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Identification of *Vibrio parahemolyticus* Isolated from Seafood via Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry

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HIGHLIGHTS

- Out of 103 isolates, 41 were conformed as *Vibrio parahaemolyticus* trough Matrix-Assisted Laser Desorption/Ionization Time of flight Mass Spectrometry (MALDI-TOF MS).
- The Polymerase Chain Reaction analysis confirmed 93% of the results obtained by MALDI-TOF MS.
- MALDI-TOF MS showed high discriminative capacity and can be used for fast identification of *V. parahaemolyticus* in seafood samples.

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Acronyms and abbreviations
MALDI-TOF MS=Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry
PCR=Polymerase Chain Reaction

ABSTRACT

Background: *Vibrio parahaemolyticus* is the most common cause of human infections of all members of the *Vibrio* genus, accounting for between 31 and 50% of the food poisoning cases. Consumption of food contaminated with *V. parahaemolyticus* can cause severe digestive infection with symptoms of watery or bloody diarrhoea, stomach pain, vomiting, fever, and dehydration. The objective of the study was to establish the reliability of Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS) for identifying *V. parahaemolyticus* isolated from seafood marketed for human consumption.

Methods: A hundred and eighty seafood samples including mussels (*Mytilus galloprovincialis*), veined rapa whelks (*Rapana venosa*), bluefish (*Pomatomus saltatrix*), horse mackerel (*Trachurus mediterraneus*), gilthead seabream (*Sparus aurata*), sea bass (*Dicentrarchus labrax*), Atlantic salmon (*Salmo salar*), whiteleg shrimp (*Litopenaeus vannamei*), Argentine shortfin squid (*Illex argentinus*), and oysters (*Ostreidae*) were tested by Polymerase Chain Reaction (PCR) and MALDI-TOF MS for the presence of *V. parahaemolyticus*.

Results: Of the tested 103 isolates, 44 (43%) samples were identified as *V. parahaemolyticus* by PCR, while 41 (40%) samples were confirmed as *V. parahaemolyticus* by MALDI-TOF MS. The PCR analysis using non-parametric *t*-test for comparison of the proportions confirmed 93% of the results obtained by MALDI-TOF MS.

Conclusion: MALDI-TOF MS showed high discriminative capacity and can be used for high reliability fast identification of *V. parahaemolyticus* in seafood samples.

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Introduction

Vibrio parahaemolyticus is a Gram-negative, non-sporous slightly curved rod-like bacterium, normally inhabiting marine and coastal areas. It belongs to the *Vibrio* spp. genus, which contains about 30 other species. The bacterium is strictly halophilic, developing, and reproducing in an environment with sodium chloride (NaCl) content between 1-9%, at an optimum of 3% (Tan et al., 2020). *V. parahaemolyticus* is found either as a free-living microorganism or as a colonizer of various aquatic animals. It is most often isolated from bivalve molluscs, crustaceans, the gastrointestinal tract of fish, thus becoming the main means of transmission of the bacterium (Tan et al., 2020).

Consumption of food contaminated with *V. parahaemolyticus* can cause severe digestive infection presenting with watery or bloody diarrhoea, stomach pain, vomiting, fever, and dehydration. Wound and ear infections, as well as septicaemia in people with comorbidities, have also been reported (Hoefler et al., 2022). *V. parahaemolyticus* is the most common cause of human infections of all members of the *Vibrio* genus, accounting for between 31 and 50% of food poisoning cases (CDC, 2019; Tan et al., 2020). The *V. parahaemolyticus* infection is more common in the warm geographical regions, mainly the coast of Asia and USA. This is accounted for by the fact that high temperatures facilitate a lot the pathogen development and reproduction. However, both global warming and dynamic increase of surface water temperature worldwide have caused the expansion of the *V. parahaemolyticus* infection in atypical latitudes (Le Roux et al., 2015). This is evidenced by reports of diseases due to *V. parahaemolyticus* in Germany, France, Spain, Italy, Finland, and Sweden (Amato et al., 2022; Letchumanan et al., 2019). However, the lack of a mandatory system to inform about diseases caused by *Vibrio* spp. in Europe hinders the reporting of the actual number of cases (Dietrich et al., 2023).

Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS) is a widely-used method worldwide for fast identification of microorganisms, including moulds (Sivanesan et al., 2023). The software compares the mass spectra of peptides and proteins obtained from microorganisms available in the database with the spectra generated from the experimental isolates. Algorithms are used for the identification of the analyzed spectra with the reference spectra (Jang and Kim, 2018). The majority of spectral patterns are generated by cellular ribosomal proteins. MALDI-TOF MS is a revolution in clinical laboratory practice since it requires less time to make the samples, less volumes are needed, lower costs are required, and has much higher level of identification compared to the classical laboratory procedures for microbial identification (Jang and Kim, 2018). Identification of microorganisms isolated from the environment is a greater challenge than in clinical microbiology due to the extremely varied habitats of microorganisms (Torsvik et al., 2002). Therefore, in the cases of potentially pathogenic isolates obtained from the environment, a method for fast and sure identification is needed. MALDI-TOF has recently been applied to identify isolates from environmental and food samples. This type of technology is of particular importance in food microbiology, which will support and improve food safety and quality (Böhme et al., 2016). The objective of this study was to establish the reliability of MALDI-TOF MS for identifying *V. parahaemolyticus* isolated from seafood marketed for human consumption. To the best of the authors' knowledge, this is the first report on the identification of *V. parahaemolyticus* isolated from seafood by MALDI-TOF in Bulgaria. Although, the conventional Polymerase Chain Reaction (PCR) is a highly sensitive and specific method for the detection of pathogenic bacteria in samples of different origins, it still has some disadvantages. It cannot be quantified and cannot differentiate between the presence of live and dead bacteria (Bonny et al., 2022).

Material and methods

Sample collection and processing

A hundred and eighty samples of various hydrobionts were collected during April-September 2021 and April-September 2022. The samples consisted of live mussels (*Mytilus galloprovincialis*) (n=20), veined rapa whelks (*Rapana venosa*) (n=20), bluefish (*Pomatomus saltatrix*) (n=10), horse mackerel (*Trachurus mediterraneus*) (n=20), gilthead seabream (*Sparus aurata*) (n=20), sea bass (*Dicentrarchus labrax*) (n=20), Atlantic salmon (*Salmo salar*) (n=20), whiteleg shrimp (*Litopenaeus vannamei*) (n=10), Argentine shortfin squid (*Illex argentinus*) (n=20), and oysters (*Ostreidae*) (n=20). Mussels and veined rapa whelks were wild catch from the Black Sea, and only part of the oysters were taken from an oyster farm in the village of Kranevo, Bulgaria. All other samples were purchased from the stores in Stara Zagora and Burgas in Bulgaria. All samples were stored in thermal insulated containers during their transportation to the microbiological laboratory of the Department of Food Quality and Safety and were processed within 12 h after their delivery.

Microbiological analysis of the samples was performed in accordance with the procedures and criteria specified in ISO 21872-1:2017 (2017). Briefly, 25 g of sample was homogenized in 225 ml of alkaline saline peptone water, (Hi Media, India). Enrichment of the samples was two-

stage with subsequent inoculation of the enriched broth in two chromogenic selective media thiosulfate-citrate-bile salts-sucrose agar and HiCrome^{TM}Vibrio Agar (Hi Media, India). For identification by PCR and MALDI Biotyper, isolates with typical *V. parahaemolyticus* phenotypic characteristics were selected.

Identification of V. parahaemolyticus by PCR

-DNA extraction

Genomic DNA was extracted by boiling method (Park et al., 2013). From typical colonies on zobell marine agar (Hi Media, India), a suspension of each isolate was prepared in 1 ml of distilled and deionized water. The suspension was placed in a thermoblock at 98 °C for 10 min, and then centrifuged at 14,000 rpm for 10 min. A portion of the supernatant was transferred to a sterile Eppendorf tube.

-PCR procedure

PCR reaction was performed by a pair of primers for *toxR* gene (*toxR*-F GTC TTC TGA CGC AAT CGT TC; *toxR*-R ATA CGA GTG GTT GCT GTC ATG) (Nelapati and Krishnaiah, 2010). The reagent mixture was with a volume of 25 µl: 2 µl DNA, 12.5 µl master mix (Hot Star Master Mix, Cytiva), 0.2 µl from each primer, and 10.1 µl distilled water. Amplification was made in prime thermal cycler (Techne, UK) according to the following protocol: initial denaturation at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 63 °C for 2 min, extension at 72 °C for 1.5 min, and final extension at 72 °C for 10 min (Nelapati and Krishnaiah, 2010). Amplified DNA fragments were confirmed by horizontal electrophoresis in 2% agarose gel (Agarose, Sigma) at 130 V for 35 min. The gel was tainted with safe stain gelred nucleic acid stain (Biotium, Fremont, CA) and visualized with UV transilluminator (Syngene, UK). Direct LoadTM PCR 100 bp low ladder (Sigma) marker was used to determine the molecular mass. *V. parahaemolyticus* (ATCC 17802) was used as a control strain.

Identification of V. parahaemolyticus by MALDI-TOF MS

Each isolate was inoculated on zobell marine agar (Hi Media, India) and incubated at 37 °C for 24 h. By direct bacterial transfer (extended direct transfer), in accordance with the manufacturer's recommendations (Bruker, Germany), a small portion of a fresh single bacterial colony was inoculated on a 96-spot polished steel target plate (MSP 96; Bruker, Germany). Each sample was spread well within the pit and allowed to dry at room temperature. One µl of saturated solution of α-cyano-4-hydroxycinnamic acid (HCCA Matrix) was dropped onto each sample and again allowed to dry at room temperature for 5-10 min before the target plate was introduced into the mass spectrometer. The matrix solution penetrates the cell to extract the intracellular proteins to be analyzed. The particles are then ionized by a laser, separated according to the mass-to-charge ratio. The ionized molecules are then accelerated in a vacuum analyzer tube to determine the ion's time of flight. When the charged particles reach the detector, a peak spectrum is automatically generated. Based on the library data inserted in the MALDI Biotyper research use only software (Server Version: 4.1.100 (PYTH)) (Bruker, Germany), the spectral peaks of the analyzed samples were compared with the available reference peaks (MBT COMPASS 4.1.14 Bruker). A statistical algorithm generates coefficients of differentiation between 0.000 and 3.000. Identification values ≥2 are valid for a species level (Bruker, 2016).

Statistical analysis

The obtained results were processed on a computer statistical program Statistica 10 (StatSoft 1984-2000 Inc. Copyright 1990-1995, Microsoft Corp.) using non-parametric *t*-test for comparison of the proportions.

Results

Following microbiological analysis of the samples, 103 typical isolates were selected for subsequent identification. The greatest number of isolates were recorded in the mussels (*Mytilus galloprovincialis*) samples and the least in bluefish (*Pomatomus saltatrix*). Only in the shrimp samples no typical isolates were found (Table 1).

After molecular analysis, 44 (43%) of 103 isolates showed the presence of *toxR-V. parahaemolyticus* gene (Figure 1). The greatest percentage of confirmed isolates were from oysters (88%) and mussels (70%), followed by veined rapa whelks (56%), horse mackerel (36%), gilthead seabream (33%), bluefish (33%), Atlantic salmon (27%), and sea bass (10%) (Table 1).

Compared to PCR, 41 (40%) of 103 isolates were conformed as *V. parahaemolyticus* by MALDI-TOF MS. Three isolates of veined rapa whelks, horse mackerel, and sea bass were confirmed as *V. parahaemolyticus* by PCR, but identified as *V. alginolyticus* by MALDI-TOF MS (Table 1). In general, the PCR analysis confirmed 93% of the results obtained by MALDI-TOF MS.

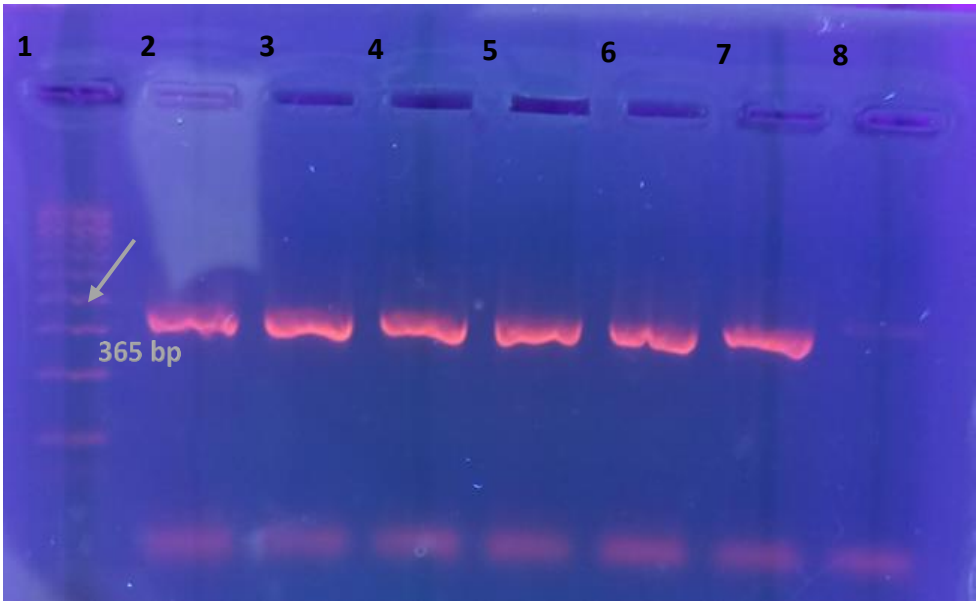

Figure 1: Presence of *toxR* gene in *Vibrio parahaemolyticus* from mussels
Key: 1: marker (100 bp); 2: positive control; 3, 4, 5, 6, 7: positive isolates; 8: negative control

Table 1: Confirmed *Vibrio parahaemolyticus* isolates by PCR and MALDI-TOF MS

Isolate origin	**Number of isolates**	***Vibrio parahaemolyticus* isolates identified by**		***Remark**
		PCR, n (%)	**MALDI-TOF MS n (%)**	
Mussels (*Mytilus galloprovincialis*)	20	14 (70)	14 (70)	-
Veined rapa whelks (*Rapana venosa*)	16	9 (56)	8 (50)	*V. alginolyticus*
Horse mackerel (*Trachurus mediterraneus*)	14	5 (36)	4 (29)	*V. alginolyticus*
Atlantic salmon (*Salmo salar*)	11	3 (27)	3 (27)	-
Sea bass (*Dicentrarchus labrax*)	10	1 (10)	0 (0)	*V. alginolyticus*
Gilthead seabream (*Sparus aurata*)	9	3 (33)	3 (33)	-
Argentine shortfin squid (*Illex argentinus*)	9	0 (0)	0 (0)	-
Oysters (*Ostreidae*)	8	7 (88)	7 (88)	-
Bluefish (*Pomatomus saltatrix*)	6	2 (33)	2 (33)	-
Whiteleg shrimp (*Litopenaeus vannamei*)	0	-	-	-
Total	**103**	**44 (43)**	**41 (40)**	

* Isolates were confirmed as *V. parahaemolyticus* by Polymerase Chain Reaction (PCR), but were determined as another species by Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS).

Discussion

A serious challenge is the identification of bacteria that inhabit the aquatic environment and especially the differentiation of closely related isolates from the environment. This also determines the need for a method for rapid identification in situations related to outbreaks of disease or identification of less common microorganisms, where classical methods and approaches have a weak degree of differentiation. All these challenges can be successfully met by applying MALDI-TOF MS (Popović et al., 2017). MALDI-TOF has the potential to identify subspecies and serotypes, which is important in food safety risk assessment due to the different pathogenic potential of isolates (Böhme et al., 2016). Therefore, this study investigated the reliability of MALDI-TOF MS for the identification of *V. parahaemolyticus* isolated from seafood, comparing the results with PCR identification. If the results of a sample did not match, the result of the PCR

analysis would be considered correct, since DNA is a specific biomarker for each microorganism.

PCR is a method with high sensitivity and specificity for the identification of pathogenic bacteria from clinical, environmental, and seafood samples (Bonny et al., 2022). A PCR method with a target *toxR* gene was applied to identify *V. parahaemolyticus*, since it is highly sensitive and accurate (Bonny et al., 2022). The *toxR* gene is present in both pathogenic and non-pathogenic isolates of *V. parahaemolyticus* (Letchumanan et al., 2014). Of the 103 tested isolates, 44 (43%) showed 368 bp amplicons identical to the positive control *V. parahaemolyticus* (ATCC 17802), while 41 (40%) were identified as *V. parahaemolyticus* by MALDI-TOF MS. In this study, PCR analysis confirmed 93% of the results obtained by MALDI-TOF MS. Discrepancy were found in three isolates that were identified as *V. alginolyticus* by MALDI-TOF MS. This discrepancy can be explained by the finding of Hazen et al. (2009) in 56-80% interspecific similarity in these *vibrios*. Cho et al. (2017) emphasized the choice of the preparation procedure for MALDI-TOF MS identification. In their study, the extended direct transfer method showed a low discriminative ability compared to the identification results in which the preparation of the isolates was performed by trifluoroacetic acid and formic acid methods described by Hazen et al. (2009) and Kuda et al. (2014), respectively. This can be accounted for by the high viscosity of the *vibrios* from the agar medium, which prevents the lysis of the bacterial membrane. Incomplete lysis results in no proteins for MALDI-TOF MS analysis and identification cannot be performed (Cho et al., 2017). The software generates a score value after comparing the spectra based on the similarities between the experimental and those in the database (Liébana-Martos, 2018). However, not all organisms are present in the databases equally, nor do the available spectra fully represent clinical strains. This means that identification by MALDI-TOF MS is highly dependent on the quality and accuracy of the database used (Liébana-Martos, 2018). The method also has difficulty identifying microorganisms with a high phylogenetic relationship (Akimowicz and Bucka-Kolendo, 2020). This could be of particular importance considering the closely related *V. parahemolyticus* and *V.alginolyticus*. Isolates differ significantly from type strains in their phenotypic and proteotypic properties, due to modifications caused by continuous changes in the environment or food sources. For effective species-level identification of food-borne pathogens isolated from food products, it is important to supplement the available databases with spectral information of strains isolated from different food matrices (Böhme et al., 2016). As an additional reason, the fact that the mass spectra of *Vibrio* spp. are mainly from protein peaks defined as abundant proteins, mainly ribosomal and nucleic acid binding proteins (Malainine et al., 2013). According to Malainine et al. (2013), protein profiling has lower discriminatory power compared to molecular typing methods. The high rate of species-level differentiation by MALDI-TOF MS fully confirms the results of other studies (Dieckmann et al., 2010; Hazen et al., 2009). The closely related *vibrios V. alginolyticus* and *V. parahaemolyticus* were identified with log (score values) ≥2.2. Although the World Health Organization (WHO) recognizes only *V. parahaemolyticus*, *V. cholerae*, and *V. vulnificus* as major human pathogens, sporadic human infections due to *V. alginolyticus, V. harveyi, V. fluvialis,* and *V. mimicus* have also been reported (Joint FAO/WHO, 2001; Popović et al., 2017). Another important factor should be noted, namely the quality of the database, which affects the accuracy of the identification. The more reference spectral peaks contained in the available library, the more accurate the identification (Pavlovic et al., 2013). Sample preparation, purity of bacterial growth, curtailing or deviation from the protocol are also critical points affecting the accuracy of the analysis (Pavlovic et al., 2013).

Although extremely accurate and reliable, the PCR method is also quite complicated by preliminary sample preparation procedures (Cherkaoui et al., 2010). The time required for DNA extraction, sample preparation and setup to a specific protocol, amplification of specific DNA regions, separation of DNA fragments by gel electrophoresis exceed several hours. The expensive consumables, the use of different equipment in each of the stages of the analysis, as well as the need for highly qualified personnel in the implementation of the methodology should also be noted. Every single isolate from this study has been identified by the MALDI Biotyper in less than 2 min, and the only preliminary preparation consisted of re-inoculation of typical isolates on non-chromogenic agar according to the manufacturer's recommendations.

Conclusion

MALDI-TOF MS analysis is fast and easy to perform. Although the method has some drawbacks related mostly to the lack of spectra for the newly identified species in the reference database, MALDI-TOF MS showed high discriminative capacity with regard to *V. parahaemolyticus*. The method can be used for high reliability and fast identification of *V. parahaemolyticus* in seafood samples.

Author contributions

R.F. and D.S. writing original draft, methodology, investigation, formal analysis, data curation,

conceptualization; P.O. writing original draft and methodology. All authors read and approved the final manuscript.

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Conflicts of interest

The authors declare that they have no conflict of interest.

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