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RESEARCH ARTICLE

Fast and accurate identification by MALDI-TOF of the zoonotic serovar E of *Vibrio vulnificus* linked to eel culture

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Abstract

Vibrio vulnificus is a zoonotic pathogen that can cause death by septicaemia in farmed fish (mainly eels) and humans. The zoonotic strains that have been isolated from diseased eels and humans after eel handling belong to clade E (or serovar E (SerE)), a clonal complex within the pathovar (pv.) *piscis*. The aim of this study was to evaluate the accuracy of MALDI-TOF mass spectrometry (MS) in the identification of SerE, using the other two main pv. *piscis*-serovars (SerA and SerI) from eels as controls. MALDI-TOF data were compared with known serologic and genetic data of five pv. *piscis* isolates or strains, and with the non pv. *piscis* reference strain. Based on multiple spectra analysis, we found serovar-specific peaks that were of ~3098 Da and ~4045 Da for SerE, of ~3085 Da and ~4037 Da for SerA, and of ~3085 Da and ~4044 Da for SerI. Therefore, our results demonstrate that MALDI-TOF can be used to identify SerE and could also help in the identification of the other serovars of the species. This means that zoonosis due to *V. vulnificus* could be prevented by using MALDI-TOF, as action can be taken immediately after the isolation of a possible zoonotic *V. vulnificus* strain.

KEYWORDS
eel, MALDI-TOF, serotyping, serovar E, *Vibrio vulnificus*, zoonotic, PCR

1 | INTRODUCTION

Vibrio vulnificus is a zoonotic pathogen of warm ecosystems whose geographic distribution is expanding to colder areas due to global warming (Amaro et al., 2020; Baker-Austin et al., 2017). This pathogen mainly survives in warm brackish to marine water associated with aquatic animals from which it can infect susceptible fish and humans either by contact or ingestion (Amaro et al., 2020; Ceccarelli et al., 2019; Haenen et al., 2014). In addition, some strains of the species can cause zoonosis, that is, cause animal diseases (mainly in farmed fish) and, from diseased animals, directly infect humans (Amaro et al., 2020; Ceccarelli et al., 2019). All these diseases are known as vibriosis, and

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although very disparate in terms of their clinical manifestation and type of infected host, they all have in common that the most severe form is a haemorrhagic septicaemia that can lead to death in less than 24–48 h (Amaro et al., 2015, 2020; Ceccarelli et al., 2019; Dalsgaard et al., 1996; Dijkstra et al., 2009; Haenen et al., 2013; Leng et al., 2019; Oliver, 2015). Since the occurrence of an outbreak in a fish farm can endanger human life, rapid and accurate subtyping of *V. vulnificus* isolates is a necessity in order to act quickly and appropriately.

Vibrio vulnificus is a highly variable species that is currently subdivided into five phylogenetic lineages plus a pathovar specifically virulent to fish (pathovar (pv.) *piscis*; Roig, González-Candelas, et al., 2018). This pathovar possesses a transferable virulence plasmid that encodes a resistance system to innate immunity in fish blood (Hernández-Cabanyero et al., 2019; Lee et al., 2008; Pajuelo et al., 2015; Roig et al., 2018). Among the different O-serovars (serovars based on O-antigen variability) described so far within pv. *piscis* (Fouz et al., 2007), serovar E (SerE) is the only one recognized as zoonotic and, therefore, represents a risk for aquaculture professionals such as fishermen, fish farmers and fish processors as well as for consumers (Amaro & Biosca, 1996; Dijkstra et al., 2009; Gauthier, 2015; Haenen et al., 2014). All SerE strains have been isolated from environmental samples, diseased eels, and from infected humans after eel handling. This serovar has had a great evolutionary success and currently constitutes a clonal complex distributed worldwide (Roig et al., 2018). Different methods have been described for the identification of SerE strains, both serological (Biosca et al., 1997) and genetic (Sanjuán & Amaro, 2007).

MALDI-TOF (matrix-assisted laser desorption-ionization—time of flight), a mass spectrometric method (MS), is increasingly used as a fast, accurate and inexpensive alternative tool for the identification of bacterial pathogens in diagnostics, including those for fish (Emami et al., 2016; Erler et al., 2015; Jansson et al., 2020; Malainine et al., 2013). This technique has already been shown to be able to distinguish subtypes of aquatic bacterial pathogens, like *Vibrio parahaemolyticus* (Hazen et al., 2009). Also, a MALDI-TOF MS database to identify *Vibrio* species from marine environments, including potentially pathogenic strains for humans, has been developed and is freely accessible (Erler et al., 2015). However, it has never been used for *V. vulnificus* subtyping.

Therefore, the main objective of this study was to evaluate the usefulness of MALDI-TOF in the identification of SerE of *V. vulnificus* and, at the same time, in the identification of other non-zoonotic serovars involved in eel vibriosis, using a specific set of isolated and reference strains of *V. vulnificus* from clinical outbreaks in four European countries, the Netherlands, Spain, Denmark and Sweden. The Danish and Swedish *V. vulnificus* strains thereby were considered field strains, used to validate the developed subtyping method.

2 | MATERIALS AND METHODS

2.1 | Bacterial strains and growth conditions

The bacterial strains used in this study are shown in Table 1 and with original codes per sample in Table S1. Strains were routinely grown for 24 h on brain heart infusion (BHI) agar (Acumedia Neogen) with 5% (v/v) defibrinated sterile sheep blood (Bio Trading Benelux) or on Tryptic Soy Agar (Oxoid) supplemented with 0.5% (wt/vol) NaCl (TSA-1) at 22°C. Bacteria were maintained as stock cultures in Marine Broth (Difco) plus 20% (v/v) glycerol at −80°C upon culture.

2.2 | Sample preparation and MALDI-TOF analysis

Two biological replicates (independently cultured bacteria in time) for each isolate or strain were used. From each biological replicate, five small colonies were taken with a 1 μl inoculation loop and dissolved by mixing by Vortex in 300 μl distilled (ds) water in Eppendorf tubes, 900 μl absolute ethanol was added, and samples were vortexed and centrifuged for 2 min at 150 *g*. The supernatant was decanted, and the samples were centrifuged for another 2 min at 150 *g*. The remaining supernatant was aspirated, and the samples were air-dried for approximately 15 min. Samples were then incubated for 2 min with 15 μl 70% formic acid. The Eppendorf tubes were ticked to dissolve the pellet. A volume of 15 μl acetonitrile was then added, and samples were centrifuged for 2 min at 150 *g*. The supernatant was transferred to a clean tube. For each biological replicate, 1 μl of supernatant was spotted 10 times on a Bruker MSP 96 target polished steel BC plate (MSP 96 target polished steel BC art no. 828000). Samples were air-dried, and 1 μl of Bruker Daltonic HCCA was pipetted onto each spot and air-dried for 5 min. The dried samples were measured using a Bruker Daltonic Microflex MALDI-TOF device. The_Standard_FAMS method (www.bruker.com) was used, and bacterial test standard BTS (Bruker Daltonic, art. no. 255343) was used for internal calibration (version 3.1, Bruker Daltonics). The automatic analysis of raw spectra was made by the software MALDI Flex Analysis. The MBT-AutoX method was used to measure the samples. Each replicate spot and a BTS spot for internal calibration were measured three times in different locations with 40 shots adding up to 240 shots per spot. In agreement with manufacturer instructions, identifications with scores between 2.0 and 2.3 were considered accurate to bacterial species level (www.bruker.com).

2.3 | Making main spectra peaks (MSPs) for subtyping

The spectrograms of the six reference isolates or strains (VvA-8 (SerA), 95-8-7 and 95-8-162 (SerI), ATCC 27562 (non pv. *piscis*), and NL-20 and NL-31 (SerE)) (Table 1) were compared manually for height of peaks. The main discriminating peak areas and peak values per strain were noted. Then, all other Dutch strains were analysed by MALDI-TOF for the discriminating peaks for the three serovars (A, E and I) of pv. *piscis*. Then, NL-7 and NL-9 (SerA); NL-6, NL-12, NL-20, NL-31 and NL-32 (SerE); and NL-4, NL-5, NL-8, NL-13 and NL-14 (SerI) isolates or strains were chosen as representatives for each of the three serovars, respectively, to make main spectra peaks (MSPs) according to instructions by Bruker (Jansson et al., 2020). These were used for reference.

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TABLE 1 Subtypes of *Vibrio vulnificus* pv. *piscis* strains as tested by two distinguishing peaks in MALDI-TOF (MT) (Figure 1) or by MT testing based on created MSPs for the Serovars (no peaks given), and by serology and multiplex SerE-specific PCR

Vv strain[a]	Source	Year of isolation	MT-height of peak in 1st area (Da)	MT-height of peak in 2nd area (Da)	Pv *piscis* (+/−)/ Serovar	Multiplex PCR profile for *Vv* (pv *piscis* SerE/ non-SerE)[b]	Subtype (based on MT testing using Serovar MSPs)
NL-1	Eel	1996	n.d.[c]	n.d.	+/I	pv *piscis* non-SerE	n.g.[d]
NL-3	Eel	1999	n.d.	n.d.	+/I	pv *piscis* non-SerE	n.g.
NL-4	Eel	2000	3083	4043	+/I	pv *piscis* non-SerE	SerI
NL-5	Eel	2002	3084	4036	+/I	pv *piscis* non-SerE	SerI
NL-6	Eel	2005	3097	4041	+/E	pv *piscis* SerE	SerE
NL-7	Eel	2005	3082	4041	+/A	pv *piscis* non-SerE	SerA
NL-8	Eel	2005	3083	4042	+/I	non-SerE	SerI
NL-9	Eel	2005	3084	4036	+/A	pv *piscis* non-SerE	SerA
NL-10	Eel	2007	n.d.	n.d.	+/E	pv *piscis* SerE	n.g.
NL-12	Eel	2007	3097	4041	+/E	pv *piscis* SerE	SerE
NL-13	Eel	2009	3082	4041	+/I	pv *piscis* non-SerE	SerI
NL-14	Eel	2009	3083	4042	+/I	pv *piscis* non-SerE	SerI
NL-32	Eel	2007	3096	4040	+/E	pv *piscis* SerE	SerE
NL-20	Human	2007	3097	4042	+/E	pv *piscis* SerE	SerE
NL-31	Eel	2005	3098	4043	+/E	pv *piscis* SerE	SerE
DK-1	Eel	1996	3083	4041	+/n.d.[e]	pv *piscis* non-SerE	SerA
DK-2	Eel	1996	3083	4041	+/n.d.	pv *piscis* non-SerE	SerI
DK-3	Eel	1996	3083	4041	+/n.d.	pv *piscis* non-SerE	SerI
DK-4	Eel	1998	3083	4041	+/n.d.	pv *piscis* non-SerE	SerI
DK-5	Eel	1998	3083	4041	+/n.d.	pv *piscis* non-SerE	SerI
DK-6	Glass Eel	2004	3083	4034	+/n.d.	pv *piscis* non-SerE	SerA
DK-7	Eel	2005	3084	4034	+/n.d.	pv *piscis* non-SerE	SerA
DK-8	Eel	2005	3083	4042	+/n.d.	pv *piscis* non-SerE	SerI
DK-9	Wild Eel	2006	3097	4041	+/n.d.	pv *piscis* SerE	SerE
DK-10	Wild Eel	2006	3097	4042	+/n.d.	pv *piscis* SerE	SerE
SE-1	Eel	1985	3097	4042	+/n.d.	pv *piscis* SerE	SerE
SE-2	Eel	1988	3083	4042	+/n.d.	pv *piscis* non-SerE	SerI
SE-3	Eel	1990	3096	4041	+/n.d.	pv *piscis* SerE	SerE
SE-4	Eel	1990	3096	4041	+/n.d.	pv *piscis* SerE	SerE
SE-5	Eel	1992	3097	4041	+/n.d.	pv *piscis* SerE	SerE
SE-6	Eel	1999	3097	4041	+/n.d.	pv *piscis* SerE	SerE
SE-7	Eel	2005	3096	4041	+/n.d.	pv *piscis* SerE	SerE
VvA-8	Eel	2001	3085	4037	+/A	pv *piscis* non-SerE	SerA
DK-95-8-7	Eel	1995	3085	4044	+/I	pv *piscis* non-SerE	SerI
DK-95-8-162	Eel	1995	3083	4043	+/I	pv *piscis* non-SerE	SerI
ATCC 27562[T]	Human	Unknown	3084	4042	–[f]	Non pv. *piscis*	Non pv. *piscis*

[a] NL: Dutch strains. DK: Danish strains; SE: Swedish strains; CECT: Spanish Collection of Type Cultures. ATCC: American Type Culture Collection. T: Type strain of the species. More details in Table S1. Reference strains in grey cells.

[b] Multiplex PCR according to Sanjuán and Amaro (2007).

[c] n.d.: Not determined, since strains could not be revived anymore from freezer collection.

[d] n.g.: No revival of strain possible anymore from freezer collection. Strain used to compare non-MT methods: serology and multiplex PCR. Non-SerE (NL-1 and NL-3) and SerE (NL-10) furthermore confirmed by Hi-MLST (Multilocus sequence typing) and/or REP-PCR (Diversilab®) in Haenen et al. (2014).

[e] The DK and SE strains were not serotyped, but just used as international field strains to test the MSPs made for *Vibrio vulnificus* pv. *piscis*.

[f] *V. vulnificus* non pv. *piscis*. Therefore, serology is not applicable.

The whole set of international strains isolated from eels (Dutch, Danish and Swedish) were subsequently tested with the new MSPs by means of MALDI-TOF. Again, only scores between 2.0 and 2.3 were considered accurate (www.bruker.com).

2.3.1 | MALDI-TOF settings

Detection: Mass range: 1000–20,000 Da, detector gain: linear, 3.8×2802 V, laser frequency 60 Hz, sample rate and digitizer settings: 0.50 GS/s, realtime smoothing off, baseline offset adjustment 0%, analogue offset: −1.2 mV. Bruker MALDI-TOF device (spectrometer): Ion source1 20 kV, Ion Source2 18.05 kV 90.3% IS1, Lens 6 kV 30% IS1, Pulsed Ion extraction 120 ns.

Laser: global attenuator offset 15%, attenuator offset 0%, attenuator range 30%, digitizer: sensitivity 100 mV, analogue offset linear −1.2 mV, analogue offset reflector 0.0 mV, digital offset linear 0 cnt, digital offset reflector 0 cnt, detector gain voltages: linear base 2600 V, reflector base 1400 V, linear boost 0 V, reflector boost 0 V.

2.3.2 | Data analysis of MALDI-TOF

Spectra were analysed in the Bruker Flex Analysis. MBT_Standard_FAMS method was used, and BTS was used for internal calibration. The MALDI-TOF spectra were treated by smoothing and baseline subtraction and saved. Spectra were considered of sufficient quality if the interval between the same peaks in different replicates was <500 ppm. Only the clear and high-intensity peaks were compared between samples, and spectra were approved if the intensity was between 20,000 and 40,000 Da.

2.4 | DNA isolation and identification by multiplex PCR

A 1 μl inoculation loop of colony material (pure cultures of the bacteria grown on TSA-1 after incubation during 24 h at 28°C) was added to 1 μl distilled water and vortexed. The samples were centrifuged at 150 *g* for 5 min, and the supernatant was aspirated and the pellet dissolved in 100 μl distilled water. The samples were heated for 10 min at 95°C. A dilution of 100× was made from the heat-treated samples and used for DNA extraction. DNA was purified using a QIAamp DNA mini kit (Qiagen) according to the manufacturers' protocol. Purified DNA was used to identify cultures at species level by PCR targeting *vvhA* gen and to subtype them by PCR targeting the gene marker for pv. *piscis* (*fpcrp*, fish phagocytosis and complement resistance protein) and the sequence marker for SerE (*vep67*) according to Sanjuán and Amaro (2007). Negative (PCR master mix) and positive (DNA from NL-20 strain) PCR controls were included. The amplified products were separated by electrophoresis on 1.8% (w/v) agarose gels and were visualized by staining with Red Safe^{T} Nucleic Acid Staining Solution (iNtRON Biotechnology, Inc.). GeneRuler 1 Kb DNA ladder (Thermo Scientific) was used as molecular weight marker.

2.5 | Serological characterization

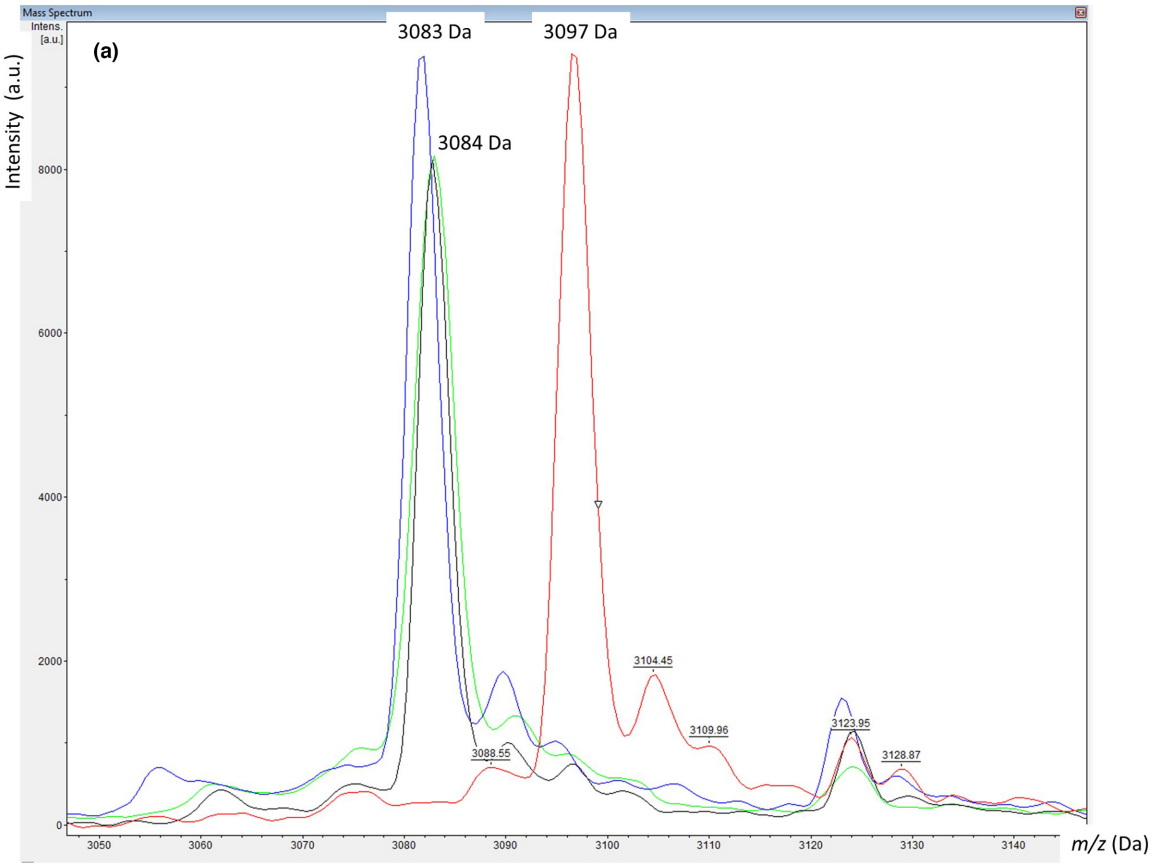
To determine serovars within pv *piscis*, rabbit antisera against formalin-killed cells of SerE, SerA and SerI strains were prepared as described previously (Sørensen & Larsen, 1986). Then, slide agglutination with whole cell suspensions containing 10^8 colony-forming units (cfu)/ml and O-antigens in PBS was performed according to Fouz and Amaro (2002).

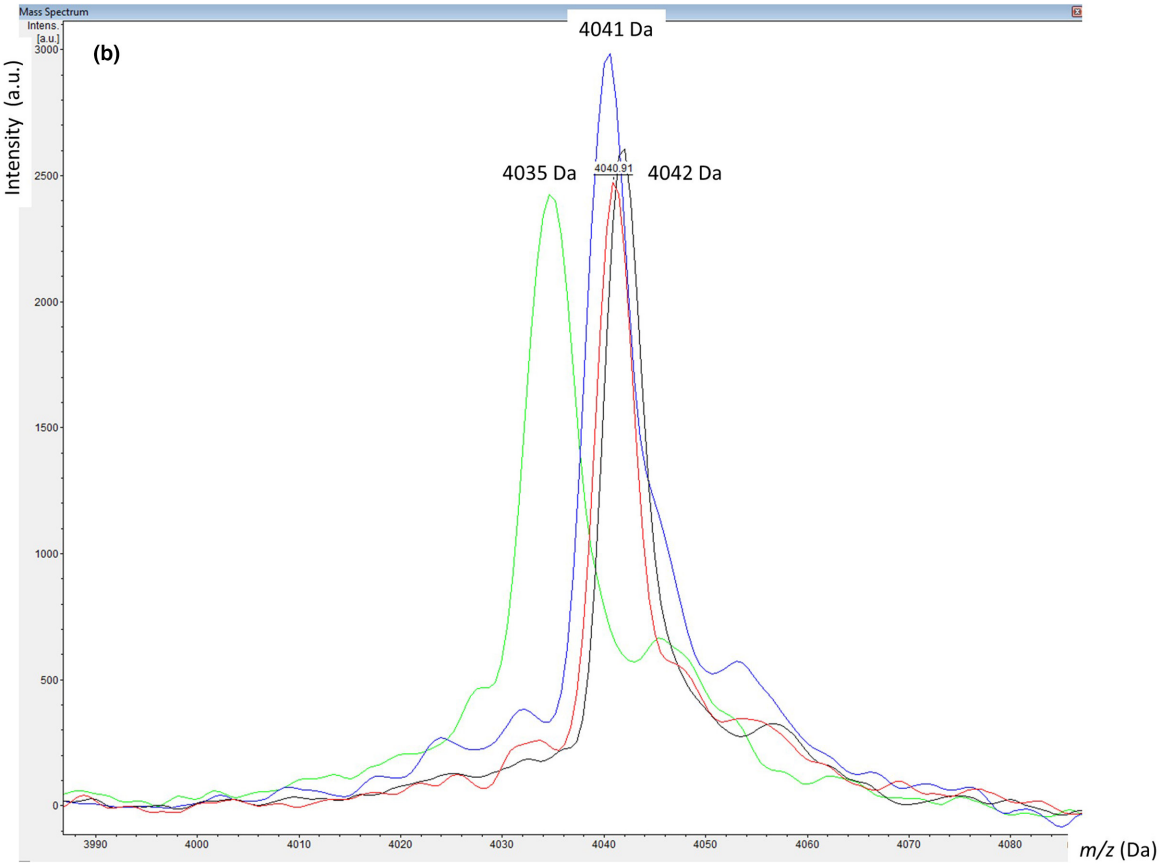
3 | RESULTS

All the *V. vulnificus* strains were analysed in duplicate, and MALDI-TOF (MT) spectra were acquired automatically in linear positive mode with a range from 1 to 20 kDa. The peak patterns in the MT-chromatogram for ten Dutch and the reference *V. vulnificus* pv. *piscis* strains (Table 1) were roughly the same per serovar. Among the non-reference Dutch isolates, four belonged to SerE, two belonged to SerA and seven to SerI, according to serology results (Table 1). Two areas with subidentifying peaks could be distinguished with values of 3083–3098 Da and 4037–4044 Da, respectively, with the SerE- and SerA-identifying peaks the most easily distinguishable from each other (Table 1, Figure 1a,b). SerE isolates or strains exhibited two specific peaks at approximately 3098 Da and 4045 Da, whereas SerA isolates or strains had them at 3085 Da and 4037 Da (Figure 1a,b). Remarkably, SerI strains gave a peak pattern that lied between SerE- and SerA-specific patterns: they shared a peak at 3085 with the SerA strains and another peak at 4044 with SerE strains (Figure 1b). The spectrogram of ATCC 27562 strain (non pv *piscis*), with peaks at 3085 Da and 4042 Da, was more similar to that of SerI than that of SerE (Figure 1).

To determine whether the detection of serovars could be automated in the MT by using subtyping MSPs, main spectra of SerE, SerA and SerI strains (Table 1) were created using Bruker Daltonics Flexanalysis and MBT Biotype 3 software. Peak weighing for

FIGURE 1 Areas in spectrogram of MALDI-TOF with discriminating peaks for serological subtyping of *Vv pv piscis* to SerA, SerE or SerI: (a) area of 3083–3098 Da and (b) area of 4037–4044 Da. Peak values (X-axis) in Da. Relative intensity (Y-axis) in arbitrary units (a.u.). (a) Red SerE 3097 Da peak, green SerA 3084 Da peak, blue SerI 3083 Da peak and black ATCC 27562 3084 Da peak. (b) Red SerE 4042 Da peak, green SerA 4035 Da peak, blue SerI 4041 Da peak and black ATCC 27562 4042 Da peak. Based on multiple spectra, the distinguishing peaks are as follows: SerA (*green*): ~3085 Da and ~4037 Da. SerE (*red*): ~3098 Da and ~4045 Da. SerI (*blue*): ~3085 Da and ~4044 Da. ATCC (*black*): ~3084 Da and ~4042 Da.





subtyping MSPs was manually optimized. The validity of using visual identification or identification by running against subtyping was further tested using 17 strains, 10 from Denmark and 7 from Sweden, with reference isolates or strains as controls (Table 1).

Mass spectrometry successfully identified and discriminated the further Dutch ($n = 10$), and the Danish ($n = 10$) and Swedish ($n = 7$) isolates into MT subtypes of *Vv* (Table 1). Among Dutch strains, two SerA, three SerE and five SerI strains were identified, in full concordance with the results obtained in serological assays (Table 1). Among Danish isolates, at least two were identified as SerA, two as SerE and four as SerI, apart from one SerA and one SerI strain based on only MSP-based testing in MALDI-TOF. However, the peak values of *Vv* strain DK-1 (Table 1) corresponded more to those of SerI than those of SerA. Furthermore, the DK-5 (identified by MALDI-TOF as SerI) strain had an MT-score below 2.0, although results of multiplex PCR and of MT corresponded well. Finally, Swedish isolates were identified to six SerE and one non-SerE by Multiplex PCR, and these results were confirmed by testing by MALDI-TOF, based on MSPs and on their peak values (Table 1).

All Dutch *Vv* pv *piscis* isolates or strains, determined by MT, were confirmed by multiplex PCR, giving the amplification double bands profile (519 pb and 344 pb bands). The four SerE isolates were also positive for the *vep67* marker (665 pb band; Table 1, Figure 2). Moreover, all the non-reference isolates were positive for the pv. *piscis* genetic marker *fpcrp*, giving the expected double bands profile (Figure 2), and eight of them, two from Denmark and six from Sweden, presented the triple band (665, 519 and 344 pb) profile after PCR amplification (Table 1). These latter eight isolates were subtyped as SerE by MALDI-TOF, as expected. The other nine strains belonged to SerA or I (Table 1).

The identification at subtype level of the non-reference isolates showed a nearly complete agreement between MALDI-TOF subtypes and PCR or serology results. Results obtained in MALDI-TOF subtyping of SerE isolates or strains correlated entirely with those obtained by PCR and for the strains that had also been tested by serology also entirely with serology.

Apart from the MT-tested Dutch *Vv* strains, three Dutch *V. vulnificus* strains (NL-1, −3 and −10) are included in Table 1, since they had been tested by serology and multiplex PCR, supported by the Hi-MLST (Multilocus sequence typing) and REP-PCR (Diversilab®) data for strains NL-1, NL-3 and NL-10 of Haenen et al. (2014), as given in the footnote of Table 1. Since these three strains were not revivable anymore out of the −80°C, no MALDI-TOF data were available including no peak values, and retesting was impossible. These strains were therefore solely used for cross-comparison among serology, multiplex PCR, Hi-MLST and REP-PCR (Diversilab®) identification approaches. The four test results per strain corresponded with each other: NL-1 and NL-3 as non-SerE (non-zoonotic) *Vv* pv. *piscis* and NL-10 as zoonotic SerE of *Vv* pv. *piscis*.

4 | DISCUSSION

Vibrio vulnificus pv. *piscis* is a primary fish pathogen that causes a fish disease known as warm water vibriosis (Amaro et al., 2015, 2020). Pv. *piscis* strains possess, as a distinguishing feature, a fish virulence plasmid (pFv) that encodes a resistance system to innate immunity in fish blood (Hernández-Cabanyero et al., 2019; Pajuelo et al., 2015). The first pv. *piscis* strains isolated (previously referred to as biotype 2, Tison et al., 1982) were all associated with eel culture (Amaro et al., 2015, 2020). These strains are grouped into three serologically homogeneous clades within phylogenetic lineage 2 (Roig et al., 2018). Among these three clades or serological groups, SerE strains stand out as the only ones clearly associated with cases of animal-to-human transmission of the disease (Amaro & Biosca, 1996; Gauthier, 2015; Haenen et al., 2014). This group has been very successful evolutionarily and has spread worldwide, both in open water and in wild aquatic animals, of which wild (glass) eels are being caught as exclusive stocking fish for eel farming (Nielsen & Prouzet, 2008). Given this scenario, it is essential to have rapid, accurate and simple methods to identify this serovar in order to make quick decisions for prevention and cure of *V. vulnificus* pv. *piscis* infections and disease in eel farming systems and humans. Therefore, it is of utmost importance to subtype the isolate as soon as possible after identification in order to avoid human casualties caused by zoonotic SerE. Until now, serological techniques or multiplex

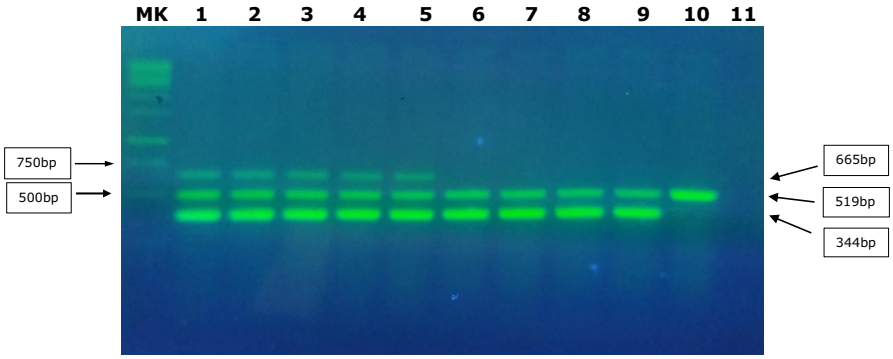


FIGURE 2 Agarose gel electrophoresis of the multiplex PCR (Sanjuán & Amaro, 2007) products obtained from pure *Vibrio vulnificus* cultures. MK, molecular weight GeneRuler 1Kb DNA ladder (Thermo Fisher Scientific). Lanes 1 to 10: DNA from pure cultures of *V. vulnificus* NL-20 positive SerE control) (1), NL-31 (2), NL-6 (3), NL-10 (4), NL-12 (5), NL-1 (6), NL-3 (7), NL-7 (8), NL-9 (9) and ATCC 27562 (10). Lane 11: negative control (no DNA).

PCR (Sanjuán & Amaro, 2007) have been used, but the serology requires specific antibodies, which are not commercially available, and this method is therefore not widely available to some laboratories, whereas the multiplex PCR requires specific equipment.

Identifying SerE using MALDI-TOF is an interesting alternative to the previous methods, as demonstrated in our study. This methodology is used in many hospital and veterinary laboratories for the identification of cultures of pathogenic strains of bacteria and fungi (Croxatto et al., 2012; Lévesque et al., 2015) as well as for the identification of bacterial fish pathogens (Jansson et al., 2020). Its main advantage is its high reproducibility since the working procedures of all laboratories using this system are standardized and the databases consulted are common. After the costly MALDI-TOF device and the database are installed in a laboratory, the costs of analysis of an individual sample from a bacterial colony grown on an agar plate are relatively low and over the years lower than those of serology or PCR. The results of MALDI-TOF analysis allow viewing detailed background data per sample, and the spectrogram of each sample can be reviewed for large peaks (Da), for rapid subtyping.

At a diagnostic fish disease laboratory, various colony types may be taken one by one from the primary culture to test immediately in MALDI-TOF. Moreover, in severe cases of eel vibriosis by *V. vulnificus* pv. *piscis*, this bacterium is predominantly present in most or all organs of the eels and will probably be the major colony on the agar plate of the primary culture. In case there would be a mixed culture, by some laboratories the multiplex PCR will be used. If *V. vulnificus* is detected by PCR, if not already clear, it is utmost important to know directly if it is the zoonotic serovar; thus, MALDI-TOF may still be used. Additionally, since serology is disappearing or not easily available at laboratories, it is very important to use this modern and reliable identification method.

In our study, the MALDI-TOF peak profiles of the pv. *piscis* strains tested were clearly associated with one of the three serological groups described, even so by using strains from various European countries. More importantly, zoonotic SerE showed a clear peak in the spectrogram at 3097–3098 Da, very easy to distinguish from the peaks corresponding to SerA and SerI in that area, which would allow identification of the strains belonging to this zoonotic group. Therefore, medical and veterinary laboratories could use the MALDI-TOF peak profiles from our study without having to perform MSPs, for manual and even rapid subtyping of *V. vulnificus*. By checking the spectrogram of the two peaks, it would be possible to judge the zoonotic potential of strains isolated, not only from diseased eel but also from the environment, from humans or even from other animal species that could act as a reservoir. Moreover, this system could most probably be implemented for the identification of lineages or clades of this species, beyond those related to eel farming. Thus, a simple and rapid protein-based tool is added to the existing serologic and molecular tools.

Our results demonstrate that MALDI-TOF can be used to identify not only SerE but also other serovars of the species. This strategy would allow rapid and accurate detection of this dangerous zoonotic pathogen to avoid human casualties.

5 | CONCLUSIONS

Our study proved that MALDI-TOF (MT) is a rather good, fast and cheap alternative method to subtype *V. vulnificus* pv *piscis* and to test, with high accuracy, if the isolated bacterium belongs to the zoonotic SerE group, based on two peaks in the MT-spectrogram.

When large numbers of samples are expected, it is advised to make MSPs for the serovars, to confirm the presence or not of strains potentially dangerous to humans.

To increase the confidence in obtained results, it is advised to do multiple testing of grown *Vv* pv *piscis* colonies by MALDI-TOF next to use other comprehensive methods, to see which serogroup dominates the results, as not all non-SerE strains were subtyped accurately by MALDI-TOF.

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CONFLICT OF INTEREST

There is no conflict of interest by anybody of the authors.

DATA AVAILABILITY STATEMENT

Data are available through the institution of the first author, WBVR.

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