Antimicrobial Resistance Patterns of Aeromonas spp. Isolated from Ornamental Fish

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Abstract

The potential risk of occurrence of new diseases associated with the trade of live animals is well known. However, little importance is still given to the problematic of the dissemination of resistance genes that pass along with the animal trade. In this study we aimed to isolate Aeromonas spp. strains from water and skin of ornamental fish and test their resistance to antibiotics. The samples were collected from a national ornamental fish importer, with the intent of obtaining a collection of Aeromonas strains. The identification of the strains was made by gyrB and rpoD gene sequencing. A total of 288 strains grouped in seven different species - Aeromonas veronii, Aeromonas media, Aeromonas jandaei, Aeromonas hydrophila, Aeromonas caviae, Aeromonas salmonicida, Aeromonas aquariorum, were isolated. The susceptibility profile was determined for 28 antibiotics commonly used. All the strains presented multiresistance to the tested antibiotics. The antibiotic susceptibility profile to tetracycline, ticloprin, carbencillin, ampicillin and erythromycin revealed resistance levels of more than 80%. Few strains resistant to aztreonam and imipenem were identified. On the other hand, all were sensitive to cepotaxime and cefepime. The results show that these Aeromonas spp. strains are potentially reservoirs of antibiotic resistance genes.

Keywords: Aeromonas spp.; Antibiotic resistance; Ornamental fish

Introduction

Bacterial disease is one of the most important diseases in ornamental fishes and a significant cause of high fish morbidity and mortality rates [1]. Many stress factors could contribute to bacterial infection in ornamental fish, namely, poor water quality, crowding, transportation and inadequate nutrition [2].

The genus Aeromonas belongs to the family Aeromonadaceae within the Gammaproteobacteria and comprises Gram-negative, nonspore-forming, motile bacilli or coccobacilli rods with rounded ends which measure 1-3.5 µm across [3]. They are facultative anaerobic, oxidase, catalase and indol-positive, able to reduce nitrate to nitrite and are, glucose-fermenting, generally resistant to the vibriostatic agent O/129 [4,5].

Members of the genus Aeromonas are found in a wide variety of ecological niches. They are able to inhabit surface water (rivers, lakes), sewage, drinking water (tap and bottled mineral), thermal water and sea water [6,7]. Some species, mainly the psychrophilic Aeromonas salmonicida and the mesophilic Aeromonas hydrophila and Aeromonas veronii are recognized causative agents of fish disease [3,8].

Infections caused by motile aeromonads are probably the most common bacterial disease of freshwater fish [9]. Resistance of Aeromonas spp. to commonly used antibiotics is an emerging problem in the ornamental fish. An increase in resistance levels of the genus Aeromonas, particularly to β-lactam antibiotics has been observed previously [10,11]. Antimicrobial resistance genes, including cassette-borne resistance genes in class I integrons, have been described as occurring in A. salmonicida and in motile aeromonads [12-14].

The objective of the present study is to isolate and identify Aeromonas spp. from the water of aquarium and the skin of imported ornamental fish and to evaluate their susceptibility to some antimicrobial agents.

Materials and Methods

Bacteria strains isolation and identification

This evaluation was conducted with samples of skin and water (30 and 14 according to the fish and tanks available, respectively) from imported ornamental fish. Water samples filtered onto nitrocellulose membranes and from fish skin were collected aseptically and incubated at 30°C for 24 h on GSP media (Oxoid, Basingstoke, UK). This media was used to isolate (typical colonies, i.e. yellow on GSP medium) and purify the strains. Bacteria strains were identified, following standard procedures, to identify Aeromonas at the genus level, and further standard biochemical classification was performed by using API 50 CH (bioMérieux) at 30°C for 48 h, following the manufacturer’s instructions. Procedures and characteristics of oligonucleotide primers for amplification and PCR-based sequencing house-keeping genes (gyrB and rpoD) are as described previously [15]. PCR products were purified with QIAquick PCR purification kit (QIAGEN, Germany), following the manufacturer’s instructions and prepared for sequencing by using the Big Dye Terminator V3.1 cycle sequencing kit and amplified sequences were sequenced with an ABI PRISM 3100 Genetic Analyser (Applied Biosystems, USA).

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Antibiotic susceptibility testing

*Aeromonas* spp. strains isolated in the present study were subjected to susceptibility testing against 28 antimicrobials commonly used. Susceptibility was determined by the disk-diffusion technique of Kirby-Bauer on Mueller-Hinton agar plates (Oxoid Basingstoke, UK) with inocula adjusted to an optical density of 0.5 McFarland standard units [16]. Disks containing ampicillin (AMP), carbenicillin (CAR100µg), amoxicillin (AML), amoxicillin/ clavulanic acid (AMC), piperacillin (PRL), piperacillin/ tazobactam (TZP), ticarcillin (TIC), ticarcillin/ clavulanic acid (TIM), cephalothin (KF), cefoxitin (FOX), cefotaxime (CTX), ceperoxazone (CPX), ceftazidime (CAZ), ceftriaxone (CRO), cefepime (FEP), aztreonam (ATM), imipenem (IMP), gentamicin (CN), kanamycin (K), tobramycin (TOB), amikacin (AK), netilmicin (NET), tetracycline (TE), ciprofloxacin (CIP), norfloxacin (NOR), erythromycin (E), trimethoprim/sulfamethoxazole (SXT) and chloramphenicol (C) were used. All disks were obtained from Oxoid.

After 24 h incubation at 30°C, organisms were classified as sensitive (S), intermediate resistant (I) or resistant (R) on the basis of the size of the zone of bacteria growth inhibition according to the guidelines of the CLSI (2010).

**Results and Discussion**

The genus *Aeromonas* has been the subject of various antimicrobial susceptibility studies over the last years. Although *Aeromonas* species are distributed throughout the world, there are geographic differences in the frequency of diseases caused by these bacteria [3].

A total number of 299 isolates were obtained from aquaria of ornamental fish shops (221 from skin and 77 from water). Using gyrB and rpoD sequencing several species of *Aeromonas* were identified, namely 110 *Aeromonas veronii* (36, 8%), 106 *Aeromonas hydrophila* (35, 5%), 43 *Aeromonas aquariorum* (14, 4%), 24 *Aeromonas cunicola* (8, 0%), 3 *Aeromonas media* (1, 0%), one *Aeromonas caviae* (0, 4%), and one *Aeromonas jandaei* (0, 4%). *A. hydrophila* has been the most common bacteria associated with aquatic animal disease. In a Malaysian aquarium shop, 60% of *A. hydrophila* were isolated from sick freshwater ornamental fish [2]. Other reports also refer to the antimicrobial susceptibility of clinical isolates of this species [17] and in a prevalence study of fish and prawn from south India market, 33.5% and 17.6% of *A. hydrophila* were isolated, respectively [18].

Strains of *Aeromonas* spp. (n = 225) characterized genetically (43 *Aeromonas aquariorum*, 67 *A. hydrophila*, 94 *A. veronii*, 16 *A. cunicola*, 3 *A. media*, 1 *A. caviae* and 1 *A. jandaei*) were tested for susceptibility to a panel of 28 antibiotics. The results are presented in Table 1 (in percentage); however, the values regarding *A. media, A. caviae* and *A. jandaei* are not included due to the small number of isolates found. Our results show the existence of differences in some of the antibiotics tested according to the species and a high incidence of resistance of *Aeromonas* isolates to β-lactams antibiotics, as 95% were resistant to amoxicillin, 96% to carbenicillin and 94% to ampicillin (Table 1). It is noteworthy that the main differences were in the isolates of *Aeromonas aquario-**

![Table 1: Susceptibility profile (%) to antibiotics of *Aeromonas* spp. (n=220) isolates.](image-url)
rum. For this specie, regarding the β-lactams antibiotics, ampicillin, carbenicillin, amoxicillin, cefalothin and cefoxitin were less effective and of the aminoglycosides antibiotics the most effective was amikacin (84%). Moreover, Aeromonas hydrophila showed values to quinolones (ciprofloxacin and norfloxacin) about 40% and on the other hand no significant difference in the values of resistance found in the remain species studied.

Identical susceptibility patterns to β-lactams antibiotics were found for the species of A. hydrophila, A. veronti, A. cunicola (Table 1), A. media, A. caviae and A. jandaiei, with exception of cefalothin and cefoxitin that for these strains were more effective. Aeromonas isolates from different sources have been reported to have a relatively high resistance to β-lactams antibiotics, usually correlated with naturally occurring phenotypes of β-lactamases production [19]. The combination of aminopenicillin and carbapenemycin with a β-lactamases inhibitor was effective in reducing resistance, as shown by the decrease in the proportion of resistant strains: 95% (amoxicillin) versus 15% (amoxicillin/clavulan acid); 73% (ticarcillin) versus 22% (ticarcillin/clavulanic acid), that was more pronounced with amoxicillin. Nevertheless, these results are in agreement with the statement above, described in others studies [8], indicating that the penicillins resistance is probably due to the action of the inducible penicillinasises susceptible to clavulanic acid.

The isolates found in this work from the species of A. hydrophila, A. veronti, A. cunicola were observed strains with sensitivity to aminopenicillins. The isolates from A. aquariorum, A. media, A. caviae and A. jandaiei did not reveal sensitivity to any of these antibiotics. The results show that by using a culture media with ampicillin for the isolation of the genus Aeromonas, we may be underestimating the presence of these microorganisms from the different environments where they are found. Previous studies related to that Aeromonas strains are 100% resistant to ampicillin, which is generally included in culture media for the isolation of aeromonads [20]; but this observation was based on studies using clinical isolates and it is possible that in a natural environment the selective constraints are different.

High resistance to first and second-generation cephalosporins (cefalothin and cefoxitin, respectively) has been detected in motile aeromonad isolates [21,22] and are in accordance with our results for the strains of A. aquariorum measured in 88% and 98% of the isolates. Decreased susceptibility to third generation cephalosporins were previously reported [19]. A previous work [23] studied the presence of Aeromonas strains in mussels from the Adriatic Sea, reported isolates of A. hydrophila, A. caviae and A. bestiarium. These authors tested the activity of cephalosporins, first and third-generation (namely, cefalothin and cefotaxime). For cefalothin, we were obtained 100% of resistance to ampicillin, which is generally included in culture media for the isolation of aeromonads [20]; but this observation was based on studies using clinical isolates and it is possible that in a natural environment the selective constraints are different.

Astrapheon, a monobactam antibiotic was effective against all species (two isolates resistant). Remarkably in this work, imipenem resistance was observed in three isolates of Aeromonas (1 A. aquariorum, 1 A. veronti and 1 A. jandaiei). Other studies also reported the incidence of strains resistant to this antibiotic [24,25]. Resistance to imipenem in non clinical strains supposed not subjected to selective pressure by use of such drug is a worrying trait as this is a last-resort antimicrobial agent used in the clinical environment. Chloramphenicol showed the highest efficacy against the bacterial strains tested (87% sensitive and 13% resistant). Tetracycline resistance was 80% for Aeromonas spp. isolated, with no differences observed in these studied species. The resistance to tetracycline has been reported to be acquired and encoded by plasmids or transposons [26-28]. Ciprofloxacin and norfloxacin resistance was more prevalent among A. hydrophila isolates (43% and 34%, respectively) than the other species. Commonly, quinolones are synthetic antibiotics used as first therapeutic options for Aeromonas infections in humans [29,30], also used in the treatment of bacterial fish diseases [31]. These drugs can persist for a long time in the environment, which could favor the emergence of resistant strains in environmental samples. The relatively high rates of resistance towards tetracycline and quinolones antibiotic might be due to extensive use of such compounds in hospital environments [30].

The results found for the aminoglycosides (gentamicin, kanamycin, tobramycin, amikacin and netilmicin) were observed, the differences between the susceptibility profiles of the A. aquariorum and of the others species. The antimicrobial agent with the most effective activity to Aeromonas spp. was amikacin (6% of resistance). The susceptibility tests with gentamicin and kanamycin revealed the highest percentages of resistance (28% and 31%, respectively). Notably, 50% of the isolates of A. aquariorum showed resistance to tobramycin.

The trimethoprim/sulfamethoxazole susceptibility tests revealed a percentage of resistance 29% and 49% for the isolates in the present study (Table 1), with the lowest values found for A. veronti and the highest for A. aquariorum. The 3 isolates of A. media and one of A. jandaiei revealed sensitivities to this antibiotic, while the isolate from A. caviae showed the resistance to trimethoprim/sulfamethoxazole. A previous work [25] on the characterization of Aeromonas spp. in samples of frozen fish reported a resistance for this antibiotic of 49%, and the isolates from A. veronti presented 25% of resistance, that are similar to the values found in the present work. A. salmonicida which is known as fish pathogenic agent was not found in this study. This fact might suggest that this specie is not frequent in ornamental fish infections, as previously reported on South African ornamental fish [32]. Mesophilic aeromonads are considered to be opportunistic pathogens, capable of producing infections in weakened fish or as secondary invaders in fish populations suffering from others diseases [15,33].

The present study revealed Aeromonas species are common inhabitants of aquatic ecosystems. Through genetic sequencing were found 288 isolates that belong to 7 different species of this genus. There is a frequent occurrence and a considerable diversity of Aeromonas spp. in ornamental fish. All the isolates tested presented multi resistance to the used antibiotics. Some strains were resistant to all aminoglycosides tested. This was verified in 3% (2 out of 67) of the isolates of A. hydrophila and 16% (7 out of 43) of A. aquariorum, collected from the water and skin. Also, there was a crossed multi resistance between aminoglycosides, quinolones, tetracycline, chloramphenicol, erythromycin and trimethoprim/sulfamethoxazole. The patterns of antibiotic resistance displayed by these organisms increase their potential health hazard and their broad distribution on different habitats is a problematic question. Therefore, these Aeromonas spp. strains showed to be potential reservoirs of antibiotic resistance genes, being of high importance to perform monitoring studies in order to evaluate and control its dissemination in aquatic environments. Thus ornamental fish can be considered a possible transmission route for aeromonads, however, further studies should be performed.

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