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Original article

Vibrio infection in freshwater fish in Poland

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Abstract

Vibrio species are common inhabitants of aquatic environments and have been described in connection with fish and human diseases.

Six *Vibrio* species were isolated from diseased freshwater and ornamental fish in Poland. The strains were identified based on morphological and biochemical characteristics and confirmed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) as *V. albensis* (n=3) from *Gymnocephalus cernua*, *Sander lucioperca*, *Paracheirodon innesi*, and *Xiphophorus hellerii*; *V. mimicus* (n=1) from *Xiphophorus maculatus*; and *V. vulnificus* (n=1) from *Nematobrycon palmeri*. This is the first time that *Vibrio* species have been isolated and described from ornamental fish in Poland. The isolates were resistant to ampicillin (83.3%), gentamicin (16.6%), ciprofloxacin (16.6%), sulfamethoxazole-trimethoprim (16.6%), and chloramphenicol (16.6%). The multiple antibiotic resistance (MAR) index was 0.00-0.08 for *V. albensis*, 0.17 for *V. mimicus*, and 0.33 for *V. vulnificus*.

Our study confirmed the presence of potentially pathogenic *Vibrio* species in freshwater and ornamental fish. Therefore, further monitoring of the presence of *Vibrio* species, mainly in ornamental fish, is necessary.

Keywords: antibiotic resistance, fish diseases, *Vibrio*

Introduction

Vibrio species are aquatic G(-) bacteria belonging to the phylum Proteobacteria, class Gammaproteobacteria, order Vibrionales, family Vibrionaceae, and genus *Vibrio*. The genus *Vibrio* contains 170 species with validly published names, including synonyms (www.bacterio.net/vibrio.html). They are important pathogenic bacteria for humans, marine and freshwater animals. Four species are responsible for most cases of disease in humans: *V. cholerae*, *V. vulnificus*, *V. parahaemolyticus* and *V. alginolyticus* (Baker-Austin et al. 2018). Other *Vibrio* species routinely isolated from human clinical samples include *V. mimicus*, *V. harveyi*, *V. fluvialis*, *V.* (*Photobacterium*) *damselae* and *V. metschnikovii*. Fish diseases caused by *Vibrio* species have been associated mainly with *V. alginolyticus*, *V. anguillarum*, *V. carchariae*, *V.* (*Photobacterium*) *damselae*, *V. ordalli*, *V. salmonicida*, and *V. vulnificus* (Manchanayake et al. 2023). Fish infected by *Vibrio* sp. usually suffer from abdominal dropsy, exophthalmia, haemorrhage, ulcers, intestinal inflammation, scale shedding, epidermal bleeding, and fin and tail rot (Austin and Austin 2007, El-Deen and Elkamel 2015, Huzmi et al. 2019). *Vibrio* species are common inhabitants of aquatic environments (Dong et al. 2015, Manchanayake et al. 2023). In Poland, the presence of the *Vibrio cholerae* has been detected in the River Bug by Stypułkowska-Misiurewicz et al. (1995). Kurpas et al. (2021) described the presence of *V. alginolyticus*, *V. cholera/mimicus*, and *V. vulnificus* in the Gulf of Gdansk. In addition, Stypułkowska-Misiurewicz et al. (2006) reported two cases of septicaemia caused by *V. cholera* non-O1, non-O139 in various regions of Poland.

Common antibiotics applied in aquaculture are amoxicillin, benzylpenicillin, co-trimazine, enrofloxacin, florfenicol, flumequine, oxolinic acid, oxytetracycline, sarafloxacin, trimethoprim, and sulfadiazine (Huzmi et al. 2019). *Vibrio* spp. are considered to be highly susceptible to all basic antimicrobials, i.e. tetracyclines (tetracycline, doxycycline), fluoroquinolones (ciprofloxacin, levofloxacin), third-generation cephalosporins (cefotaxime, ceftazidime, ceftriaxone), aminoglycosides (amikacin, streptomycin), and folate pathway inhibitors (trimethoprim-sulfamethoxazole) (Baker-Austin et al. 2009). Resistance of fish pathogenic bacteria to commonly used antibiotics has been reported throughout the world (Kumarage et al. 2022).

Identification of *Vibrio* species is traditionally based on biochemical reactions and sequencing analysis of the 16S rRNA and *rpoG* genes (Cheng et al. 2015). A number of studies have shown that MALDI-TOF-MS (matrix-assisted laser desorption ionization-time of flight mass spectrometry) can rapidly and accurately identify bacteria to the species level (Dieckmann et al. 2010). Results suggest that MALDI-TOF-MS is a powerful tool for rapid and accurate classification and identification of *Vibrio* species (Dieckmann et al. 2010, Cheng et al. 2015).

In view of the above, in this study we described the isolation and characterization of *Vibrio* species isolated from diseased freshwater and ornamental fish.

Materials and Methods

Sample collection and bacteriological analysis

Bacteria were isolated between July and September 2022 from diseased ornamental and freshwater fish, at the Department of Biology and Fish Diseases, Faculty of Veterinary Medicine, University of Life Sciences in Lublin, Poland (Table 1). The samples were taken once (fish were anesthetized with tricaine methane-sulfonate (MS-222) at 100 mg l^{-1}), and then the fish were euthanized with MS-222 at 200 mg l^{-1} in a water bath. For this reason, the approval of the Ethics Committee was not required. Samples were taken by swab from the liver, kidney, skin, and other affected areas (if present, e.g. ulcers). The isolates were streaked directly onto tryptic soy agar (TSA) (Sigma-Aldrich, USA), and the plates were incubated at 28°C for 48 h. The colonies obtained were distinguished based on their size, shape and colour and purified by streaking on fresh TSA plates. All isolates were stored at −80°C with the Microbank system (Pro-Lab Diagnostics, UK) until use.

The morphological characteristics of the colonies were visualized by Gram staining according to the standard procedure. A motility test was carried out to evaluate the capacity of colonies to swim in semi-liquid medium. For this purpose, LB (Invitrogen, Thermo Fisher Scientific, USA) plates with 0.3% (v/v) bacteriological agar (BioMaxima, Poland) were prepared. Plates were inoculated centrally with a fresh overnight bacterial culture in LB medium. The swimming zone was measured as the diameter of the zone travelled by the bacteria from point-inoculation after 24 h incubation (temperature 30°C).

The ability of *Vibrio* strains to produce haemolysins was tested on blood agar medium containing 5% (v/v) defibrinated sheep blood (BD Difco, USA). Each isolate was plated and incubated (37°C for 24 h), after which the plates were checked for signs of alpha-, beta, or gamma-haemolysis.

Matrix Assisted Laser Desorption/Ionisation – Time of Flight Mass Spectrometry (MALDI-TOF-MS)

MALDI-TOF-MS was conducted to identify *Vibrio* isolates to species level (Erler et al. 2015). The cellular proteins of all 6 strains were extracted using a standard ethanol/formic acid procedure, according to the manufacturer's recommendations for the UltrafleXtreme MALDI-TOF mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany).

Using an inoculation loop, 2 or 3 fresh bacterial colonies were completely suspended in 300 µl of ultrapure water (water for chromatography, LC-MS Grade, Sigma-Aldrich, USA) and vortexed. Next, 900 µl of absolute alcohol (≥99.8%, Stanlab, Poland) was added to the samples. The mixture was vortexed and centrifuged (13,000 x *g* for 2 min), and the supernatant was removed. The bacterial pellets were allowed to air-dry at room temperature for a few minutes. Once dried, the pellets were re-suspended in 50 µl of 70% formic acid (Sigma-Aldrich, USA) and vortexed (1 min). Then 50 µl of acetonitrile (≥99.99, Sigma-Aldrich, USA) was added to the samples, which were mixed thoroughly and centrifuged (same conditions). The resulting extract was spotted (1 µl) onto a 384-well MALDI target plate (Bruker, Germany) in 3 replicates, overlaid with 1 µl of HCCA matrix solution (10 mg/mL; α-cyano-hydroxicinnamid acid disolved in 50% acetonitrile/0.1% trifluoroacetic acid), and dried. A bacterial test standard (containing *Escherichia coli* DH5 alpha; Bruker, Germany) was used to calibrate the mass spectrometer and validate it to run. Mass spectra of bacteria were analysed using MALDI Biotyper 3.1. The interpretative score criteria were applied as recommended the manufacturer: a score ≥ 2.300 indicates highly probable species-level identification; a score from 2.00 to 2.299 indicates probable species and secure genus identification; a score between 1.700 and 1.999 indicates probable genus identification; and a score < 1.700 indicates unreliable identification.

A dendrogram showing the similarity between the main spectra of individual *Vibrio* strains was generated using dedicated tools implemented in MALDI Biotyper 3.1 software (Bruker, Germany). Hierarchical cluster analysis was performed using the unweighted pair group method with arithmetic mean (UPGMA).

Mass spectral profiles (MSP, Main Spectrum) were generated on the basis of mass spectra obtained for a given strain, taking into account all masses present in the spectra and their intensities.

Matching and grouping were performed using standard settings, i.e. maximum mass error tolerance for each spectrum – 2000 ppm, requested mass error for MSP – 200 ppm, requested minimum peak frequency – 25%, maximum requested number of peaks – 70.

Biochemical characterization

The commercial API-20NE and API-20E (for *V. vulnificus*) systems (BioMérieux, France) were used, following the manufacturer's instructions. Briefly, single bacterial colonies from an 18–24 hour culture on TSA medium were suspended in 0.85% NaCl solution (turbidity was equivalent to 0.5 McFarland standard). Each of the 20 wells of the test strips was filled with the prepared bacterial suspension. Mineral oil (anaerobic reaction conditions) was added to the marked wells. The test strips were incubated at 29°C for 24 h. After incubation and the addition of reagents, colour changes in the test wells were recorded based on the colour code included in the commercial kit. The API strips were read after 48-hr incubation too.

Antibiotic susceptibility

The antibiotic susceptibility of each *Vibrio* isolate was determined using the Kirby–Bauer disc diffusion assay (Bauer et al. 1966) on Mueller-Hinton agar medium (Sigma-Aldrich, USA) with commercially available disks (Oxoid UK, Biomaxima Poland). Discs with 12 antimicrobial agents from different groups were tested: ampicillin (AM, 10 µg), trimethoprim-sulfamethoxazole (SXT, 25 µg), cefuroxime (CXM, 30 µg), cefotaxime (CTX, 30 µg), meropenem (MEM, 10 µg), imipenem (IPM, 10 µg), amikacin (AK, 30 µg), gentamicin (CN, 10 µg), chloramphenicol (C, 30 µg), piperacillin-tazobactam (TZP, 100/10 µg), and tetracycline (TE, 30 µg). After the discs were applied, the plates were incubated at 32°C for 18 h. *Escherichia coli* strain ATCC 25922 was used as a quality control agent in the test.

Based on the inhibition zones surrounding the discs (mm), all strains tested were classified as resistant (R), intermediate (I) or sensitive (S) to the tested agents, according to the CLSI guidelines for *Vibrio* species – Clinical and Laboratory Standards Institute document M45 [CLSI 2015]. The index of multiple resistance to antibiotics (MAR) for the strains was determined using the formula MAR = a/b (Krumperman 1983), where *a* is the number of antibiotics to which the microorganism was resistant, and *b* is the total number of antibiotics for which the strain was assessed for susceptibility. MAR index values of >0.2 designate a high-risk source of contamination where antibiotics are often used.

Table 1. Clinical signs associated with *Vibrio* infections in diseased fish.

Species/ isolates	Host	Clinical signs of disease	Isolated from fish organs
V. albensis (n=4)			
01	Eurasian ruffe *Gymnocephalus cernua*	Skin discoloration haemorrhagic spots	Liver
02	European pike-perch *Sander lucioperca*	Skin discoloration haemorrhagic spots	Liver
03	Neon tetra *Paracheirodon innesi*	Ocular opacity, weakened condition, change of gill cover movement (accelerated movement)	Liver
04	Swordtail fish *Xiphophorus hellerii*	Skin discoloration haemorrhagic spots and superficial ulcers	Skin – ulcers
V. mimicus (n=1)			
01	Southern Platyfish *Xiphophorus maculatus*	Emaciation, lethargy, swimming disorders, caudal fin with frayed edges	Trunk kidney
V. vulnificus (n=1)			
01	Emperor tetra *Nematobrycon palmeri*	Lethargy, debilitated condition, no appetite, increased respiration	Liver

Results

Clinical signs of infected fish were nonspecific and are summarized in Table 1. Of the 76 samples analysed, a total of 23 colonies with characteristics of the genus *Vibrio* were identified by MALDI-TOF-MS, and 4 isolates (from *Gymnocephalus cernua*, *Sander lucioperca*, *Paracheirodon innesi*, and *Xiphophorus hellerii*) were confirmed as *V. albensis* (score values: 2.003, 1.986, 2.13, 1.982), one isolate (from *Xiphophorus maculatus*) was confirmed as *V. mimicus* (score value: 1.759), and one isolate (from *Nematobrycon palmeri*) was confirmed as *V. vulnificus* (score value: 1.921) (Table 1, Table 2). In the dendrogram based on MALDI-TOF-MS, the *Vibrio* isolates were differentiated into two clusters (Fig. 1). *V. albensis* (01, 02, 03 and 04) and *V. mimicus* 01 were closely related to the *V. albensis* and *V. mimicus* reference strains (cluster 1) and distantly related to the *V. harveyi* and *V. vulnificus* reference strains. *V. vulnificus* 01 was closely related to the *V. vulnificus* and *V. harveyi* reference strains (cluster 2) and distantly related to the *V. albensis* and *V. mimicus* reference strains (Fig. 1).

During the Gram staining and subsequent microscopic observations, all *Vibrio* isolates were found to be Gram-negative curved rods. In the API-20NE system, 7-digit profiles 7474744, 7474744, 7436744, 7676745, 7070745 and 7432445 were shown for the 6 *Vibrio* strains. All isolates showed a positive oxidase reaction, nitrate reduction, indole production, gelatine liquefaction, β-galactosidase production, and assimilation of potassium gluconate and malic acid, while negative results were obtained for arginine dihydrolase and assimilation of L-arabinose, cupric acid, adipic acid, and phenylacetic acid. All isolates exhibited swimming motility in 0.3% LB agar, with zone diameters ranging from 28 to 85 mm after 24-hr incubation (Table 3). Three isolates, i.e. *V. albensis* 01, *V. albensis* 02 and *V. albensis* 04, were able to cause partial haemolysis of erythrocytes in blood agar, producing a green zone around the colony (alpha-haemolysis). The green colour is due to the oxidation of haemoglobin to methaemoglobin by the hydrogen peroxide produced by the bacteria. None of the strains induced complete (beta-) haemolysis. The remaining strains (*V. albensis* 03, *V. mimicus* 01, and *V. vulnificus* 01) are non-haemolytic organisms (gamma-haemolysis) (Table 3). In the API-20E system, *V. vulnificus* 01 produced oxidase, β-galactosidase, lysine decarboxylase, ornithine decarboxylase, indole, and gelatinase, but not arginine dihydrolase, tryptophan deaminase, H_2S, or acetoin. It fermented D-glucose and amygdaline but not D-mannitol, inositol, D-sorbitol, L-rhamnose, D-saccharose, D-melibiose or L-arabinose, and it did not utilize citrate (API-20E identification number: 5346005, % id 99.9 – excellent identification) (data not shown).

All 6 *Vibrio* isolates showed high sensitivity to the 12 antibiotics used. Five of them (83%) were resistant to ampicillin, and only *Vibrio albensis* 03 showed intermediate resistance to this agent. The isolates were also resistant to CN (1 of 6), CP (1 of 6), C (1 of 6), and SXT (1 of 6), and showed intermediate resistance to CTX

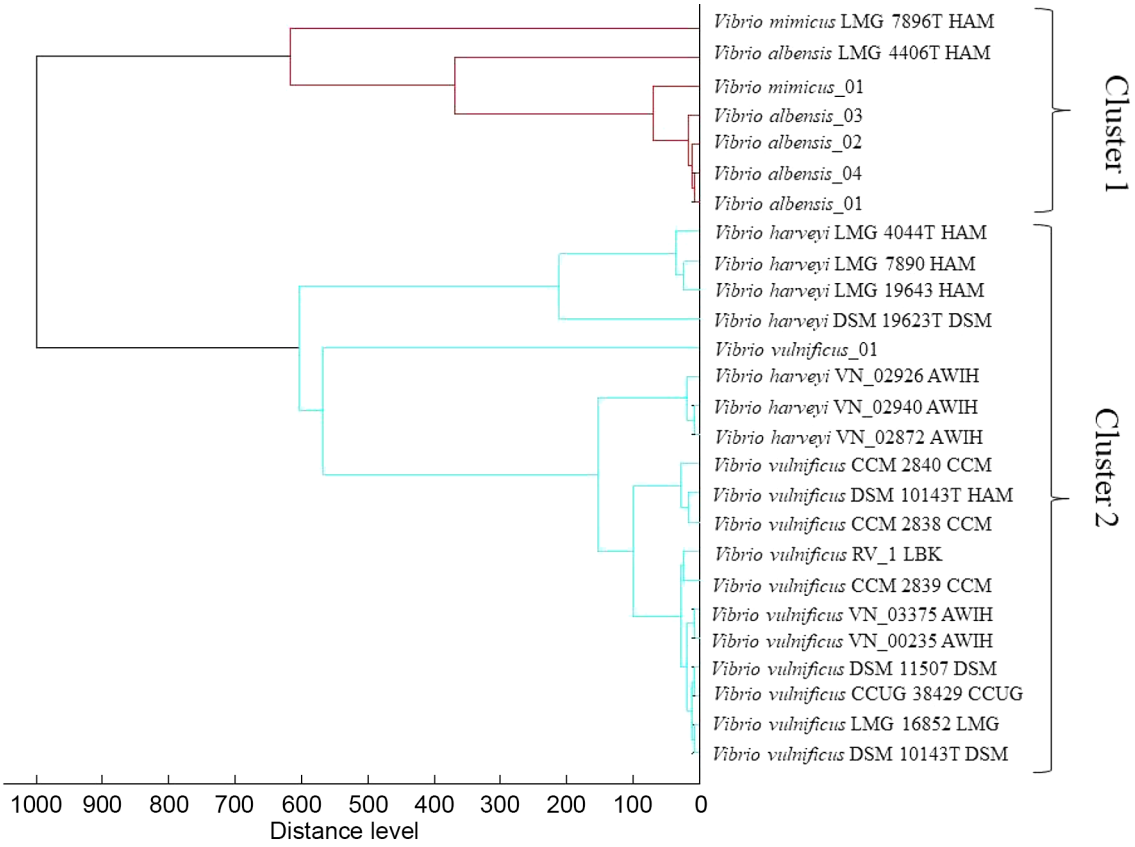

Fig. 1. Dendrogram generated using Bruker's MBT Compass Explorer based on MSP spectra, showing similarity between the *Vibrio* isolates tested in this study (*n=6*) and standard strains whose mass spectra were available in the reference database.

Table 2. Identification results using MALDI-TOF-MS for *Vibrio* isolates.

Isolate	Matched pattern	Score value	Consistency
V. albensis 01	*V. albensis* LMG 4406T HAM	2.003	B
V. albensis 02	*V. albensis* LMG 4406T HAM	1.986	B
V. albensis 03	*V. albensis* LMG 4406T HAM	2.13	B
V. albensis 04	*V. albensis* LMG 4406T HAM	1.982	B
V. mimicus 01	*V. mimicus* LMG 7896T HAM	1.759	B
V. vulnificus 01	*V. vulnificus* CCM 2838 CCM	1.921	B

(1 of 6), AK (2 of 6), CIP (2 of 6) and C (1 of 6). In addition, 50% of them (3 of 6) displayed intermediate resistance to IPM and TE. All tested strains were susceptible to piperacillin-tazobactam, cefuroxime, and meropenem (Table 3). The highest MAR index among the 6 isolates was observed for *V. vulnificus* 01; it was 0.33, as shown in Table 4.

Discussion

Vibriosis is a very important bacterial disease in fish and can cause economic losses (El-Deen and Elkamel 2015, Osunla and Okoh 2017). Manfrin et al. (2001) pointed out that importing ornamental fish poses a potential risk by introducing pathogens for fish and humans. This study is the first report documenting the presence, identification, and antibiotic resistance of *Vibrio* sp. in freshwater and ornamental fish in Poland. The diseased fish presented variability in their external symptoms, i.e. noticeable skin discoloration, haemorrhaging, ulcers, swimming disorders, eroded fins, lethargy, and increased respiration.

V. albensis (also known as non-O1 serovar *Vibrio cholerae*) is a luminescent organism that biochemically resembles *V. cholerae*. Non-O1 and non-O139 *V. cholerae* serotypes (also called non-agglutinable, NAG serotypes) have been implicated as etiologic agents of diseases in humans. NAG serotypes have

Table 3. Morphological and biochemical properties of six *Vibrio* isolates.

Characteristics	*V. albensis* 01	*V. albensis* 02	*V. albensis* 03	*V. albensis* 04	*V. mimicus* 01	*V. vulnificus* 01
Gram reaction	-	-	-	-	-	-
Shape	curved rods	curved rods	curved rods	curved rods	curved rods	curved rods
Motility - swimming (ability/z.d)	+/28	+/28	+/29	+/71	+/68	+/85
Hemolytic test	α-hemolysis	α-hemolysis	γ-hemolysis	α-hemolysis	γ-hemolysis	γ-hemolysis
Oxidase reaction	+	+	+	+	+	+
Nitrate reduction: $NO_3 \rightarrow NO_2$	+	+	+	+	+	+
Indole production	+	+	+	+	+	+
Glucose fermentation	+	+	-	+	+	+
Arginine dihydrolase	-	-	-	-	-	-
Urease production	-	-	-	+	-	-
Aesculin hydrolysis	+	+	+	+	-	+
Gelatin liquefaction	+	+	+	+	+	+
β-galactosidase production	+	+	+	+	+	+
D-Glucose	+	+	-	+	+	-
L-Arabinose	-	-	-	-	-	-
D-Mannose	-	-	+	+	-	+
D-Mannitol	+	+	+	+	-	-
N-Acetyl-glucosamine	+	+	+	+	+	-
D-Maltose	+	+	+	+	+	-
Potassium gluconate	+	+	+	+	+	+
Capric acid	-	-	-	-	-	-
Adipic acid	-	-	-	-	-	-
Malic acid	+	+	+	+	+	+
Trisodium citrate	-	-	-	+	+	+
Phenylacetic acid	-	-	-	-	-	-
API-20NE code number	7474744	7474744	6436744	7676745	7070745	7132445
API-20NE identification	nd	nd	nd	nd	*V. cholerae*	*V. vulnificus*
API-20NE % identification	nd	nd	nd	nd	99.5	99.3

+, indicates positives results; -, indicates negative results; +/-, indicates inconclusive result; z.d, indicates zone diameter in mm; nd, not detected

Table 4. Antibiotic resistance of *Vibrio* species isolated from freshwater and ornamental fish.

Antibiotics	*V. albensis* 01	*V. albensis* 02	*V. albensis* 03	*V. albensis* 04	*V. mimicus* 01	*V. vulnificus* 01
Ampicillin (10 µg)	R	R	I	R	R	R
Piperacillin-tazobactam (100/10 µg)	S	S	S	S	S	S
Cefuroxime (30 µg)	S	S	S	S	S	S
Cefotaxime (30 µg)	S	S	S	I	S	S
Meropenem (10 µg)	S	S	S	S	S	S
Imipenem (10 µg)	S	S	I	S	I	I
Amikacin (30 µg)	S	S	S	S	I	I
Gentamicin (10 µg)	S	S	S	S	S	R
Tetracycline (30 µg)	S	S	S	I	I	I
Ciprofloxacin (5 µg)	S	S	I	I	S	R
Sulfamethoxazole-trimetho-prim (1.25/23.75 µg)	S	S	S	S	S	R
Chloramphenicol (30 µg)	S	S	S	S	R	I
MAR index	0.08	0.08	0.00	0.08	0.17	0.33

R, indicates resistant species; I, indicates intermediate species; S, indicates sensitive species; MAR, multiple antimicrobial resistance

been reported to cause sporadic cases of gastroenteritis or extraintestinal infections, wound infections, ocular infections, and urinary tract infections. In Europe, human infections with NAG serotypes are rare, and reported cases are sporadic (Stypulkowska-Misiurewicz et al. 2006, Araj et al. 2019). They are most commonly isolated from environmental sources, and have been reported in cases of fish diseases (Kiiyukia et al. 1992, Dong et al. 2015, Rehulka et al. 2015, Kiani et al. 2016, Zago et al. 2017, Kolada et al. 2022). Fish infected by *Vibrio albensis* usually suffer from petechial haemorrhages on the surface of the body (Austin and Austin 2007). *V. albensis* is a fish pathogen that is not well-described. It has usually been identified as a foodborne pathogen that could cause serious illness in humans. Previous studies indicated that fish normally serve as reservoirs and vectors of these zoonotic bacteria (Senderovich et al. 2010, Dong et al. 2015, Rehulka et al. 2015). In our study, four *V. albensis* strains were isolated from diseased freshwater fish. MALDI-TOF-MS was used to confirm four isolates as *V. albensis*, but the API-20NE test did not identify them. All strains were negative for arginine dihydrolase, L-arabinose, capric acid, adipic acid, and phenylacetic acid. The data from this study suggest that the API-20NE test is not an acceptable method for the identification of the *V. albensis* strains tested in our study.

V. mimicus is closely related to *V. cholera* and causes gastroenteritis in humans, characterized by diarrhoea, nausea, vomiting, abdominal pain, and fever. Moreover, it is found to be distributed in seawater, brackish water, and freshwater, and to cause diseases with high mortality in fish (Geng et al. 2014, Zhang et al. 2014). The infection is characterized by the presence of bleeding and regularly-shaped ulcers. *V. mimicus* has also been isolated from ornamental fish from various countries, i.e. Iran, China, Singapore, and Thailand (Kiani et al. 2016, Zago et al. 2017). In our study, one *V. mimicus* strain was isolated from a diseased ornamental fish, *Xiphophorus maculatus*. MALDI-TOF-MS was used to confirm our isolate as *V. mimicus*, but the API-20NE test identified it as *V. cholera* with 99.5% identification (presumptive identification). The result of identification based on the API-20NE test did not coincide with the MALDI-TOF -MS method, due to the high similarity of strains (*V. mimicus* and *V. cholerae*). Similar cases of inconsistency in species identification of *Vibrio* isolates from ornamental fish are known from the literature (Zago et al. 2017).

V. vulnificus is a heterogeneous bacterial species that comprises virulent and avirulent strains. Based on phenotypic characteristics, *V. vulnificus* strains were grouped into three biotypes. Biotype 1 is ornithine decarboxylase- and indole-positive, biotype 2 is ornithine decarboxylase- and indole-negative, and biotype 3 is cellobiose-negative (Tison et al. 1982, Bisharat et al. 1999, Warner and Olivier 2008). In addition, Esteve et al. (2007) described wild-type *V. vulnificus* biotype 2, serovar A strains, which were non-motile due to the lack of a sheathed polar flagellum. The literature indicates that three biotypes are potentially pathogenic for humans (Baker-Austin et al. 2018). Human infections caused by *V. vulnificus* have been reported in northern Poland (Aksak-Wąs et al. 2021). Research by Kurpas et al. (2021) confirmed the presence of potentially pathogenic *V. vulnificus* in the Gulf of Gdansk. In our study, MALDI-TOF-MS was used to confirm one isolate, from a diseased ornamental fish *Nematobrycon palmeri*, as *V. vulnificus*. The API-20E and API-20NE tests identified *V. vulnificus* 01 as *V. vulnificus* with 99.9% identification (excellent identification) and 99.3% identification (very good identification), respectively. The isolate was positive for ornithine decarboxylase and indole production, and it fermented D-mannitol and D-sorbitol.

Antibiotics are widely used to prevent or treat bacterial diseases in aquaculture. Antimicrobial resistant bacteria cause over 33,000 human deaths annually in the European Economic Area (Cassini et al. 2019). Monitoring the spread of antibiotic resistance is important for assessing the role of *Vibrio* species. In this study, *Vibrio albensis* (01-04) and *V. mimicus* 01 isolates showed a similar drug resistance pattern, being sensitive/intermediate to AK, CXM, CTX, CIP, CN, IPM, MEM, TZP, SXT, and TE. All strains (except *V. albensis* 03) were resistant to AM, and *V. mimicus* 01 was additionally resistant to C. The MAR index for *V. albensis* and *V. mimicus* strains ranged from 0.00 to 0.17. A MAR index value ≤ 0.2 is considered to be indicative of strains originating in animals in which antibiotics are seldom or never used (Krumperman 1983). This resistance pattern was similar to those reported by Lupiani et al. (1993). Noorlis et al. (2011) reported high MICs for TE, furazolidone, bacitracin, vancomycin, cephalothin, and erythromycin against *V. cholera* isolates from fish, with a MAR index ranging from 0.13 to 0.93. In this study, the *V. vulnificus* 01 strain was resistant to commonly used drugs (AM, CN, CIP and SXT), with MAR index of 0.33. Bacteria having an MAR index higher than 0.2 originate from a high-risk source of contamination where antibiotics are used. In a study by Al-Dulaimi et al. (2019), *V. vulnificus* isolates showed high rates of antibiotic resistance against two or more antibiotics. According to the literature, *Vibrio vulnificus* has shown resistance to amoxicillin, fosfomycin, nitrofurantoin, penicillin V, polymyxin B, sulfanilamide, sulfadimethoxine (Tison et al. 1982), AK, CN, TE, apramycin, azithromycin,

cephalexin, streptomycin, nalidixic acid, trimethoprim (Baker-Austin et al. 2009), C (Shaw et al. 2014), AK, CTX, MEM cefepime, cefoxitin, ceftazidime, ceftriaxone, cephalothin and cefepime (DaSilva et al. 2021).

Conclusions

The study showed that *Vibrio* spp. including *V. albensis*, *V. mimicus* and *V. vulnificus*, are present in diseased freshwater and ornamental fish in Poland. All *Vibrio* isolates can be successfully identified to the species level by MALDI-TOF-MS. The biochemical protocols proposed for the API 20NE strips seem to be of limited importance for the identification and differentiation of *V. albensis* and *V. mimicus* strains, but are an adequate method for the identification of *V. vulnificus*. This study suggests that multiple-antibiotic-resistant *Vibrio* species, especially *V. vulnificus*, can be recovered from diseased ornamental fish, and these sources may be a reservoir responsible for pathogenic bacteria. Further monitoring of the presence of *Vibrio* species, mainly in aquarium fish, is necessary.

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