RESEARCH ARTICLE

INTERNATIONAL MICROBIOLOGY (2004) 7:207–211
www.im.microbios.org

INTERNATIONAL MICROBIOLOGY

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Received 3 June 2004
Accepted 11 July 2004

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Resistance to β-lactam antibiotics in *Aeromonas hydrophila* isolated from rainbow trout (*Oncorhynchus mykiss*)

Summary. Bacterial infections caused by members of the genus *Aeromonas*, with a relatively high antibiotic resistance, are among the most common and troublesome diseases of fish raised in ponds with recirculation systems. In this study, carried out at an experimental aquaculture station in northern Portugal, 51 strains identified as belonging to the genus *Aeromonas* were isolated from 20 rainbow trout (*Oncorhynchus mykiss*) skin and kidney samples, as well as from raceway water samples. Macro- and microscopic examination of the fish tissues revealed lesions or cellular alterations in skin and kidney that seemed to correlate with the presence of those isolates. The sensitivity of all isolated strains to different groups of β-lactam antibiotics (penicillins, cephalosporins, monobactams and carbapenems) was evaluated using the disc diffusion method. The highest rates of resistance were to amoxicillin, carbenicillin and ticarcillin. Unexpected resistance to imipenem, an antibiotic of clinical usage, was also detected, which suggests that resistance may have been transferred to the *Aeromonas* population from the environment. [**Int Microbiol** 2004; 7(3):207–211]

Key words: *Aeromonas hydrophila* · antibiotic resistance in fish · rainbow trout (*Oncorhynchus mykiss*)

Introduction

The genus *Aeromonas* comprises a group of gram-negative, facultatively anaerobic bacteria that are pathogenic for aquatic and terrestrial animals and have also been associated with a wide spectrum of infectious diseases in humans [1,8,17,26]. Apart from the psycrophilic fish pathogen species *Aeromonas salmonicida*, many other mesophilic aeromonads are considered to be opportunistic pathogens, capable of producing infections in weakened fish or as secondary invaders in fish populations suffering from other diseases [7]. The widespread distribution of *Aeromonas* species in the aquatic environment combined with the stress induced by intensive culture practices predisposes fish to infections. Aeromonads can be the cause of fatal hemorrhagic septicaemia [21] and red pest and red sore in fish [7], but they present a risk of infection for human handlers and consumers [3,9,13,17]. The prevention and treatment of both human and fish diseases by the extensive use of antimicrobial agents have undoubtedly contributed to an increase in the frequency of resistant strains [28]. Additionally, since antimicrobial agents are released into the surrounding water during treatment of bacterial fish diseases, there is a direct, negative impact on the aquaculture environment [2,33].

Numerous studies have attempted to elucidate the occur-

rence and persistence of antibiotic resistance, mostly in marine aquaculture production systems, predominately in the production of salmonids [15,18]. An increase in resistance levels of the genus *Aeromonas*, particularly to β-lactam antibiotics, has been observed [4,25,30,32]. This evolution towards increasing levels of resistance is, in part, attributed to the production of different β-lactamases, for instance inducible β-lactamases active against penicillins, cephalosporins, and carbapenems [16,25,27]. Although most studies tested clinical isolates, β-lactamases from the environmental microbiota have recently been described [14,31].

The purpose of the present study was to evaluate the susceptibility to β-lactam antibiotics of *Aeromonas* isolated from trout and to correlate the histopathological alterations of skin and kidney tissues with the presence of resistant bacteria.

Materials and methods

Sampling site and collection. This evaluation was conducted with 20 rainbow trouts (*Oncorhynchus mykiss*) raised at the Fish Experimental Unit of University of Trás-os-Montes e Alto Douro (UTAD), Vila Real, Portugal. At the beginning of the experimental work, 250 fish, with an average weight of 150 g, were housed in a raceway of 20 m^3 supplied with filtered freshwater in an open, flow-through system (16± 2ºC) at a flow rate of 10 l/min. The most important physicochemical variables (dissolved O_2, pH and nitrogenated compounds) were monitored throughout the trial and maintained at levels described as optimum for the species. Fish were exposed to the natural photoperiod. They were fed twice daily with a commercial diet (40% crude protein; 17% crude lipids) until apparent satiation. Fish weight and fish consumption were registered every 3 weeks. Samples of two fish and water were randomly collected every 3 weeks, from mid-January until August 2001.

Necropsy and histopathological examination. Fish were measured and examined for external lesions (Fig 1). The organs subjected to analyses included skin, muscle, kidney, liver, spleen, intestine, gill and heart. All major tissues were carefully examined, and samples for bacterial and histological examination were collected. The tissue samples were fixed in 10 % buffered formalin and routinely embedded in paraffin wax. Serial sections of 3 μm were stained with hematoxylin and eosin and examined by microscopy.

Bacterial strains isolation and identification. Samples of skin and kidney were collected aseptically and incubated at 28 ºC for 24 h on GSP media (Oxoid, Basingstoke, UK) and MacConkey agar (Oxoid). These media were used to isolate and purify the strains. Bacterial strains were identified using standard biochemical classification techniques (API 20NE, BioMérieux 20050, Marcy l'Etoile, France). Selected strains were subjected to genetic identification by restriction fragment length polymorphism (RFLP) analysis of PCR-amplified 16S rDNA following a previously described protocol [6,11].

Antibiotic susceptibility tests. Susceptibility was determined by the disk method of Kirby-Bauer on Mueller-Hinton agar plates (Oxoid) with inocula adjusted to an optical density of 0.5 McFarland standard units. After 24 h of incubation at 28ºC, organisms were classified as sensitive (S), intermediate (I), or resistant (R) according to NCCLS 2002 guidelines [23]. The antibiotic-containing disks were obtained from Oxoid and consisted of the following: piperacillin (PRL_{100}), piperacillin+tazobactan (TZP_{110}), amoxicillin (AML_{10}), amoxicillin + clavulanic acid (AMC_{30}), ticarcillin (TIC_{75}), ticarcillin + clavulanic acid (TIM_{85}), ampicillin (AMP_{10}), carbenicillin (CAR_{100}), cephalothin (KF_{30}), cefotaxime (CTX_{30}), cefoperazone (CFP_{30}), cefepime (FEP_{30}), aztreonam (ATM_{30}) and imipenem (IMP_{10}).

Results

Isolation and identification of bacterial strains. From a total of 51 isolates obtained, only three were biochemically identified as *Aeromonas sobria* (water origin) while the remaining 48 were identified as *A. hydrophila*, nine of them from water, 30 from animal skin, seven from the kidney, and two from liver. Five of the *A. hydrophila* strains that showed resistance to imipenem were identified genetically, by RFLP-16S rDNA analysis, and biochemically.

Susceptibility to β-lactam antibiotics. The results show that there is a high incidence of resistance by *Aeromonas* isolates to carboxipenicillins, as 76% were resistant to ticarcillin and 82% to carbenicillin (Table 1). The combination of an aminopenicillin and a carboxipenicillin with a β-lactamases inhibitor was effective in reducing resistance, as shown by the decrease in the proportion of resistant strains: 88% (amoxicillin) versus 35% (amoxicillin + clavulanic acid); 76% (ticarcillin) versus 35% (ticarcillin + clavulanic acid). Resistance to a first-generation cephalosporin (cephalothin) was measured in 65% of the isolates. Aztreonam, a monobactam antibiotic, was more effective against these bacteria, with only 29% of the isolates being resistant. It should be noted that the occurrence of resistance was independent of the source of the isolate, water, kidney or skin. The strains isolated from kidney and skin lesions, 33.3% (3/9) and 20.0 % (6/30), respectively, were identified as *A. hydrophila* and were resistant to imipenem, an antibiotic belonging to the carbapenem group. All of the strains isolated from kidney (n = 9) were resistant to carbenicillin, ampicillin and cephalotin. Interestingly, the resistance profiles of *A. sobria* strains isolated from water (n = 3) were identical to those of *A. hydrophila* strains from the same origin.

Gross lesions. Macroscopic lesions were predominantly on the skin (Fig. 1) and varied in size and type, with hemorrhage and necrosis on the skin and base of the fins, but also reddish/gray ulcers with necrosis extending to the muscle. Some animals had a distended abdomen with serosanguinous fluid and hepatic petechiation.

Histopathology. On histological examination, skin and muscle sections presented an acute-to-chronic dermatitis and myositis with a rich neutrophilic infiltrate. In liver, a nonpurulent multifocal hepatitis and cholangiohepatitis were observed

Fig. 1. Skin ulcers in rainbow trout (*Oncorhynchus mykiss*) raised at the Fish Experimental Unit of University of Trás-os-Montes e Alto Douro (UTAD), Vila Real, Portugal (Scale ca. 0.6×).

Table 1. Percentage of susceptibility to β-lactam antibiotics of all isolated *Aeromonas hydrophila* strains. R, resistant; I, intermediate; S, sensitive. Corresponding antibiotic concentrations are listed in Materials and methods

Antibiotic	R	I	S
Piperacillin	24	0	76
Piperacillin+tazobactan	24	0	76
Amoxicillin	88	7	5
Amoxicillin+clavulanic acid	35	35	30
Ticarcillin	76	0	24
Ticarcillin+clavulanic acid	35	7	58
Ampicillin	65	5	30
Carbenicillin	82	0	18
Cephalothin	65	5	30
Cefotaxime	12	0	88
Cefoperazone	24	12	64
Cefepime	54	0	46
Aztreonam	29	0	71
Imipenem	19	16	65

(Fig. 2). Kidney lesions were characterized by small foci of necrosis and the presence of free melanin from ruptured melanomacrophage centers (Fig. 3). Gill samples revealed a chronic branchitis with an abundant eosinophilic infiltrate. The remaining organs did not show any consistent lesions.

Discussion

The genus *Aeromonas* has been the subject of various antimicrobial susceptibility studies over the last 30 years [5,25]. Although *Aeromonas* species are distributed throughout the world, there are geographic differences in the frequency of diseases caused by these bacteria [19]. The type of lesions described in the present study are consistent with the pattern of infections reported by other authors [24,29]. However, multiple-antibiotic resistance was found in strains isolated from fish tissues showing macroscopic lesions (Fig. 1) and histopathological disorders (Figs. 2 and 3). In the present study, we observed that the majority of *Aeromonas* isolates are resistant to amoxicillin, carbenicillin and ticarcillin (see Table 1), whereas cefotaxime, piperacillin, alone and associated with tazobactan, and aztreonam were more effective (see Table 1). Nevertheless, these values are generally above those described by other authors [19]. However, approximately 20% of the *A. hydrophila* isolates have shown resistance to cefotaxime, and the aztreonam resistant strains represented only 6%. Note that 65% of *A. hydrophila* strains were resistant to ampicillin, a lower value than that obtained with amoxicillin (see Table 1), although both antibiotics usually have the same effect. *Aeromonas* strains are considered naturally resistant to ampicillin, which is generally included in culture media for the isolation of aeromonads; but this observation was based only on studies using clinical isolates and it is of course possible that in a natural environment the selective constraints are different.

It is noteworthy that the antibiotic imipenem, a carbapenem used in this study, was not as effective as would be expected since this antibiotic is one of the most potent single antimicrobial agents available for therapy. In fact, the rate of susceptible isolates was less than 65%. In previous studies, *Aeromonas* resistance to imipenem had ranged from 3% [22] to 14% [19]. Overman and Janda [25] reported that 65% of *A. jandaei* strains were resistant to imipenem; however, the strains were isolated from clinical specimens and thus resistance might have been stimulated due to prior exposure to β-lactam antibiotics. In fact, the *A. hydrophila* strains isolated from the trout shown in Fig. 1 and isolates from liver and kidney (Figs. 2 and 3, respectively) were resistant to carbapenem. Our results suggest that this trait is also being distributed throughout the population of environmental aeromonads. Moreover, a major concern of antibiotic usage is the acquisition of multiple-antibiotic resistance, which, in addition to the present study, has been reported in fish pathogens and other bacteria from aquaculture environments frequently associated with the abuse and misuse use of antibiotics [10,

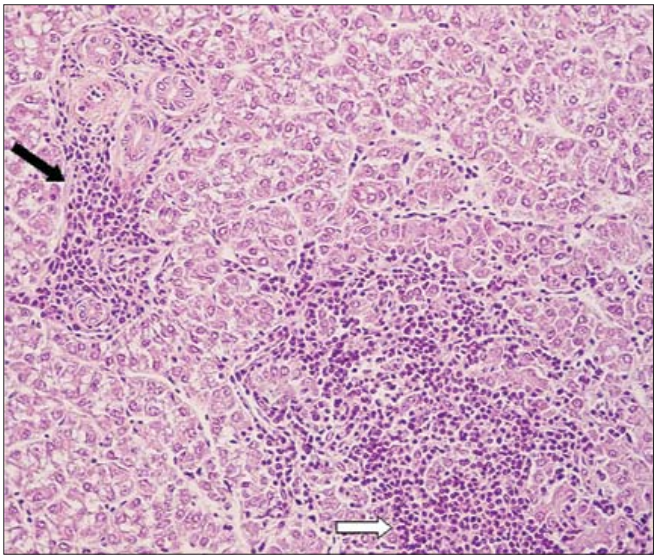

Fig. 2. Paraffin wax section of a liver. Non purulent multifocal hepatitis and cholangiohepatitis: severe infiltration of round cells (lymphocytes and macrophages) in the portal area (black arrow) and in the hepatic parenchyma (open arrow). Haematoxylin and eosin (20×).

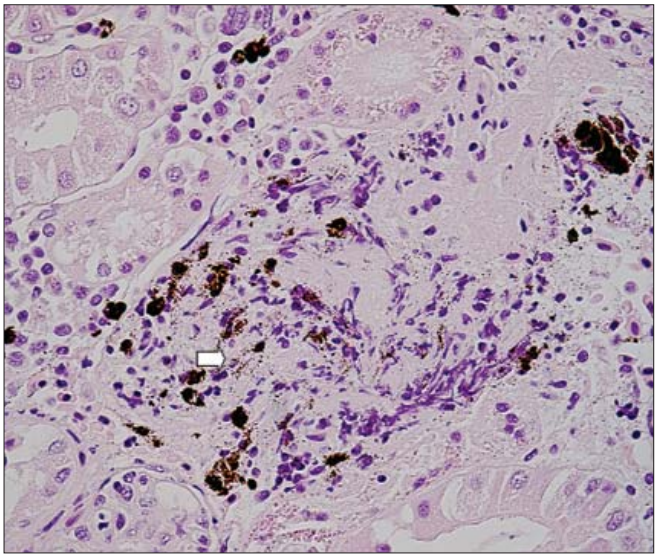

Fig. 3. Paraffin wax section of a kidney. Focus of necrosis with presence of free melanin (black arrow) from ruptured melanomacrophages, also observed in tubular epithelial cells. Haematoxylin and eosin (40×).

22,32]. Several molecular mechanisms are involved in β-lactam resistance [12,20]. Further work to investigate the possible mechanisms of resistance to β-lactam antibiotics is in progress.

To conclude, although it has been showed that strains of *A. hydrophila* isolated from trout are more sensitive to antibiotics than those isolated from clinical specimens, the environmental incidence of resistance to β-lactam antibiotics seems to be increasing, and is of particular concern with respect to imipenem. The aquaculture environment may thereby constitute a reservoir for bacterial resistance to clinically relevant antibiotics.

Acknowledgements. The authors wish to thank Dr. A. Duarte (University of Lisbon) for her comments on this manuscript. S. Guedes-Novais acknowledges the Portuguese Science and Technology Foundation for a grant (SFRH/BM/4460/2001). This work is being funded by the Technology and Science Foundation of Portugal (Project: POCTI/CVT/42025/2001).

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Resistencia a antibióticos β-lactámicos en *Aeromonas hydrophila* aislados de truchas arco iris (*Oncorhynchus mykiss*)

Resumen. Las infecciones bacterianas causadas por miembros del género *Aeromonas*, que muestran una resistencia a antibióticos relativamente elevada, figuran entre las enfermedades más comunes de peces criados en tanques con sistemas de recirculación. En este estudio, realizado en una estación de acuicultura experimental en el norte de Portugal, 51 cepas identificadas como *Aeromonas* se aislaron de muestras de la piel y el riñón de 20 truchas (*Oncorhynchus mykiss*), así como de muestras de agua. Un examen macroscópico y microscópico del tejido de los peces reveló lesiones y alteraciones celulares en piel y riñón, que parecían correlacionarse con la presencia de esos aislados. Mediante el método de difusión en disco se evaluó la sensibilidad de todas las cepas aisladas a diferentes grupos de antibióticos β-lactámicos (penicilinas, cefalosporinas, monobactámicos y carbapenemos). Las mayores incidencias de resistencia fueron a amoxicilina, carbenicilina y ticarcilina. También se detectó una resistencia inesperada a imipenem, un antibiótico de uso clínico, lo que sugiere que esta resistencia puede haberse transferido a la población de *Aeromonas* desde el ambiente. [**Int Microbiol** 2004; **7**(3):207–211]

Palabras clave: *Aeromonas hydrophila* · resistencia a antibióticos en peces · trucha arco iris (*Oncorhynchus mykiss*)

Resistência a antibióticos β-lactâmicos em *Aeromonas hydrophila* isoladas de trutas arco íris (*Oncorhynchus mykiss*)

Resumo. Infecções bacterianas causadas por membros do gênero *Aeromonas*, que mostram resistência relativamente elevada a antibióticos, figuram entre as enfermidades mais comuns em peixes criados em tanques com sistema de recirculação. Neste estudo realizado numa estação de piscicultura experimental no norte de Portugal, 51 estirpes identificadas como *Aeromonas* foram isoladas de amostras de pele e rins de 20 trutas (*Oncorhynchus mykiss*), e de amostras de água. Exames macro e microscópicos do tecido dos peixes revelaram lesões e alterações celulares na pele e nos rins que pareciam correlacionar-se com a presença desses isolados. Foi avaliada a sensibilidade de todas as estirpes isoladas a diferentes grupos de antibióticos β-lactâmicos (penicilinas, cefalosporinas, monobactâmicos e carbapenemos) através do método de difusão em discos. A incidência maior das resistências recaíram sobre: amoxicilina, carbenicilina e a ticarcilina. Também foi detectada uma resistência inesperada ao imipenemo, um antibiótico de uso clínico, o que sugere que esta resistência pode ter sido transferida à população de *Aeromonas* a partir do ambiente. [**Int Microbiol** 2004; 7(3):207–211]

Palavras chave: *Aeromonas hydrophila* · resistência a antibióticos em peixes · truta arco íris (*Oncorhynchus mykiss*)