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Investigation of *Vibrio cholerae, V. parahaemolyticus,* and *V. vulnificus* in clandestine animal products and water effluent samples in northern Tocantins, Brazil

Pesquisa de *Vibrio cholerae, Vibrio parahaemolyticus* e *Vibrio vulnificus* em produtos de origem animal clandestinos e em amostras de efluentes hídricos no norte do Tocantins

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Highlights

565 isolates were suggestive of *Vibrio* spp. by traditional microbiology methods.
103 isolates were identified as *Vibrio* spp. by uniplex PCR.
16S rRNA sequencing identified the isolates as from other genera.

Abstract

Vibrio spp. are responsible for several diseases, including cholera, which affect public and environmental health. Waterborne and foodborne diseases can cause serious harm to the society because of their potential to infect large numbers of people in a short time. The presence or absence of pathogenic microorganisms in a wide variety of samples, whether in the food, health, or environmental domains, has become possible because of the spread of molecular biology-based approaches. Compared to conventional culture methods, these methodologies have advantages, mainly in terms of sensitivity and relative high speed of analysis. Considering the risk to public health, and given the importance of the range of diseases caused by these pathogens, the aim of this project was to identify *Vibrio cholerae, V. parahaemolyticus,* and *V. vulnificus* among bacterial isolates from clandestine animal products commercialized in northern Tocantins and from water effluents from the Lontra River in Araguaína. In total, 199 isolates from animal products and 366 from water samples were evaluated. Among them, 25 and 78 isolates from animal products and water samples were confirmed as *Vibrio* spp. using uniplex PCR, respectively, and 10 of them were sequenced. The sequencing results questioned the specificity

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of the biomolecular assays performed given that the isolates were identified as *Aeromonas* spp., *Escherichia* coli, and *Morganella* spp. Under the tested conditions, performed assays can lead to false-positive results for *Vibrio* spp.
Key words: Public health. PCR. Foodborne diseases. Food safety.

Resumo

Espécies de *Vibrio* spp. são responsáveis por diversas doenças, como a cólera, de importância em saúde pública e ambiental. Estes patógenos possuem veiculação hídrica e também podem ser transmitidas por alimentos, causando sérios danos à sociedade devido ao seu potencial de infectar um grande número de pessoas e em um curto espaço de tempo. A determinação da presença ou ausência de microrganismos patogênicos numa grande variedade de amostras, seja no domínio alimentar, sanitário ou ambiental, tornou-se possível graças à difusão de abordagens baseadas na biologia molecular. Em comparação aos métodos de cultura convencionais, estas metodologias apresentam vantagens, nomeadamente em termos de sensibilidade da análise e de rapidez relativa. Considerando o risco à saúde pública, visto a importância da amplitude das doenças causadas por estes patógenos, objetivou-se neste trabalho identificar as espécies de *Vibrio cholerae, V. parahaemolyticus* e *V. vulnificus* em isolados oriundos de produtos de origem animal clandestinos e de amostras de efluentes hídricos do rio Lontra em Araguaína, Tocantins. Foram avaliados 565 isolados sugestivos de *Vibrio* spp., dos quais, 103 isolados foram confirmados utilizando metodologia PCR-Uniplex para espécies de Vibrio spp. e, entre estes, selecionados 10 isolados para sequenciamento genético. O resultado do sequenciamento confirmou as espécies *Aeromonas* sp., *Escherichia coli* e *Morganella* sp. como espécies para os isolados utilizados. Nas condições realizadas, não foi possível estabelecer a especificidade das técnicas PCR-Uniplex e PCR-Multiplex que fossem capazes de determinar as espécies de *Vibrio* spp. estudadas.
Palavras-chave: Doenças Transmissíveis por Alimentos. PCR. Saúde pública. Segurança dos Alimentos.

Vibrio is a diverse genus of ubiquitous bacteria present in marine and estuarine aquatic environments worldwide (Baker-Austin et al., 2018). Most species are nonpathogenic, but *V. cholerae, V. parahaemolyticus,* and *V. vulnificus* are the species most commonly reported to cause diseases in humans as a result of exposure to contaminated water or consumption of contaminated raw or undercooked food (Baker-Austin et al., 2018). Cholera, a serious diarrheal disease that has been a defining part of human history, is caused by *V. cholerae,* and can be fatal if left untreated (Moura et al., 2018).

Vibrio species that do not cause cholera, such as *V. parahaemolyticus* and *V. vulnificus,* can cause vibriosis, a group of diseases with adverse symptoms that pose a risk to the life of individuals with underlying diseases (Baker-Austin et al., 2018). The Centers for Disease Control and Prevention (CDC) estimates that vibriosis affects approximately 80,000 people and causes approximately 100 deaths annually (Centers for Disease Control and Prevention [CDC], 2023). In Brazil, there is no active surveillance system for *Vibrio* spp., and few studies conducted in the country have evaluated the pathogenic potential

of these microorganisms using molecular methodologies, mainly focusing on the isolation and identification of bacterial strains (Martins et al., 2021).

In 2020, the RDC 12/2001, a Brazilian law that established microbiological standards for food, suggested that ready-to-eat foods made from raw fish and comparable foods should be tested for the presence of *V. parahaemolyticus.* However, this law is no longer in force and no microbiological standards for pathogenic Vibrio spp. exist in the country (Resolução RDC nº 12, 2001).

According to Oliva et al. (2016), the existence of virulence genes in strains isolated from water bodies suggests that these environments serve as reservoirs of potentially virulent strains that can be harmful to human health. The spread of virulence genes endangers public health and can reduce the effectiveness of the antibiotics currently used to treat infections caused by *Vibrio* spp.

The high diversity of *Vibrio* spp. makes it difficult to accurately identify less frequently isolated species; therefore, phenotypic and biochemical tests are often insufficient for reliable discrimination at the species level (Canellas & Laport, 2021).

Since the consumption of food and exposure to water contaminated by *Vibrio* spp. may pose a risk to public health, this study verified the presence of pathogenic *Vibrio* spp. in samples of illegal animal products and water effluents from a river in the northern region of the state of Tocantins, northern Brazil, using biomolecular tools to identify microorganisms, meeting the demand for the introduction of new methodologies for the investigation of these microorganisms in the northern region of the country, and contributing to a better understanding of the diversity and evolution of bacteria of this genus.

A total of 565 isolates suggestive of *Vibrio* spp. were obtained from food from animal products (FAP), including ground beef, fresh pork sausage, shrimp, free-range chicken, and fish fillet samples, collected from street stalls and from water samples from the Lontra River, city of Araguaína, in the northern region of the state of Tocantins, between March 2019 and December 2020. These isolates were obtained in partnership with other research projects of the Postgraduate Program in Animal Health and Public Health in the Tropics of the Federal University of Northern Tocantins (UFNT).

The FAP samples were subjected to qualitative analysis for *Vibrio* spp., according to the methods recommended by the American Public Health Association (APHA), as described in the 5th edition of the Compendium of Methods for the Microbiological Examination of Foods (Silva et al., 2017). In addition, a molecular assay for confirming the identity of suggestive isolates obtained on thiosulfate-citrate-bile salts-sucrose (TCBS) agar plates was included. Analyses were performed at the Food Microbiology Laboratory of the UFNT.

The 199 isolates obtained from FAP samples that had been identified as *Vibrio* spp. by multiplex polymerase chain reaction (PCR) were then analyzed by uniplex PCR to individually identify those that corresponded to *V. cholerae, V. parahaemolyticus,* and *V. vulnificus,* as described by Izumiya et al. (2014).

Between September 2021 and June 2022, five surface water samples were collected from each of eight locations along the Lontra River. The selected collection points were located near streams that flow into the river and the regions where effluents from the municipal sewage system are discharged, which are strategic regions for possible points of microbiological contamination. Water samples were analyzed using the same methodology described for FAP samples.

Subsequently, isolates from the FAP and water samples were inoculated into Brain Heart Infusion (BHI) broth and plated on standard count agar (PCA). Bacteria were then inoculated in BHI broth for the extraction of genomic DNA (gDNA) by simple boiling, as described by Ribeiro et al. (2016).

A total of 565 suggestive isolates were submitted for individual confirmation of the species *V. cholerae, V. parahaemolyticus,* and *V. vulnificus* using uniplex PCR, as previously mentioned. Uniplex PCR mixtures included gDNA (≈ 50 ng), 100 nM of each DNTP, 2.5 µL of 10× buffer, 75 mmol•L^{-1} of $MgCl_2$, 20 pmol•L^{-1} of each primer (of the corresponding species), 2.5 U of Taq DNA polymerase (Invitrogen), and ultrapure water to a final volume of 25 µL.

Amplification was performed in a thermocycler (BioRad) under the following conditions: an initial denaturation cycle at 95 ºC for 2 min; 35 cycles of denaturation at 95 ºC for 20 s, annealing at 50 ºC for 30 s, and extension at 72 ºC for 60 s; and a final extension cycle at 72 ºC for 10 min. The amplified products were subjected to 2% agarose gel electrophoresis, stained with 20 mg•L^{-1} ethidium bromide solution for 20 min, and visualized under ultraviolet light. Those isolates with amplification products of 160, 794, and 373 bp were considered positive for *V. cholerae, V. parahaemolyticus,* and *V. vulnificus,* respectively (Izumiya et al., 2014).

Among the 103 isolates confirmed by uniplex PCR, 10 isolates, including strains from the three species, were selected and subjected to partial amplification of the 16S rRNA gene using primers 27f (5'-GAGTTTGATCMTGGCTCAG-3') and 1492r (5'-GGYTACCTTGTTACGACTT-3') (Osborne et al., 2005), and sequenced. The amplification conditions were as follows: an initial denaturation cycle at 94 ºC for 5 min; 35 cycles of denaturation at 94 ºC for 1 min, annealing at 58 ºC for 1 min, and extension at 72 ºC for 1 min; and a final cycle of extension at 72 ºC for 10 min.

For DNA sequencing, the Sanger method (ABI 3500 Genetic Analyzer; Applied Biosystems, Foster City, CA, USA) was used in both directions. The PCR product of the 16S rRNA gene was purified using a PureLink genomic DNA purification kit (Invitrogen). The quality of the 16S rRNA sequences was assessed using BioEdit v. 7.2.5 (Hall, 1999), and consensus sequences were generated using CAP 3 (Huang & Madan, 1999). Identification was performed using the Basic Local Alignment Search Tool from the National Center for Biotechnology Information (NCBI).

Using multiplex PCR, 199 isolates from FAP samples, suggestive of *Vibrio* spp., were identified. However, only 25 of them were confirmed as pathogenic *Vibrio* spp. using uniplex PCR (Table 1). In addition, 366 isolates suggestive of *Vibrio* spp. from water samples, which were phenotypically identified

through colony isolation and differentiation in TCBS culture medium, were also analyzed by uniplex PCR. The results showed that only 78 isolates could be confirmed as *Vibrio* spp. Among them, 18 isolates were confirmed as *V. cholerae,* 6 as *V. parahaemolyticus,* and 54 as *V. vulnificus.*

Table 1
Uniplex and multiplex PCR assay results for identifying pathogenic *Vibrio* spp. among bacterial isolates from animal product and water samples from the northern region of the state of Tocantins, north of Brazil

Sample origin	No. suggestive isolates	*V. cholerae*		*V. parahaemolyticus*		*V. vulnificus*	
		Multiplex	*Uniplex*	*Multiplex*	*Uniplex*	*Multiplex*	*Uniplex*
Chicken	8	3	-	1	-	4	3
Fish	4	2	-	2	-	0	2
Minced meat	45	30	-	6	-	9	8
Shimp	65	13	-	25	1	17	5
Pork sausage	77	29	6	24	-	24	-
Water	366	-	18	-	6	-	54
Total	**565**	**77**	**24**	**58**	**7**	**54**	**72**

The number of confirmed *Vibrio* spp. from FAP by uniplex PCR decreased in comparison with that obtained by multiplex PCR. Notably, multiplex PCR may present several difficulties, including low sensitivity, specificity, and/or preferential amplification of specific targets, in relation to traditional PCR (Polz & Cavanaugh, 1998).

Genetic sequence analysis results from the 10 isolates chosen from among the 25 and 78 isolates from the FAP and water samples identified as *Vibrio* spp., respectively, are shown in Table 2. Unexpectedly, the uniplex PCR molecular assay, which used the species-specific primers described by Izumiya et al. (2014), presented nonspecific reactions that resulted in fragments of the same size as those expected for pathogenic *Vibrio* species. Therefore, the results from the uniplex PCR assay should not be used to confirm the identity of the isolates, given that genetic sequencing revealed that the amplification targets were not specific; therefore, no *Vibrio* spp. were identified.

The variations in the biochemical characteristics of *Vibrio* spp. have questioned the reliability of those identification methods based on phenotypes, which are time-consuming and laborious procedures, and rest on cultures for the detection of such microorganisms (Kim et al., 2015).

Kim et al. (2015) observed that counts on TCBS agar plates were lower than the sum of three *Vibrio* spp. counts obtained by real-time PCR using different primer sets.

However, this disparity can be explained by the fact that, despite being selective for *Vibrio* spp., TCBS may inhibit the growth of bacterial cells damaged by selective agents (Kim et al., 2015).

Table 2
Identification of suspected pathogenic *Vibrio* spp. isolated from animal products and water samples in the northern region of the state of Tocantins, Brazil by 16S rRNA gene sequencing

Sample origin	Uniplex PCR result	16S rRNA sequence identification
Sausage	*V. cholerae*	*Escherichia coli*
Water	*V. cholerae*	*Aeromonas* spp.
Water	*V. cholerae*	*Morganella* spp.
Water	*V. parahaemolyticus*	*Morganella* spp.
Shimp	*V. parahaemolyticus*	*Aeromonas* spp.
Shimp	*V. vulnificus*	*Morganella* spp.
Minced meat	*V. vulnificus*	*Morganella* spp.
Water	*V. vulnificus*	*Aeromonas* spp.
Water	*V. vulnificus*	*Morganella* spp.
Water	*V. vulnificus*	*Morganella* spp.

Molecular biology techniques, particularly PCR, have been developed as alternatives to culture-based identification methods for the detection of pathogenic *Vibrio* spp. in food and clinical microbiology. Such techniques have proven to be of great value for environmental surveillance, especially because *Vibrio* spp. are capable of remaining in the environment in a "viable but non-cultivable" state as an adaptation mechanism, not being detected by conventional cultivation methods, but rather by molecular strategies (Vezzulli et al., 2020).

One of the impacts of validating a biomolecular assay for the identification of pathogenic *Vibrio* spp. is to provide a reliable methodological database since previously reliable reference methods for detecting these microorganisms have shown unreliable, as demonstrated here (Table 2).

A reliable diagnosis of pathogenic species of the genus Vibrio is crucial. In the present study, a wide range of samples from different sources were used, and several methodologies proposed in the literature were followed; therefore, it may be considered an extensive and valid methodological database for future studies on Vibrio spp. from the northern region of Brazil.

The isolation and identification of *Vibrio* spp. were not specific when TCBS agar plates were used for selection. Phenotypic isolation and identification are frequently used to confirm *Vibrio* spp. pathogenicity; however, the results of this

study demonstrate that such identification could lead to false-positive results for *Vibrio* spp. in clandestine FAP and water samples; therefore, new culture media of different brands should be used in further research.

The multiplex and uniplex PCR protocols used, under the conditions presented, were not specific or sensitive enough to correctly identify pathogenic *Vibrio* spp. among the isolates obtained from FAP and water samples. PCR adaptations, that is, changes in PCR protocols, often involve the incorporation or elimination of primer sets, depending on the availability of reference genomic sequences. However, these changes alone cannot guarantee true positive results, and further studies must be performed before establishing an accurate PCR protocol for the identification of *Vibrio* spp.

Further studies are needed to validate research on pathogenic *Vibrio* spp. in food and water samples to create a rapid tool for identifying these microorganisms for monitoring food and water safety.

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