. cholera*, as a means of preventing cholera disease due to ingestion of contaminated food.

Food borne diseases are defined as disorders caused by the ingestion of chemically or microbiologically contaminated food or water. Contamination may be due to a problem that occurs during the processing, handling, preservation, transport, distribution or commercialization of food or water. Such diseases are a major cause of morbidity and mortality all over the world because of their increasing occurrence, the existence of new transmission vectors, the appearance of new vulnerable groups, the antimicrobial resistance of some pathogens and the socioeconomic impact of health costs and food production. The occurrence of Food borne diseases is a sign of the sanitary quality of foodstuff (Flores and Herrera, 2005; Luz-Zamudio *et al*., 2011). Food borne diseases can be classified etiologically as follows: (1) infections originating from the ingestion of food contaminated with live microorganisms that results in bacterial invasion and multiplication, leading to alterations in the host tissue caused by the bacteria in the foodstuff (examples include

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Salmonella spp., *Shigella* spp. and *Escherichia coli*) and (2) food intoxication, not including food hypersensitivity, caused by microbial metabolites produced in animal or plant tissues by bacteria such as *Staphylococcus aureus, Clostridium perfringens* or *Bacillus cereus* (Kopper *et al*., 2009; Luz-Zamudio *et al*., 2011). Other Food borne diseases are caused by a combination of intoxication and infection, known as toxi-infections; these diseases are caused by ingesting a non-invasive pathogenic microorganism capable of producing toxic compounds in the host, as is the case for the toxin produced by *Vibrio cholerae* and *Vibrio parahaemolyticus* (Kopper *et al*., 2009).

Microbiological agents, such as viruses, bacteria and parasites, are responsible for most of the foodborne diseases in underdeveloped countries, with a wide diversity of bacteria (Argilagos *et al*., 2010). A few examples of these bacteria include the following: *Salmonella* spp., *Listeria monocytogenes*, *Yersinia enterocolitica*, *Campylobacter jejuni*, *Escherichia coli*, *Bacillus cereus*, *Staphylococcus aureus*, *Clostridium perfringens* and species of the genus Vibrio cholerae O1 including, vulnificus, parahaemolyticus and cholerae No O1. Many factors are implicated in the outbreak of foodborne diseases, including a growing and more mobile population, new food consumer habits, conditions of culinary elaboration, the feeding of livestock with contaminated foodstuffs, handling during elaboration processes, storage and conservation (Tamara *et al*., 2008). Detection, investigation and control of foodborne disease outbreaks are some of the key challenges facing the public health system, as this requires obtaining medical and food lab information for the raw materials or even for the hands of workers implicated in the food handling (Flores and Herrera, 2005).

Vibrio: The Vibrio genus is classified within the family *Vibrionaceae*, as well as *Aeromonas*, *Plesiomonas* and *Photobacterium*. It is characterized as Gram negative and either aerobic or facultative anaerobic and contains motile bacilli while lacking a capsule and spore-forming abilities. These bacilli are glucose fermenters and the majority of them do not produce gas. Their nutritional requirements are limited and some species are halophilic, with NaCl requirements of at least 0.5%. Of the 36 recognized species, 12 are potential human pathogens (*V. cholerae*, *V. mimicus*, *V. metschnikovii*, *V. cincinnatiensis*, *V. hollisae*, *V. damsela*, *V. fluvialis*, *V. furnissii*, *V. alginolyticus*, *V. parahaemolyticus*, *V. vulnificus* and *V. carcharias*), which can be differentiated using various biochemical assays (Table 1) (Crespo Casal, 2002; Rojas *et al*., 2006; Romero, 2007). Some of these pathogenic agents are foodborne disease producers and have been classified by some researchers as food risk bacterial groups according to their health risk. Therefore, some members of this genus, namely Vibrio cholerae O1 and *V. vulnificus*, are classified as group I (severe food risk), whereas *V. cholerae* No O1and*V. parahaemolyticus* are classified as group III (moderate food risk) (Tamara *et al*., 2008).

Vibrio are found in marine and aquatic habitats in association with multiple vertebrate and invertebrate species (fish, shellfish and zooplankton) that inhabit those ecosystems. Non-halophilic species can also be isolated from the water of lakes and rivers. Human beings are incidental hosts that can contaminate water and food resources (Crespo Casal, 2002). Marine foodstuffs, such as bivalve mollusks, are highly perishable and are easily infected with pathogenic microorganisms; because of their ability to filter lots of water, they also bio-accumulate toxic substances and microorganisms present in their environment. Examples of such organisms include V*ibrionaceae* family members, such as *Vibrio cholerae*, Vibrio parahaemolyticus and Vibrio vulnificus, whose contamination is a worldwide public health problem due to the consumption of raw or poorly processed fish and shellfish (Lopez-Hernandez *et al*., 2014).

Vibrio cholerae: *V. cholerae*is the most pathogenic species within its genus and is responsible for cholera epidemic breakouts and pandemics. It is a 0.5-1.0 μm, motile, uniflagellate, oxidase-positive, Gram-negative bacilli with other biochemical characteristics that differentiate it from *Aeromonas*, *Pseudomonas* and other *Enterobacteriaceae* (Table 2). Its antigenic structure is similar to that of *Enterobacteriaceae*, with an H flagellar antigen and an O somatic antigen. Depending on which O antigens present, *V. cholerae* can be classified into one of four groups: O1, O2, O3 or O139. *V. cholerae* O1 strains are further classified into three serotypes: Inaba, Ogawa or Hikojima. According to their phenotypic characteristics, metabolic properties and susceptibility to bacteriophages and antimicrobial substances, they can be classified into two biotypes: "classical or El Tor" (Crespo Casal, 2002; Rodriguez Solis *et al*., 2001; Romero, 2007).

The *V. cholerae* biotype "El Tor" genome has been sequenced and is composed of two circular chromosomes with 2,961,146 base pairs on chromosome 1 and 1,072,314 base pairs on chromosome 2. Most of the genes code for proteins involved in the replication and repair of DNA, transcription, translation and cell wall biosynthesis, as well as catabolism and anabolism. Moreover, most of the genes that code for proteins involved in pathogenicity, such as the synthesis of cholera toxin, the toxin co-regulated pilus, lipopolysaccharides and the extracellular protein secretion machinery, are situated on chromosome 1. Chromosome 2, on the other hand, contains approximately 59% open reading frames encoding hypothetical proteins and proteins of unknown function (Fernandez and Alonso, 2009).

The natural reservoir for Vibrio is aquatic ecosystems (wells, rivers, estuaries and the ocean), where they live in

Table 1: Biochemical characteristics of different *Vibrionaceae* found in seafood (Kaysner and DePaola, 2001, 2004)

Analysis	*V. alginolyticus*	*V. cholerae*	*V. fluvialis*	*V. furnissii*	*V. hollisae*	*V. metschnikovii*	*V. mimicus*	*V. parahaemolyticus*	*V. vulnificus*
AgarTCBS	A	A	A	A	NC	A	V	V	V
Agar mCPC	NC	M	NC	NC	NC	NC	NC	NC	A
Oxidase	+	+	+	+	+	-	+	+	+
Arginine dihydrolase	-	-	+	+	-	+	-	-	-
Lysine decarboxylase	+	+	-	-	-	+	+	+	+
Growth in NaCl (w/v):									
0%	-	+	-	-	-	-	+	-	-
3%	+	+	+	+	+	+	+	+	+
6%	+	-	+	+	+	+	-	+	+
8%	+	-	V	+	-	V	-	+	-
10%	+	-	-	-	-	-	-	-	-
Growth at42°C	+	+	V	-	Nd	V	+	+	+
Formation of acid from:									
Saccharose	+	+	+	+	-	+	-	-	-
D-cellobiose	-	-	+	-	-	-	-	V	+
Lactose	-	-	-	-	-	-	-	-	+
Arabinose	-	-	+	+	+	-	-	+	-
D-Mannose	-	+	+	+	+	+	+	+	+
D-Mannitol	+	+	+	+	-	+	+	+	V
Voges-Proskauer	+	V	-	-	-	+	-	-	-
Gelatinase	+	+	+	+	-	+	+	+	+
Urease	-	-	-	-	-	-	-	V	-

A: Yellow colonies, V: Green colonies, M: Purple colonies, NC: Poor or absent growth, Nd: Not determined, V: Variable, TCBS: Thiosulfate-Citrate-Bile Salts-Sucrose, mCPC: Modified Cellobiose-Polymyxin B-Colistin Agar

Table 2: Differentiation and identification of *V. cholerae* from *Aeromonas, Pseudomonas* and other *Enterobacteriaceae* based on biochemical characteristics (Kaysner and De Paola, 2004; Rojas *et al*., 2006; CDCa,b)

Analysis	% positive
Gram-negative staining, unsporulated	100
String mucoid	100
Oxidase	100
Kligleriron agar	K/A no gas, no H_2S
Triple sugar iron agar	A/A no gas, no H_2S
Glucose[a] (acid generation)	100
Glucose (gas production)	0
Sucrose (acid production)	100
Lysine[a]	99
Arginine[a]	0
Ornithine[a]	99
Growth in 0% NaCl[b]	100
Growth in 1% NaCl[b]	100
Voges-Proskauer[c]	75
Nitrate to Nitrite	99
Methyl red	99
Indole production	99

K/A: Alkalinity/acidity, A/A: Acidity/acidity, a: Modified by addition of 1% NaCl; b: Base nutrient broth, c: The isolates of serotype O1 *V. cholerae* biotype "El Tor" are Voges-Proskauer positive, whereas the "classical" biotype strains are negative

a non-pathogenic, viable but non-culturable (VBNC) state due to the pH, salinity and non-favorable temperature; however, they are able to recover their pathogenic potential and produce cholera toxin when conditions change (Romero, 2007; Senderovich *et al*., 2010). Several studies have shown that Vibrio can proliferate when associated with eukaryotic organisms such as copepods (crustacean), Chironomidae (insects, Diptera) and fish (*V. cholerae* is a commensal organism in the gut of fish where it produces proteases and chitinases that help fish to digest their prey). These organisms serve as a vector and reservoir for intermediate hosts and this has led to the hypothesis that fish, copepods (crustaceans) and chironomids, which are eaten by and dispersed by migratory waterfowl, result in the global distribution of bacteria between different bodies of water (Senderovich *et al*., 2010). Cholera outbreaks have been associated with the ingestion of raw or poorly processed crab, fish and shellfish. Moreover, transitional carriers and chronically ill patients, considered to be natural reservoirs, can transmit *V. cholerae* via the fecal-oral route through contaminated food or water (Romero, 2007).

Pathogenic bacteria present different factors that favor the establishment of virulence factors that cause cholera. These factors can be extracellular proteins, as is the case for *V. cholerae* O1 and O139. One of these proteins is the cholera toxin (CT), which is not heat-resistant and acts within the small intestines to cause a massive secretion of fluids into the intestinal lumen. Cholera toxin is composed of two covalently bonded polypeptides (subunits A and B); subunit B binds to host cellular receptors allowing subunit A to cross the cell membrane and antagonize the cell (Madigan *et al*., 2004; DGE, 2012). Subunit A has a MW of 27,200, whereas subunit B has a MW of 11,600 and multimerizes into a pentamer. Subunit B has a conserved site that binds specifically with its receptor, the GM1 ganglioside, in the epithelial cytoplasmic membrane. This binding allows subunit A to enter the cell and activate adenylate cyclase, which promotes the conversion of intracellular ATP to 3',5'-cyclic AMP (cAMP), thus altering the intracellular transport of ions, resulting in diarrhea. The increase in cAMP causes the active secretion of water, chloride ions and bicarbonate ions from mucosa cells into the lumen of the small intestines, generating a massive loss of fluid and extreme dehydration, which can lead to death (Madigan *et al*., 2004; DGE, 2012). Cholera toxin is encoded by the ctxA and ctxB genes, whose expression is controlled by ToxR, a trans-membrane protein that regulates not only the production of toxin but also other virulence factors such as the toxin co-regulated pilus (TCP),outer membrane proteins and pili required for adherence and colonization of the small intestines, a regulator protein (ToxR) and cytotoxins (RTX), as well as hemolysin, haemagglutinin and neuraminidase, which are encoded by the genes hlyA, mshA, hapA and nanH, respectively (Madigan *et al*., 2004; Crespo Casal, 2002; Fernandez and Alonso, 2009; ANLIS, 2010).

Additionally, there are other *Vibrio cholerae* strains capable of producing toxins, such as Zot (occludens zone toxin), that break cellular mucosa bonding (occludens zone) and maintain the integrity of the membrane, resulting in the drainage of the luminal content and altered ionic equilibrium, which causes diarrheal episodes (Rodriguez Solis *et al*., 2001; Crespo Casal, 2002; ANLIS, 2010; DGE, 2012).

Cholera: Cholera is an acute diarrheal infectious disease caused by the ingestion of *Vibrio cholerae* via contaminated food and/or water (OMS, 2015). Toxigenic species of *V. cholerae* O1 and O139 give rise to epidemic and pandemic outbreaks and the severity of the clinical symptoms depends on the species serotype and biotype, the infectious dose and the subject (Crespo Casal, 2002; Senderovich *et al*., 2010; OMS, 2015). Although *V. cholerae* No O1/No O139 strains have not been linked with epidemic cholera events, they have been associated with gastroenteritis non-sepsis outbreaks with no fatal consequences. The symptoms of cholera are vomiting, a rice water-like diarrhea, fluid leakage reaching rates of 1 L/h in adults and 300 mL/kg in children, muscle spasms, abdominal pain and low intensity fever (Romero, 2007; Senderovich *et al*., 2010). The treatment of this illness is based on the following: (a) prevention and treatment of dehydration; (b) the application of intravenous fluids in the most severe cases; (c) early and appropriate feeding during the acute diarrhea phase; (d) breast feeding; (e) use of antidiarrheal medication and (f) antibiotic use, where appropriate, typically tetracycline and doxycycline (Romero, 2007). This treatment should be considered an appropriate use of antibiotics, despite the fact that such strains have not been observed in the clinic. Some *V. cholerae* isolates have been identified with multiple resistances to antibiotics due to transfer genetic elements such as plasmids and integrons (Fernandez and Alonso, 2009). According to the World Health Organization (WHO), numerous cases of cholera have been reported worldwide. A total of 117,570 cases were reported in Africa in 2012, with a death rate of 1.7%. In Asia, 7,367 cases were reported, with a death rate of 0.4%, but in Europe and Oceania, only 18 and 5 cases, respectively, with non-fatal consequences were reported. In the Americas, 120,433 cases were reported, with a 0.8% mortality rate (WHO, 2013). In 2012, the countries within the American continent with the highest number of cases and death rates were the Dominican Republic (7,919 cases and 68 deaths), Haiti (112,076 cases and 894 deaths) and Cuba (417 cases and 3 deaths). Mexico reported only 2 cases, with no fatal consequences (WHO, 2013).

Cholera is considered a public health problem in Mexico and primarily affects the infant population, though its incidence and prevalence correlates with the patient's socioeconomic status. According to data from the National Council for Evaluation of Social Development Policy in Mexico (Coneval), the states with a low socioeconomic

status are Chiapas, Oaxaca, Guerrero, Puebla, Tabasco, Durango, Yucatán, Campeche, Veracruz, Hidalgo, San Luis Potosí, Guanajuato and Michoacán. These data could explain why gastrointestinal diseases, cholera included, are endemic in certain regions and why cholera prevention is failing (Cortez *et al*., 2011). A study performed by Giono *et al*. (1995) characterized 26,922 *V. cholerae* strains isolated in Mexico between 1991 and 1993 to determine biotype and serotype. They found that 100% of the "El Tor" biotypes were sensitive to most antibiotics except for furazolidone, streptomycin and sulfisoxazole. Additionally, a dramatic change in the serotype was observed in this time frame. In 1991, 99.5% of the strains were Inaba serotypes; however, in 1992, 95% of the strains were Ogawa toxigenic serotypes. These findings correlate with the observation that on the American continent, the *V. cholerae "*El Tor" biotype prevails. Interestingly, the Inaba serotype is frequently present at the beginning of a pandemic and is late replaced by the Ogawa serotype, as has been observed in both Mexico and Latin America (Cortez *et al*., 2011).

The last outbreak in Mexico was documented in September 2013,when the Mexico Liaison National Center for International Health Regulations reported to the WHO an outbreak of 10 confirmed cases of autochthonous toxigenic Ogawa *Vibrio cholerae* O1 infections. In October of the same year, 171 cases, one resulting in death, were reported in the states of Hidalgo, Mexico, Distrito Federal, Veracruz and San Luis Potosí and it was established that river water was the cause of the contamination. Mexico's health authorities reinforced epidemiological monitoring activities, ensuring access to sufficient potable water and basic sanitization at the community level. Genetic studies linked this strain with the strain circulating in Asia, which was imported to Haiti in 2010 and characterized it based on its virulence and antibiotic resistance (MSSSI, 2013).

Microbiological analysis of *V. cholerae* in the laboratory

One of the key objectives of a microbiology laboratory is to use precise methods to detect, isolate and identify microorganisms related to clinically relevant infections. Currently, microbial identification is performed using traditional methods, as these are more accessible and cost efficient because they are based on phenotypical characteristics (observable characteristics such as morphology, development and biochemical and metabolic properties). When a culture is viable, it is possible to isolate the microorganism, identify it, determine its antimicrobial sensitivity and characterize its epidemiological markers. In practice, phenotypical methods have some limitations, specifically when identifying certain microorganisms. Genotypic or molecular methods can reduce some of these limitations, as they reduce analysis time, are more sensitive and do not require culturing, allowing for the analysis of viable, but not culturable, microorganisms. Genotypic methods use genes as molecular signatures in taxonomic or phylogenetic studies, with 16S rRNA analysis being the initial marker and, in many cases, the most suitable marker to perform a more precise identification. This type of analysis is not universally implemented because of its high cost and need for highly specialized training; thus, it is primarily used at reference facilities. It is important to mention that phenotypic identification is culture-independent and identification methods do not provide absolute results; they only indicate the genus and/or species to which a microorganism is most likely to belong. Moreover, proteomic approaches are also used for microorganism identification, with techniques based on electrophoresis and mass spectrometry; matrix-assisted laser desorption/ionization time of flight(MALDI-TOF) spectrometry and TOF mass spectrometry are two of the most commonly used techniques. These techniques are beyond the scope of this article, although they are important to the field of microbiological diagnosis and will surely impact the future of microbial detection in future years (Fernandez *et al*., 2010; Bou *et al*., 2011; Garcia *et al*., 2012; Palomino and Gonzalez, 2014).

Within the scientific literature, diverse protocols have been described that are similar with respect to the types of media and phenotypical and molecular identification techniques reported for identification of *V. cholerae* in foodstuff, water and clinical samples. Previously reported methodologies include the Bacteriological Analytical Manual (BAM) published by Kaysner and DePaola (2004) of the U.S. Food and Drug Administration (FDA), (http://www.fda.gov/Food/FoodScienceResearch/LaboratoryMethods/ucm2006949.htm); *V. cholerae* Diagnosis Methods, edited by the Centers for Disease Control and Prevention (CDC) http://www.cdc.gov/cholera/laboratory.html (Perilla *et al*., 2004); the Manual for the Laboratory Identification and Antimicrobial Susceptibility Testing of Bacterial Pathogens of Public Health Importance in the Developing World, jointly edited by the WHO and the CDC (Caffer *et al*., 2007); the Manual de Procedimientos Aislamiento, identificación y caracterización de *Vibrio cholerae* from the Centro Regional de Referencia del WHO-PAHO Global Salm Surv para América del Sur. Instituto Nacional de Enfermedades Infecciosas ANLIS Dr.C.G.Malbrán http://repositorio.anlis.gov.ar/xmlui/handle/123456789/549 and the Detection, Isolation and Identification of *Vibrio cholerae* from the Environment (Anwar *et al*., 2012), among others.

When a sample has been collected, it must be stored and transported at 10-15°C for no more than 8 h (Anwar *et al*., 2012). *V. cholerae* can be detected and isolated from foodstuff through several traditional and/or molecular methods, such as conventional or real-time polymerase chain reaction (PCR) or a pre-enriched media (e.g., peptone-water). Traditional methods for isolation and identification have been improved using specific media that allows for identification directly from samples cultured in thiosulphate citrate bile salts sucrose (TCBS) agar,

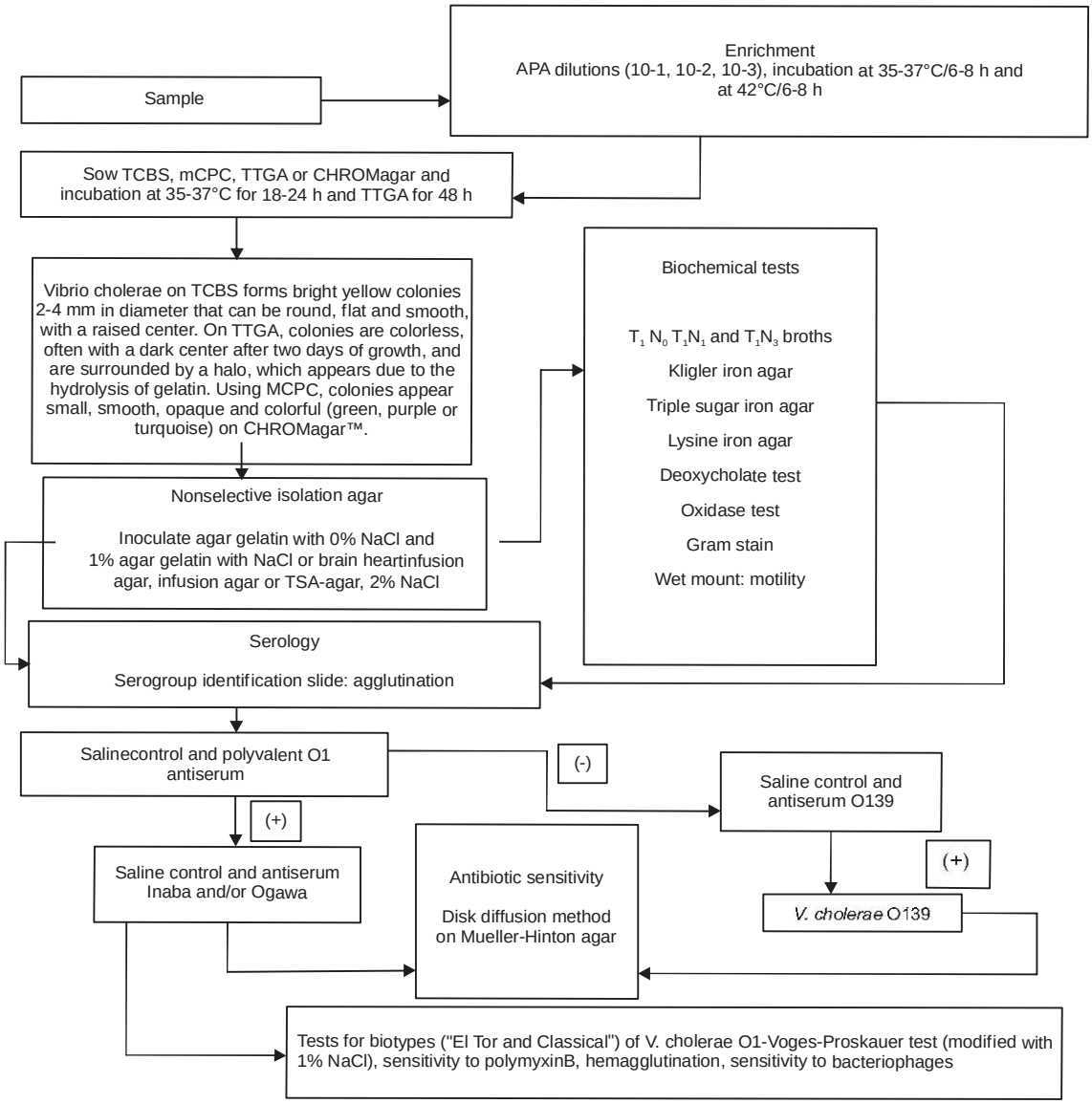
Monsur's taurocholate tellurite agar (TTGA), CHROMAgar™ or an alkaline peptone-water pre-enrichment media (highly recommended), followed by seeding on one or a combination of these agars. Cultures are then incubated prior to confirmation studies. Presumptive colonies of *V. cholerae* are confirmed by biochemical analysis and phenotypic characteristics (Table 1 and 2) or by PCR. Colonies are then sero-grouped (serology) as O1, O139 or non-O1/non-O139 by agglutination assays using antiserum for the O1 and O139 antigens orby PCR using O1 and O139 genomic-specific codification control primers. The "El Tor", or classical biotype, is determined using an antibiotic sensitivity assay, particularly for isolates in the O1 sero-group. Once *V. cholerae* strains have been isolated, they can be kept in nutrient agar with 0.5% NaCl if covered with mineral oil and stored in glycerol at -70°C. Stock cultures should be regularly propagated for isolation, which will improve viability and purity (Kaysner and DePaola, 2004; Perilla *et al*., 2004; Caffer *et al*., 2007; Anwar *et al*., 2012; CDC, 2016a; b). Figure 1 illustrates the general flux of the traditional *V. cholerae* isolation and detection process.

To detect and identify *V. cholerae*, the most commonly used molecular method is conventional or real-time polymerase chain reaction (PCR). Classical methods are sensitive and specific but require several days to complete, whereas molecular methods are faster and have a better limit of detection, specificity and sensitivity. For these reasons, molecular methods are more valuable not only for the detection of Vibrio but also for the detection of the causal agents of other Food borne diseases (Koch *et al*., 2001; Kaysner and DePaola, 2004; Anwar *et al*., 2012; Palomino and Gonzalez, 2014). PCR is a technique based on amplifying a DNA fragment in an exponential manner using sequential heat denaturation cycles and a thermo stable DNA polymerase. The amplified nucleic acid segment is specific because its ends are recognized by specifically designed synthetic oligonucleotides that bind to complementary sequences at the 5' end of each template DNA strand. Information related to the presence or absence of sequences complimentary to the oligonucleotides and the distance at which primers bind the DNA is important when considering a template (Rodriguez *et al*., 2001; Caffer *et al*., 2007; Tamay de Dios *et al*., 2013). The specificity, efficiency and accuracy of PCR are directly related to the diverse components that are found within the reaction: the reaction mixture (triphosphate deoxyribonucleosides, monovalent and divalent ions, buffers, primers and DNA template), the cycling program and the DNA polymerase (Bolivar *et al*., 2014).

The three different steps in the PCR cycling program are (1) denaturation, where DNA strands are heat-separated at 94-95°C; (2) annealing, where "primers or initiators" bind to complementary sites on the DNA sample at the optimal melting temperature, or where primers hybridize (determined by base composition), generally between 50 and 65°C and (3) polymerization or extension after hybridization of the primers occurs, typically at 72°C, performed by DNA polymerase. Finally, the number of cycles used depends on the degree of specificity and amplification required but is typically between 25 and 35 cycles (Bolivar *et al*., 2014; Caffer *et al*., 2007; Tamay de Dios *et al*., 2013).

For conventional PCR, the presence of a specific piece of genetic material is determined by electrophoresis and by the visualization of DNA under UV fluorescence after staining with ethidium bromide. The size of the DNA is determined by comparing it to a DNA ladder. Because PCR occurs quickly, it has been modified into a technique termed real-time PCR, which does not require electrophoresis. In this method, amplification and detection of genetic material are simultaneously obtained using fluorochrome intercalating agents (SYBR GREEN-I) and specific probes (TaqMan, molecular beacon probes and fluorescence resonance energy transfer (FRET)) that consist of two different types of fluorochromes, a donor and an acceptor, which perform FRET and produce fluorescence when bound to the target genetic material. Another instrument detects the reaction and generates a curve of intensity versus temperature. The curve's peak shows the melting point (termed the melting peak) at which 50% of the DNA is in the form of a double chain; the fluorescence emission produced in the reaction is proportional to the amount of formed DNA. This procedure allows one to identify and record the amplification reaction kinetics at all times. Thus, each microorganism generates a specific fusion temperature that indicates its presence (Costa, 2004; Vanegas and Rojas, 2004; Sorribes, 2008; Tamay de Dios *et al*., 2013). Real-time PCR has been useful in microbiology laboratories in recent years because it allows for the easy detection of food pathogens with minimal manipulation, reducing the interpretation of the results. Real-time PCR can be performed using instruments and reagents from BAX Q7 (Du Pont Qualicon), iQCheck (Bio-Rad), TaqMan Pathogen Detection kits (Applied Biosystems), Roche/Biotecon Diagnostics Light Cycler (Roche) and Warnex (AES Chemunex) (Sorribes, 2008).

There are two types of PCR techniques: simple PCR, which uses a pair of primers to produce one fragment and multiplex PCR, which uses more than one primer to amplify multiple target sequences. Nested and semi-nested PCR are based on the amplification of a previously amplified fragment using internal primers to increase specificity and sensitivity and RT-PCR is used to amplify RNA sequences (Caffer *et al*., 2007; Bolivar *et al*., 2014; Palomino and Gonzalez, 2014). Several studies and protocols have been published in recent years that describe the extraction and detection of *V. cholerae* DNA from foodstuff with or without pre-enrichment (pure culture) using sensitive and specific molecular techniques such as the variations of PCR discussed above. These studies and protocols focus on the detection of toxins or other



Alkaline peptone water (APA), Thiosulfate-citrate-bile salts-sucrose (TCBS) agar, Modified Cellobiose-Polymyxin B-Colistin (mCPC), Monsur's taurocholate tellurite gelatin agar (TTGA), T_1N_0 (1% tryptone and 0% NaCl), T_1N_1 (1% tryptone and 1% NaCl), T_1N_3 (tryptone 1 and 3% NaCl), Trypticase Soy Agar (TSA)

Fig. 1: General flowchart analysis for the traditional isolation and identification of *V. cholerae* from food samples (Kaysner and DePaola, 2004; Perilla *et al*., 2004; Anwar *et al*., 2012; CDC, 2016a, b)

virulence factor genes such as group antigens (ctx, tcpA, tcpI,rtxA, ompW and stn/o). Such protocols include the Detection of Enterotoxigenic *Vibrio cholerae* in Foods by the Polymerase Chain Reaction (Koch *et al*., 2001); the Bacteriological Analytical Manual(BAM)-FDA http://www.fda.gov/Food/FoodScienceResearch/LaboratoryMethods/ucm072649.htm (Kaysner and DePaola, 2004); Genotypic detection of the cholera toxin gene by polymerase chain reaction in the Bacteriological Analytical Manual (BAM)-FDA http://www.fda.gov/Food/FoodScienceResearch/LaboratoryMethods/ucm070830.htm;Protocols CDC (2016c) Centers for Disease Control and Prevention CDC Laboratory Methods for the Diagnosis of *Vibrio cholerae*, http://www.cdc.gov/cholera/laboratory.html; Manual de Procedimientos Aislamiento, identificación and caracterización de Vibrio cholera (Caffer *et al*., 2007); Centro Regional de Referencia del WHO Global Salm Surv para América del Sur http://repositorio.anlis.gov.ar/xmlui/handle/123456789/549 and Detection, Isolation and Identification of Vibrio cholerae from the Environment (Anwar *et al*., 2012).

To analyze genetic material using PCR, a sample must contain free DNA in solution (not degraded and with little or no inhibitory substances). The analysis may start from

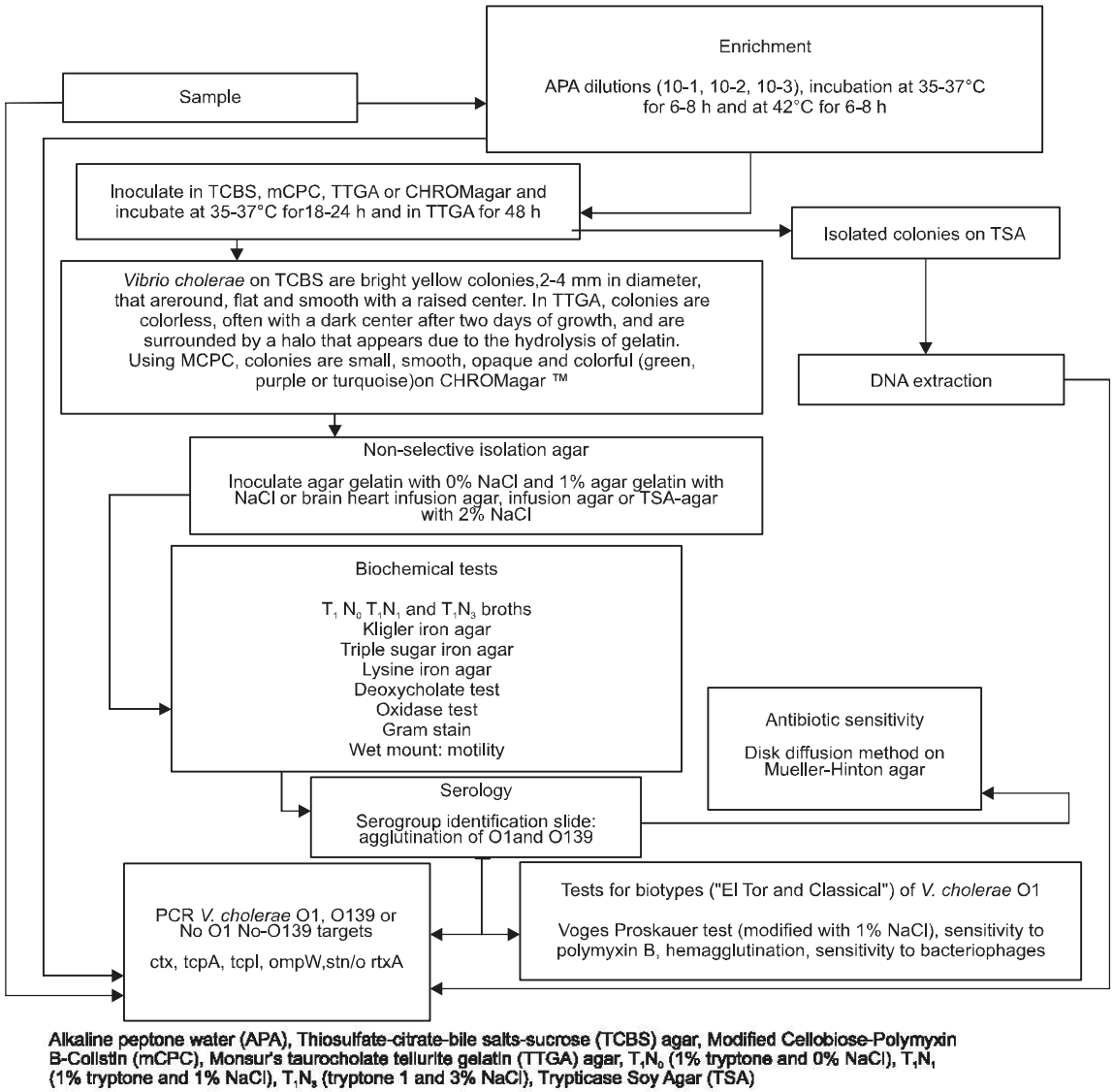


Fig. 2: Flow chart analysis for the detection, isolation and identification of *V. cholerae* from food samples by phenotypic and molecular methods (Koch *et al*., 2001; Kaysner and DePaola, 2004; Perilla *et al*., 2004; Caffer *et al*., 2007; ANLIS, 2010; Anwar *et al*., 2012; CDC, 2016a-c)

an isolated sample, from a pure culture (high DNA concentration) or from complex food samples, where the microorganism count is low and DNA purification is difficult. Therefore, if studying the microorganism is required and strains are able to be cultured, it is recommended to dilute the sample in an enrichment media (selective or not, according to the objective of the PCR study) and incubate under adequate conditions to increase the concentration of microorganisms before proceeding to cellular lysis. There are cases in which enrichment is not possible, such as when microorganisms cannot be cultured or when the study of a complex microbiota is required. In these particular cases, it is recommended to extract the DNA from the whole sample (Caffer *et al*., 2007). Figure 2 illustrates a general flow chart for conventional and molecular PCR used to detect *V. cholerae* in food samples.

It should be noted that in the case of toxins or other virulence factors, the presence of the gene does not mean the protein is being expressed; thus it is important to use biochemical and/or immunological techniques as well to detect protein expression (Mendez and Perez, 2004). In addition to factors that limit the use of PCR and other molecular methods to detect food pathogens, such as inhibitory compounds that inhibit DNA polymerase, other errors can be introduced during nucleic acid extraction and the amplification process, which can result in the underestimation of the bacterial count and lead to false

negatives (Carrillo *et al*., 2011; Palomino and Gonzalez, 2014). In addition, the early detection of hazardous genetic information is considered useful because it opens the possibility to the prevention, control and treatment of Food borne diseases caused by various pathogens (Mendez and Perez, 2004).

Conclusions: Food borne diseases are considered a serious public health problem due to their negative social and economic consequences. The incidence of Food borne diseases in the population is inversely related to food safety and hygienic quality. *Vibrio cholerae* is the causative agent of cholera, a Food borne disease related to the consumption of raw or poorly processed foodstuff, especially seafood. Cholera is considered a highly fatal illness if not treated early. To reduce the incidence of cholera and other diseases, industrial and regulatory government offices have focused on implementing methodologies for the rapid detection of pathogenic microorganisms that guarantee food safety for the health of consumers. Currently, there are traditional microbiological methods and "fast" molecular methods used in diverse laboratories all over the world to detect, isolate and identify *V. cholerae* in foodstuff. Traditional methods are commonly used with a high degree of confidence, but they can be laborious, expensive and time consuming. Additionally, they are sometimes unreliable when viable, but non-culturable, cells are used. Molecular PCR protocols have been developed to improve these processes. These protocols allow for the analysis of a higher number of samples in a shorter period of time, are easy to perform and show a high degree of sensitivity and specificity with a lower limit of detection. In spite of these benefits, these techniques are expensive and in some cases, results should be compared to those obtained by a traditional or reference-based methodology. Currently, generating and maintaining food safety is of primary importance to both food processors and government agencies due to factors including the globalization of food production and distribution, changes in disease-producing microorganisms, demographic mobility, consumer habits and new food product development. These efforts have resulted in more exhaustive regulation and supervision that is based on analytical methodologies performed to detect Food borne disease-causing microorganisms, thus reducing the incidence of Food borne illness and favoring public health by ensuring that the food supply is both nutritious and safe.

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