

Bacterial flora associated with diseased freshwater ornamental fish

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Abstract

Introduction: Ornamental fish can suffer from different bacterial diseases. Among them the most prevalent are infections caused by *Aeromonas*, *Shewanella*, *Citrobacter*, *Plesiomonas*, *Edwardsiella*, and *Pseudomonas*. But there is a broad spectrum of rarely identified bacteria which may be causative agents of diseases. The aim of the study was to determine the species of bacteria pathogenic for fish which are prevalent in aquariums. **Material and Methods:** Bacteria were isolated from infected ornamental fish from pet shops and private aquariums in the Lublin region in 2015 and classified to species using MALDI-TOF MS. **Results:** A total of 182 isolates from ornamental fish were identified. The most frequent bacteria found in diseased fish were *Aeromonas veronii* (30.8% of total number of strains), *A. hydrophila* (18.7%), *Shewanella putrefaciens* (7.1%), *Citrobacter freundii* (7.1%), *Pseudomonas* spp. (7.1%), *Shewanella baltica* (4.9%), and *Plesiomonas shigelloides* (3.3%). **Conclusion:** Isolated bacterial species are facultative pathogens for fish and humans and may be isolated from fish without apparent symptoms of the disease.

Keywords: ornamental fish, fish pathogens, MALDI-TOF MS method, species identification.

Introduction

The ornamental fish trade is a developing worldwide industry. According to data of the European Pet Food Industry Federation, there are 4,500 species of ornamental freshwater fish and 1,450 species of marine ornamental fish. More than 50% of ornamental fish originate from Thailand, Japan, China, Malaysia, and Sri Lanka (20). The European Union (EU) is one of the largest importers of ornamental fish (<http://www.ornamentalfish.org/wp-content/uploads/EU-Trade-Stats-Report-2015>) and the Czech Republic and the Netherlands are reported among the largest European global exporters of ornamental fish. Statistics show that in the EU, Poland holds the seventh place in terms of number of ornamental pet fish (data from 2014, <http://www.fediaf.org/facts-figures/>), estimated at 280,000, which means that aquariums are popular in Polish households. The worldwide development of the ornamental fish trade favours the wide spread of their pathogens, such as bacteria, parasites, fungi, and viruses, which are often transported with fish. This increases the risk of human infection. The bacteria most frequently described as fish pathogens are

Aeromonas (28), *Edwardsiella* (5, 21), *Pseudomonas* (3, 25), *Shewanella* (13), *Mycobacterium* (30), *Streptococcus* (25), and *Flavobacterium* (25), of which some are common in Polish waters (12, 15). However, there is a risk that fish imported from distant countries are reservoirs of atypical bacteria for Polish environments. These microorganisms can spread uncontrollably during the transfer of fish from one aquarium to another. It results in the emergence of new types of infections in aquatic organisms. These factors contribute to the limited state of knowledge about ornamental fish diseases and their causative agents.

The aim of this study was identification of bacterial species isolated from freshwater ornamental fish obtained from pet shops and private aquariums in the Lublin region.

Material and Methods

Sampling of freshwater ornamental fish. A total of 62 diseased ornamental fish, belonging to 27 different species, were sampled and transported alive to the laboratory of the Department of Fish Diseases

and Biology, Faculty of Veterinary Medicine, University of Life Sciences in Lublin, for bacteriological examination during the period from January 2015 to December 2015. The species investigated were *Danio rerio* (n = 10), *Puntigrus tetrazona* (n = 5), *Carassius auratus* (n = 3), *Xiphophorus helleri* (n = 3), *Poecilia reticulata* (n = 5), *Poecilia sphenops* (n = 2), *Symphosodon discus* (n = 1), *Trichogaster trichopterus* (n = 4), *T. lalius* (n = 1), *Ancistrus leucostictus* (n = 2), *Gymnocorymbus ternetzi* (n = 2), *Pterophyllum scalare* (n = 2), *Trigonostigma heteromorpha* (n = 2), *Xiphophorus maculatus* (n = 3), *Labidochromis caeruleus* (n = 3), *Paracheirodon innesi* (n = 2), *Chromobotia macracanthus* (n = 1), *Microgeophagus ramirezi* (n = 1), *Betta splendens* (n = 2), *Tanichthys albonubes* (n = 1), *Macropodus opercularis* (n = 1), *Crossocheilus oblongus* (n = 1), *Pterygoplichthys gibbiceps* (n = 1), *Hyphessobrycon bentosi* (n = 1), *Pangio kuhlii* (n = 1), *Pseudotropheus saulosi* (n = 1), and *Cichlasoma synspilum* x *Cichlasoma citrinellum* (n = 1). Fish presenting at least one clinical sign of disease (skin ulcerations, abdominal distension, scale protrusion, haemorrhages on skin and fins, fin rot, distortion of body, or lack of appetite) were collected from pet shops and private aquariums located in the Lublin region of Poland.

Bacterial isolation. All diseased fish were euthanised using an overdose of ethyl 3-aminobenzoate methanesulfonate (MS-222, Sigma Aldrich, USA). Samples were taken from the skin ulcers, kidneys, and gills, and streaked on tryptic soy agar (TSA). Plates were then incubated at 28°C for 48 h. The strains were subcultured at least twice, or until a homogeneous cell culture was observed.

Identification of species by MALDI-TOF mass spectrometry. MALDI-TOF-MS (Matrix-Assisted Laser Desorption/Ionisation Time of Flight Mass Spectrometry) analysis was used for identification of bacterial species. Isolates were prepared for mass spectrometry according to a standard protocol recommended by the Bruker company.

The isolates from TSA culture were used for mass spectrometry measurements after 48 h of incubation. Bacterial cells from one single colony were suspended in 300 µL of ultra-pure water. The samples were mixed by vortexing vigorously for 1 min, after which 900 µL of LC (liquid chromatography) grade ethanol was added. The samples were stored at -20°C until use. These mixtures were centrifuged at 13,000 x g for 2 min and residual supernatants were discarded. A total of 50 µL of 70% formic acid was added to the pellet and mixed to resuspend the bacteria for 1 min. Then 50 µL acetonitrile was added and the samples were mixed. The solutions were centrifuged at 13,000 x g for 2 min. The supernatants were transferred to a new tube immediately. Processed samples were placed in a 384 steel plate, allowed to dry, and covered with a matrix solution (α -cyano-4-hydroxy cinnamic acid, HCCA)

(Bruker Daltonics, Germany) and air-dried at room temperature.

The protein analysis by MALDI-TOF-MS was performed in an UltrafleXtreme mass spectrometry unit (Bruker Daltonics, Germany). Prior to analysis, the mass spectrometer was externally calibrated with a Bacterial Test Standard (Bruker, Germany) containing previously prepared extract of *Escherichia coli* DH5 alpha. Spectra were recorded in the positive linear mode at a laser frequency of 20 Hz within a mass range from 2 to 20 kDa. Parameter settings for the instrument were ion source 1 at 25 kV, ion source 2 at 18.5 kV, lens at 8.5 kV, pulsed ion extraction of 250 ns, and no gating, under control of FlexControl software (version 3.0, Bruker Daltonics). In order to assess the reproducibility of MALDI-TOF-MS identification, every strain studied was tested in duplicate (*i.e.*, on two different MALDI-TOF-MS plates run on two different days and two different batches of culture medium).

The MALDI-TOF Biotyper (version 3.0, Bruker Daltonics) has been used to identify microorganisms. For MALDI-TOF-MS analysis, we adapted the score values proposed by the manufacturer: a score ≥ 1.999 indicated species identification, a score of 1.7–1.999 generally meant a reliable identification of bacteria, and < 1.7 signified no identification. An isolate was considered correctly identified by MALDI-TOF-MS when $\geq 3/4$ spectra had a score ≥ 1.999 for species or ≥ 1.7 for genus identification.

Results

Protein profiles of 210 isolates were studied by the MALDI-TOF MS method. In total 182 isolates were successfully identified. The most frequent bacteria found in diseased fish were *Aeromonas veronii* (n = 56; 30.8% of total number of strains) and *A. hydrophila* (n = 34; 18.7%), followed by *Shewanella putrefaciens* (n = 13; 7.1%), *Citrobacter freundii* (n = 13; 7.1%), *Pseudomonas* spp. (n = 12; 6.6%), *Shewanella baltica* (n = 9; 4.9%), and *Plesiomonas shigelloides* (n = 6; 3.3%) (Fig. 1). Among the genus *Pseudomonas*, eight species were identified: *P. alcaligenes* (n = 1), *P. putida* (n = 5), *P. mosselii* (n = 1), *P. marginalis* (n = 1), *P. monteilii* (n = 1), *P. koreensis* (n = 1), *P. oleovorans* (n = 1), and *P. nitroreducens* (n = 1). The following strains were rarely identified: *Acinetobacter junii* (n = 4; 2.2%), *Aeromonas caviae* (n = 4; 2.2%), *A. lwoffii* (n = 2; 1.1%), and *A. media* (n = 2; 1.1%). Colonies defined as rare species were also observed: *Listonella anguillarum* (n = 2; 1.1%), *Budvicia aquatica* (n = 2; 1.1%), *Bacillus cereus* (n = 1; 0.5%), *Providencia rettgeri* (n = 1; 0.5%), *Edwardsiella tarda* (n = 1; 0.5%), *Rhodococcus erythropolis* (n = 1; 0.5%), *Brevibacterium casei* (n = 1; 0.5%), *Citrobacter murlinae* (n = 1; 0.5%), and *Serratia ureilytica* (n = 1; 0.5%).

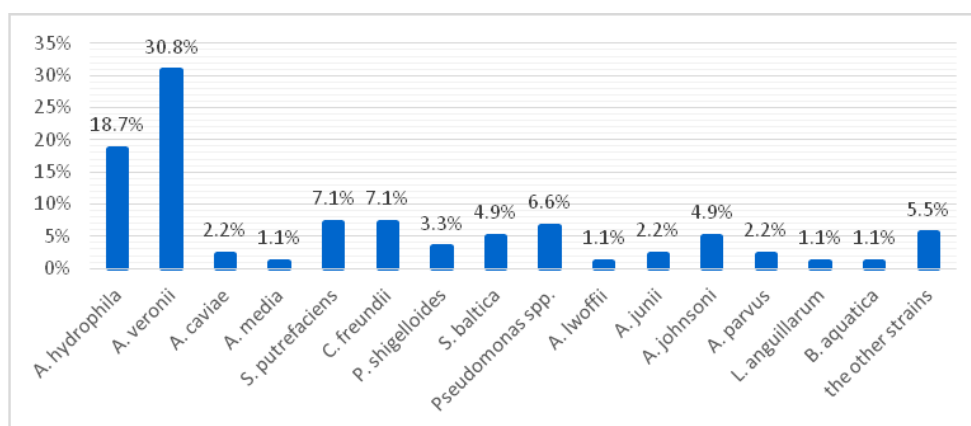


Fig. 1. Percentage of bacteria species isolated from 62 ornamental fish in Lublin region in 2015. The group of “the other strains” comprises samples which contain a single identified species: *A. scleromae*, *B. cereus*, *B. casei*, *C. braakii*, *C. murliniae*, *C. testosteroni*, *E. tarda*, *P. rettgeri*, *R. erythropolis*, and *S. ureilytica*

Discussion

Aeromonads are probably the most commonly encountered bacterial pathogens of freshwater fish. Several species may be responsible for motile *Aeromonas* infections and/or motile *Aeromonas* septicaemia in ornamental fish. In our study, bacterial infections associated with motile *Aeromonas hydrophila* were reported in 14 species of fish (*D. rerio*, *X. helleri*, *X. maculatus*, *A. leuconosticus*, *P. gibbiceps*, *C. auratus*, *G. ternetzi*, *P. tetrazona*, *P. sphenops*, *P. reticulata*, *P. innessi*, *T. lalius*, *P. saulosi*, and *M. ramirezi*). Infections caused by *A. veronii* were reported in 15 species (*D. rerio*, *X. helleri*, *P. reticulata*, *L. ceruleus*, *X. maculatus*, *P. kahli*, *A. leuconosticus*, *P. gibbiceps*, *T. trichopterus*, *C. auratus*, *G. ternetzi*, *P. tetrazona*, *P. sphenops*, *P. innessi*, and *P. saulosi*). *A. caviae* and *A. media* were recorded in *P. reticulata* and *P. saulosi*, whereas *A. media* were revealed in *T. trichopterus* and *M. opercularis*. The most frequently identified species in our study, *A. veronii* and *A. hydrophila*, were mainly isolated from ulcers.

Among the *Pseudomonas* species recovered from diseased fish, *P. anguilliseptica* is considered the most significant pathogen for cultured fish (3). *Pseudomonas* spp. is commonly isolated from ornamental fish (23, 25). In our study, diseases caused by different species of the genus *Pseudomonas* affected seven fish species (*D. rerio*, *X. helleri*, *L. caeruleus*, *X. maculatus*, *T. trichopterus*, *G. ternetzi*, and *P. innesi*). *Pseudomonas* spp. is a member of the normal bacterial flora of fresh water, often acting as an opportunistic pathogen to fish under stress conditions. A number of factors contribute to outbreaks of bacterial diseases, of which poor water quality, overcrowding, and stress are most common in aquaria (23).

Inappetence, skin ulcers, darkening of the skin, exophthalmia, and ascites were reported in outbreaks of *S. putrefaciens* infection (1, 13, 14). *S. putrefaciens* and *S. baltica* are considered inhabitants of marine and brackish water (22); however, in this study, 26 strains of

Shewanella were isolated from 11 fresh water fish species (*D. rerio*, *P. reticulata*, *X. maculatus*, *A. leuconosticus*, *T. trichopterus*, *C. auratus*, *P. tetrazona*, *C. macracanthus*, *M. opercularis*, *P. saulosi*, and *M. ramirezi*) with symptoms as mentioned above. Relatively recently, *S. putrefaciens* was reported for the first time in freshwater fish in Poland (14). Now, both these *Shewanella* species seem to be common pathogens of ornamental and pond-raised fish in Poland (22).

Edwardsiellosis has rarely been identified as a significant pathogen to ornamental fish. *E. ictaluri* was identified as causing disease in the green knife fish (*Eigenmannia virescens*), rosy barbs (*Puntius conchonius*), and zebrafish (*D. rerio*) (5). Both *E. ictaluri* and *E. tarda* were isolated from fighting fish (*Betta splendens*) (27). Edwardsiellosis caused by *E. tarda* is a disease with many symptoms, like loss of pigmentation, exophthalmia, and spiral movement (21). In this study, abnormal swimming in a parrot cichlid and lesions in internal organs were observed. *E. tarda* and also *A. caviae* were identified in this case of disease.

Cases of diseases caused by *Citrobacter* spp. have been described on more than one occasion in literature (19, 25). In our study, strains of this genus were isolated from *G. ternetzi*, *S. discus*, *M. opercularis*, *T. albonubes*, *P. sphenops*, and *P. scalare*, in this last fish in coexistence with the *C. braakii* strain. Emaciation and apathy were the main symptoms of coinfections of *Citrobacter* spp. Although *C. freundii* and *C. braakii* are commonly isolated fish pathogens (19), *C. murliniae* had not yet been described in the context of fish infections. In our study, *C. murliniae* was recovered from ulcers of *C. auratus* with other bacterial species, such as *A. veronii*, *A. hydrophila*, *C. freundii*, and *S. ureilytica*. *S. ureilytica* is a novel species in the genus *Serratia*, first isolated from freshwater fish in India (4). There is no available information about its pathogenicity for aquatic organisms; however, the genus is classified as a fish pathogen.

There are reports of pathogenicity of *P. shigelloides* to fish (17). *P. shigelloides* can occur as natural intestinal flora of fish, but in case of stress conditions the following symptoms are observed: darkening of body, haemorrhaging, fin rotting, ascitic fluid in the abdominal cavity, and lesions in internal organs (17). In this study, coinfection with *Aeromonas* spp. was observed. Six strains of *P. shigelloides* were isolated from the two fish species: *D. rerio* and *T. trichopterus*.

The presence of the atypical bacteria: *P. rettgeri*, *B. aquatica*, and *R. erythropolis* among the isolates is interesting. These species were isolated from the liver and kidneys of *S. discus* with emaciation and darkening of skin. *B. aquatica* was isolated from surface and sewage water (24), but it is considered to be non-pathogenic for humans and animals. There is no description of disease caused by *R. erythropolis* in ornamental fish. Available reports of *R. erythropolis* infections in fish are related to its presence in Atlantic salmon (*Salmo salar* L.) (18). Recent reports about *P. rettgeri* pertain to its occurrence in *Lagocephalus* pufferfish known as fugu (26).

L. anguillarum (earlier known as *Vibrio anguillarum*) is described as the causative agent of vibriosis among marine and freshwater fish (9). In this study *L. anguillarum* was isolated from *C. macracanthus* with no specific symptoms.

Many of these bacteria have pathogenic significance for humans. Species of *Aeromonas* are responsible for infections of the skin, urinary tract, and posttraumatic wounds (16). *Citrobacter* spp. is a causative agent of brain abscess and meningitis in neonates (2) and intestinal and urinary tract infections have also been reported (10). *Shewanella* spp. can cause dangerous infections: one case of monomicrobial *S. putrefaciens* infective endocarditis and several cases of osteomyelitis have been diagnosed (6, 7, 11). *P. shigelloides* and *P. rettgeri* have been associated with gastroenteritis and diarrhoea in travellers who have ingested contaminated water or food (8). Species representing the genus *Pseudomonas* were isolated from blood, urine, and sputum of affected patients (29). All these pathogens can cause bacteraemia, especially in immunocompromised persons.

The obtained results indicate that infections with *Aeromonas* are the most common in aquaria and in many cases coexist with infections with other pathogenic bacteria. In most cases, several types of bacteria were isolated from one individual. In this situation, determination of the causative agent of disease can be difficult without conducting a challenge test. The biodiversity of microorganisms inhabiting aquarium tanks in Lublin and infecting fish is high and includes many strains of bacteria. The presence of exotic, atypical bacteria in aquaria increases the risk of infections among people who take care of ornamental fish.

In conclusion, numerous bacterial strains colonising freshwater ornamental fish in the Lublin region of Poland were identified. The identification of

Aeromonas spp., *S. putrefaciens*, *P. shigelloides*, *Acinetobacter* spp., and *Citrobacter* spp. in ornamental fish is important due to the zoonotic potential of these bacteria.

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