Antibiotic susceptibility of strains of *Aeromonas salmonicida* isolated from spanish salmonids

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**ABSTRACT**

The antibiotic susceptibility of a group of *Aeromonas salmonicida* strains, isolated from Atlantic salmon (*Salmo salar*) and Brown trout (*Salmo trutta*) in some fish farms and rivers of Spain, has been studied. A high percentage of resistant strains was observed, particularly for some antibiotics that often are used in aquaculture, like nalidixic acid and oxytetracycline. Similar results were obtained with chloramphenicol, greatly used some years ago in aquaculture but forbidden in recent years. In contrast, the major part of isolates was highly susceptible to amoxicillin and flumequine or florfenicol, florfenicol being the antibiotic with the highest efficacy. Antibiotic resistance in the isolated *Aeromonas salmonicida* increases with the age of the animals on which the bacteria has been isolated, while no differences were found in the fish species or origin (fish farm or river).

**Key words** : Antibiotic resistance, *Aeromonas salmonicida*, outbreak, carrier, risk.

**RÉSUMÉ**

Sensibilité aux antibiotiques d’*Aeromonas salmonicida* isolés de salmônids sauvages ou d’élevage en Espagne.

L’étude présente les résultats de sensibilité et résistance aux antibiotiques de souches d’*Aeromonas salmonicida* isolées de Saumon d’atlantique (*Salmo salar*) et de Truite commune prélevées dans divers fleuves et établissements piscicoles d’Espagne. Les résultats mettent en évidence qu’il existe une forte proportion de souches résistantes aux divers antibiotiques utilisés en aquaculture comme l’acide nalidixique, l’oxytétracycline ou le chloramphenicol, ce dernier étant interdit depuis quelques années. Au contraire on a observé une haute sensibilité des souches à l’amoxicilline, la flumequine. La résistance aux antibiotiques des souches d’*Aeromonas salmonicida* augmente avec l’âge des animaux chez lesquelles elles ont été isolées.


**Introduction**

Furunculosis is a worldwide bacterial disease of salmonids in both fish-farm production and wild fish. This disease, caused by *A. salmonicida*, can affect salmonid and non salmonid species in fresh, and salt water (22, 25, 2). A main problem associated with furunculosis outbreaks is that a part of the surviving animals becomes carriers, which usually are persistent in the populations of both production farms and wildlife species (14, 18, 19, 23). The control of furunculosis outbreaks in aquaculture is made through chemotherapy and recently by vaccines (17, 8, 5, 7). The use of antibiotics has some associated problems such as the increase of asymptomatic carriers and the emergence of antibiotic resistant strains (13, 3).

In endemic furunculosis areas, antibiotic treatment is necessary at critical points of the outbreaks. The most used antibiotics in the last several years have been quinolones, like flumequine and oxolinic acid or nalidixic acid, oxytetracyclines or potent sulphonamides (2). However, their continuous use has led to the emergence of resistant *A. salmonicida* strains (13). In order to solve this problem, other antibiotic molecules have been studied and in some cases utilized in fish treatments, like florfenicol (12, 20, 21), or fluoroquinolones (16).

One important problem linked to the use of antibiotics in aquaculture is that fishes are an important component of human food in many countries, which means that some type of resistance transmission through the human food-chain could be possible. In Spain, antibiotics have largely been used in the control of furunculosis outbreaks, but resistance studies have not been carried out. In order to get information about this problem in our country, a study to evaluate the susceptibility, to the most used antibiotics in aquaculture, of the *A. salmonicida* strains isolated from a group of Spanish salmonids during the last 3 years has been designed. Likewise, in the study we tried to evaluate the changes in the susceptibility to chloramphenicol after some years without using it.

**Materials and methods**

**DESIGN OF THE STUDY**

The study was carried out in a group of *A. salmonicida* strains isolated from September 2001 to March 2004 in 6 salmonid rivers of the north of Spain and in two fish farms, where Atlantic salmon and Brown trout are raised. The used *A. salmonicida* strains were got from animals that were sampled in outbreaks and also in the seasonal controls of the...
rivers and fish farms carried out by the Laboratory of Ietiopathology of the Veterinary Faculty of the University of Zaragoza. Selected animals were transported to the laboratory at a temperature of 4°C in a short time (less than 10 hours). Fish characteristics, sampling point, date, and environmental conditions were noted in the data-base.

**ISOLATION AND IDENTIFICATION OF A. SALMONICIDA**

Necropsy was performed in every sampled animal. Bacteriological culture was made from kidney, liver, spleen and lesions of the affected animals on Tryptone Soya Agar (TSA) (Difco). Incubation of the cultures was performed at 22°C for 48-72 hours (2, 6). Identification was made using microscopy, staining procedures, pigment production and API20E and API20NE biochemical tests (bioMérieux) (2). Every isolated *A. salmonicida* received a reference code.

**IN VITRO STUDIES OF ANTIBIOTIC RESISTANCE IN A. SALMONICIDA ISOLATES**

In order to evaluate the susceptibility to antibiotics of the *A. salmonicida* isolates, the Kirby-Bauer disk diffusion method was used. One ml of a bacterial suspension was plated on TSA (6) and the disk diffusion protocol was carried out. Plates were incubated at 22°C for 48 hours.

We selected the antibiotics that have been largely used in Spain during the last several years and also some other interesting antibiotics, like chloramphenicol, which have not been used during the last twelve years. Antibiotics assayed were amoxicillin (25 μg), doxycycline (30 μg), erythromycin (15 μg), nalidixic acid (30 μg), cotrimoxazole (1.25-23.75 μg), flumequine (30 μg), chloramphenicol (30 μg), enrofloxacin (5 μg), oxytetracycline (30 μg), novobiocin (5 μg) and florfenicol (30 μg), purchased from Becton Dickinson Co., bioMérieux, Mast Diagnostics and Bio-Rad.

Because there is not an international agreement concerning the interpretation of the results of antibiotic susceptibility assays of bacteria associated with fish diseases, the breakpoints for each antibiotic were those defined by the manufacturer. According to it, we defined three results: resistant (R), intermediate (I) and susceptible (S). However, the susceptible category was divided into susceptible (S) and highly susceptible (HS) according to the following criteria:

- Susceptible (S)- The inhibitory zone is greater than 1-fold the intermediate inhibitory zone
- Highly susceptible (HS)- The inhibitory zone is greater than 2-fold the intermediate inhibitory zone

**DATA-BASE**

The data-base was designed using the EPI INFO 3.2. computer program (4)

The results were organized and analyzed in order to get information about the frequency and distribution of the general and individual resistance and susceptibility to the evaluated antibiotics.

**Results**

In the study we used 31 strains of *A. salmonicida* that were isolated in the period 2001-2004. The different strains were isolated from all age groups, fish species (atlantic salmon and brown trout) and also from rivers and fish farms.

Our results indicates that a high proportion of resistance is present in the isolated *A. salmonicida* strains. A total of 41.9% of the tests presented a resistant (R) result, while only 27.9% of the results were high susceptibility (HS) and 16.4% were susceptible (S) (Table I).

As is shown in Table II, florfenicol was the antibiotic with greater activity against *A. salmonicida* isolates, because all of them were highly susceptible to this drug. The strains of *Aeromonas salmonicida* also presented high susceptibility to amoxicillin, 83.9 %. Flumequine and enrofloxacin were also valuable antibiotics, but flumequine seemed to be more effective because 61.3 % of the strains were highly susceptible. In contrast, the major part of *A. salmonicida* isolates was resistant to chloramphenicol, nalidixic acid, doxycycline and oxytetracycline, and 61.3 % of strains were also resistant to novobiocin. Only 25.8 % of *A. salmonicida* strains were resistant to cotrimoxazole, but only 9.7% were highly susceptible.

The distribution of the resistance to antibiotics in the *A. salmonicida* strains according to the animal species, age group and origin (fish-farm or river) was studied. No differences in the values of resistance and susceptibility were found between either fish species from which isolates were obtained. In a same manner, no differences were found concerning the antibiotic susceptibility of the strains and the origin of fishes, fish farm or river. At this level, only small differences can be observed for antibiotics like cotrimoxazole (data not shown).

The distribution of *A. salmonicida* isolates according to their antibiotic susceptibility and the age of the fishes shows that strains isolated from salmonids younger than 1 year old have a high percentage of high susceptibility (HS) to some antibiotics, while older animals presented high levels of resistance (Table III).

**Discussion**

One important characteristic of *A. salmonicida* is the ability to produce, after one outbreak presentation, the presence of a persistant carrier status, which means this microorganism can persist for a long time period in the infected animals and also in the organic material of sludge and sediments, as was previously observed (18, 9, 2, 3).

The use of antibiotics in furunculosis outbreaks control solves the clinical disease in a large number of cases, but the infection of the animal tissues is usually not removed. That means that the use of antibiotics can promote the carrier status if this practice is not applied under surveillance programs to control the antibiotic efficacy at all times, as has been suggested by some authors (2, 3, 15).
The high percentage of resistant isolates to some antibiotics indicates that *A. salmonicida* antibiotic resistance is also an increasing problem in Spain, as has previously been described for other countries (10, 11, 3). This situation makes necessary, as requested by some international organisms (European Association of Fish Pathologists, European Union), an harmonisation of antibiotic susceptibility tests and, according to this, international harmonisation in approvals of antibiotics use (24, 1).

In this work, it has been demonstrated that although the use of chloramphenicol has been forbidden in Spain for the last years, the major part of isolates was resistant to this antibiotic. As it was seen in our previous work in the health surveillance program for salmonids, the level of resistance to chloramphenicol was very similar some years ago which suggests that the resistance to antibiotics could be persistent during long time periods after their use.

In contrast, all *A. salmonicida* strains were highly susceptible to florfenicol. We believe that this result and those found by other authors (11, 20, 21) must be related to the recent application of this antibiotic in a systematic way in aquaculture. However, it is important to establish programmes of florfenicol resistance surveillance, because, in 1991, it was described that this antibiotic was a bacteriostatic agent at the used dose and that it was necessary to greatly increase the dose in order to have bactericidal activity (12). This result strongly suggests that the treatment with florfenicol at low or medium doses could solve the clinical disease, but could also promote the carrier status in salmonids.

In order to obtain a good bactericidal activity in fish treatment, it was also proposed that the administration of cotrimoxazole, enrofloxacin, oxolinic acid or amoxicillin would be better (16, 11). In this work, we have determined the susceptibility of *A. salmonicida* isolates for some of these antibiotics and have shown that only 9.7 % of the strains were highly susceptible to cotrimoxazole, while this percentage for enrofloxacin is 45.2 %. In addition, and in agreement with other authors (13), the major part of strains was highly susceptible to amoxicillin, which is an antibiotic useful only in exceptional situations in aquaculture. Therefore, amoxicillin could be a good antibiotic to use in future programs to control furunculosis outbreaks if good administration protocols for fish are designed and legal conditions for its use are adopted. A similar suggestion can be assumed with flumequine, because 61.3 % of the strains were highly susceptible to this antibiotic.

The high levels of resistance to antibiotics in older animals of our study was an expected result because the likelihood to have had contact with antibiotics increase with the age.

<table>
<thead>
<tr>
<th>Level of Resistance</th>
<th>Number of test with the result (total number of tests)</th>
<th>Frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>R</td>
<td>143 (341)</td>
<td>41.9</td>
</tr>
<tr>
<td>I</td>
<td>47 (341)</td>
<td>13.8</td>
</tr>
<tr>
<td>S</td>
<td>56 (341)</td>
<td>16.4</td>
</tr>
<tr>
<td>HS</td>
<td>95 (341)</td>
<td>27.9</td>
</tr>
</tbody>
</table>

**TABLE I.** Frequency (%) of resistance and susceptibility in the isolated *A. salmonicida* strains.

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>R</th>
<th>I</th>
<th>S</th>
<th>HS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amoxicillin</td>
<td>0</td>
<td>6.5</td>
<td>9.7</td>
<td>83.9</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>96.8</td>
<td>0</td>
<td>0</td>
<td>3.2</td>
</tr>
<tr>
<td>Cotrimoxazole</td>
<td>25.8</td>
<td>22.6</td>
<td>41.9</td>
<td>9.7</td>
</tr>
<tr>
<td>Doxycycline</td>
<td>87.1</td>
<td>9.7</td>
<td>3.2</td>
<td>0</td>
</tr>
<tr>
<td>Enrofloxacin</td>
<td>0</td>
<td>9.7</td>
<td>45.2</td>
<td>45.2</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>6.5</td>
<td>64.5</td>
<td>29</td>
<td>0</td>
</tr>
<tr>
<td>Florfenicol</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>Flumequine</td>
<td>0</td>
<td>0</td>
<td>38.7</td>
<td>61.3</td>
</tr>
<tr>
<td>Nalidixic acid</td>
<td>93.5</td>
<td>3.2</td>
<td>0</td>
<td>3.2</td>
</tr>
<tr>
<td>Novobiocin</td>
<td>61.3</td>
<td>35.5</td>
<td>3.2</td>
<td>0</td>
</tr>
<tr>
<td>Oxytetracycline</td>
<td>90.3</td>
<td>0</td>
<td>9.7</td>
<td>0</td>
</tr>
</tbody>
</table>

**TABLE II.** Distribution (%) of the *A. salmonicida* isolates and their antibiotic susceptibility.
problem in the aquaculture of Spain. The resistance to antibiotics could be an important problem in the future, not only in animal health but also in public health as a result of the possible transmission of the resistance to humans by fish carriers consumption.

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