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Antimicrobial resistance and its genetic determinants in aeromonads isolated in ornamental (koi) carp (Cyprinus carpio koi) and common carp (Cyprinus carpio)

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Abstract
The aim of this study was to evaluate antimicrobial susceptibility of Aeromonas spp. isolates from common carp and koi carp coming from randomly chosen farms. The isolates were tested for susceptibility to 8 antimicrobial agents using the standard agar dilution susceptibility test. In all isolates, PCR was used to detect the presence of tet(A-E) genes, integrase genes, and gene cassettes. From the total 72 isolates of motile aeromonads sampled from koi carp, 36 isolates (50%) were resistant to oxytetracycline, 18 (25%) to ciprofloxacin, 5 (7%) to chloramphenicol, 5 (7%) to florfenicol, and 11 (15%) to trimethoprim. Among 49 isolates of motile aeromonads collected from common carp, 20 (41%) were resistant to oxytetracycline, 3 (6%) to...
chloramphenicol, and 3 (6%) to florfenicol. The resistance of aeromonads isolated from koi
carp was significantly higher to ciprofloxacin (P = 0.00024). The presence of class 1 integrons
was detected in these isolates only (P = 0.00024). Tet genes were detected in 40% (48/121) of
isolates, with tet(E) being the most dominant. Our results demonstrated a significant difference
in the incidence of resistant isolates collected from koi carp and common carp (P = 0.00042).
This difference can be ascribed to a distinct antibiotic policy established on consumer fish
farms versus ornamental fish farms. The potential risk for resistant bacteria to spread and
transmit infection to humans should be considered in cases of technological crossover between
the two types of fish farms.

Keywords: Aquaculture; Aeromonas spp.; Antimicrobial resistance; tet resistance genes;
Integrons

1. Introduction

The Czech Republic is a traditional producer of common carp (Cyprinus carpio). Its annual
production is approximately 18,000 tons, half of which is exported. Recently, the development
of ornamental (koi) carp (Cyprinus carpio koi) farming has appeared in the country, both on
small-scale farms and large production farms. Intensive carp farming is associated with risk for
the incidence and spread of infectious diseases commonly associated with therapeutic and
prophylactic use of antibiotics. In general, two or three agents have been granted marketing
authorisations in EU countries (Smith, 2008). In the Czech Republic, for example,
oxytetracycline and flumequine are permitted, with the former used extensively on common
carp farms. On the other hand, the regulation of antibiotic use is not enforced on such
ornamental fish farms as koi carp operations. Therefore, the ornamental fish producers tend to
administer antibiotics in a non-systematic and uncontrolled manner, thus making the selection
and spread of antibiotic-resistant bacteria possible. The results of existing studies show that
administering antibiotics in “aquacultures” leads to higher antibiotic resistance across the entire
microbial water ecosystem (Schmidt et al., 2000; Sørum, 2006) and thus also to higher risk for transmitting genetic resistance determinants to bacteria that are pathogenic for fish. Indicator bacteria suitable for studying the incidence and development of antibiotic resistance on fish farms include motile aeromonads. These bacteria are interconnected with the water ecosystem, colonize fish, and can cause various infectious processes in them (Austin and Austin, 2007). Sporadically, aeromonads have been proven to cause human infections (Janda et al., 1994; Ko et al., 2000).

To date, very little has been published on the susceptibility of bacteria isolated from common and koi carp worldwide (Guz and Kozińska, 2004; Taylor, 2003), while no data are available at all about bacterial susceptibility in the Czech Republic. The aims of this study were to identify the levels of antimicrobial resistance and to define resistance determinants in motile aeromonads isolated from koi and common carp coming from randomly chosen farms in the Czech Republic.

2. Material and methods

2.1 Bacterial cultures

A total of 138 koi and common carp were tested in 2005 (7 localities) and 2006 (7 localities). Fish were subjected to bacteriological examination for differential diagnosis of KHV (koi herpes virus). No increase of morbidity or mortality was observed. Gills and skin swabs from euthanized fish were cultured on Columbia agar (CM331, Oxoid Ltd., Basinstoke, UK), supplemented with 5% sheep blood, in order to isolate *Aeromonas* spp. If the fish had ulcerous changes on skin or gills, samples were taken from these ulcers preferentially. Inoculated agars were incubated at 28 ± 2 °C for 24–48 hours. Primary bacterial cultures thus obtained were pre-identified according to morphology of colonies, Gram reaction, motility, catalase and oxidase production, and resistance to vibriostatic agent O/129 (Rahman et al., 2002). Presumptive cultures of *Aeromonas* spp. were examined using API 20E test (bioMérieux, France). Esculin hydrolysis and growth on triple sugar iron agar slant were used as
supplementary tests. Phenotypic identification was done using Aerokey II (Carnahan et al., 1991). Identified cultures were stored in cryoprotective medium at −80°C and tested for susceptibility to antimicrobials and the presence of resistance determinants. For these tests, no more than 1 Aeromonas spp. isolate from each fish was used. Quality control was done with reference strains Escherichia coli ATCC 25922 and Aeromonas salmonicida subsp. salmonicida ATCC 33658.

2.3 Determination of susceptibility to antimicrobials

Susceptibility tests were performed by agar dilution method in accordance with Clinical and Laboratory Standards Institute (CLSI; formerly NCCLS) guidelines (CLSI, 2006) and using the following antibacterial substances: chloramphenicol, ciprofloxacin, florfenicol, oxolinic acid, oxytetracycline, trimethoprim, spectinomycin, and streptomycin (Sigma Aldrich, Prague, Czech Republic). Antimicrobial agents were incorporated into Mueller-Hinton agar (Oxoid Ltd., Basingstoke, UK), with each plate containing the agent in log2 doubling dilutions of the following ranges: chloramphenicol, florfenicol (2–64 µg/ml); ciprofloxacin, oxolinic acid and trimethoprim (0.25–16 µg/ml); oxytetracycline (1–64 µg/ml); streptomycin (0.5–64 µg/ml); and spectinomycin (2–1024 µg/ml). Frozen Aeromonas spp. isolates and control strains were inoculated on nutrient agar (CM3, Oxoid Ltd., Basingstoke, UK) and incubated at 28 ± 2°C for 24 hours. Growth was re-suspended in 2 ml sterile PBS using a sterile cotton-tipped swab at a concentration of 1.5 x 10^8 CFU/ml, in accordance with 0.5 McFarland standard. The turbidity was adjusted with a photometer (Densi-La-Meter, LIAP, Latvia). Plates were inoculated using a multipoint inoculator (Trios, Czech Republic), which delivered 10^5 organisms per spot. Inoculated plates were incubated at 28 ± 2°C for 24 to 28 hours.

The cultures were compared with a growth control plate containing no antibiotic, and minimum inhibitory concentration (MIC) for each agent was determined as the lowest concentration of an antimicrobial that inhibited the growth of a given culture. Each isolate was examined three times and each batch of media was checked using the aforementioned control strains. The values obtained were used to calculate MIC_{50}, MIC_{90} and the MIC range. Criteria published in
Standards for susceptibility tests for bacteria isolated from animals (CLSI, 2008) were used to evaluate susceptibility of isolates to chloramphenicol, ciprofloxacin, florfenicol, oxytetracycline, and trimethoprim. Cut-off values for interpreting MICs for oxolinic acid, streptomycin, and spectinomycin were not determined.

2.4 Detection of resistance genes, integrons and integron-associated genes

All isolates were tested by PCR for tet(A-E), the class 1 and 2 integrase genes intI1 and intI2, respectively, the variable region of class 1 and class 2 integrons (designed by primers 5CS-3CS and Hep74-Hep51), gene cassettes inside the integron structure, and the sulI gene which is an essential part of the class 1 integron. This test technique has been previously described by Dolejska et al. (2007) and Wilkerson et al. (2004).

2.5 Data analysis

The results obtained were statistically evaluated by chi-square test using MS Excel® software with the Analyse-it module. Differences with \( p \leq 0.05 \) were considered as significant.

3. Results

3.1 Susceptibility to antimicrobials

In total, 121 presumptive isolates were identified as A. sobria (60 isolates), A. hydrophila (55 isolates) and A. veronii subsp. sobria (6 isolates). MICs obtained for 8 antimicrobial agents are demonstrated in Table 1. Seventy-three of these isolates (60\%) were resistant to one or more antibiotics. Significant differences were detected between the prevalence of resistant isolates in koi carp and common carp \( (P = 0.0042) \). Of 72 isolates of motile aeromonads sampled from koi carp, 36 (50\%) were resistant to oxytetracycline, 18 (25\%) to ciprofloxacin, 5 (7\%) to chloramphenicol, 5 (7\%) to florfenicol, and 11 (15\%) to trimethoprim. Among 49 isolates of motile aeromonads collected from common carp, 20 (41\%) were resistant to oxytetracycline, 3 (6\%) to chloramphenicol, and 3 (6\%) to florfenicol. Comparison of MIC results indicated significant difference in susceptibility to ciprofloxacin \( (P = 0.00024) \). Significant difference
was detected also between the isolates of the two origins in the prevalence of simultaneous resistance to more than one antibiotic ($P = 0.02559$).

3.3 Tet genes, integrons and integron-associated genes

All isolates of aeromonads were screened for the presence of $tet$(A-E) genes. $Tet$(E) was identified in 33% (40/121) of these isolates. Moreover, $Tet$(E) was detected in combination with $tet$(A) (3 isolates), $tet$(D) (3 isolates), and both $tet$(A) and $tet$(D) (2 isolates). One isolate contained the $tet$(D) gene. The isolates with detected $tet$ genes had MICs for oxytetracycline in a range from 1.0 to >64 mg/L. No $tet$(A-E) genes were proven in 12 (21%) of 56 oxytetracycline-resistant isolates.

The $intI1$ gene was found in 17 (14%) of 121 isolates. The presence of class 1 integrons was detected only in the isolates from koi carp ($P = 0.00024$). These $intI1$ positive isolates were significantly related to elevated MICs for spectinomycin (in a range from 32 to >1028 mg/L). MICs for streptomycin of these isolates ranged from 1 to >64 mg/L. All of these $intI1$-positive isolates contained the $sul1$ gene. Four types of class 1 integrons with the following size of the variable region gene cassettes were determined: 1 kb with an $aadA2$ gene cassette, 1.6 kb with a $dhfr12$-$aadA2$ gene cassette, 1.7 kb and 2 kb with an $aadA1$ gene cassette. One isolate with the $intI1$ and $sul1$ genes and a $dhfr12$-$aadA2$ cassette was negative for the variable region detected by 5CS-3CS primers. All isolates were negative for the $intI2$ gene.

4. Discussion

The aim of this study was to evaluate susceptibility of Aeromonas spp. isolates from common and koi carp coming from randomly chosen farms. Standard methods for testing susceptibility of bacteria isolated from fish have been published only recently (CLSI, 2006). However, no criteria for evaluating susceptibility in bacteria isolated from aquatic animals have yet been accepted and published. Moreover, the needs of veterinarians and epidemiologists vary (Bywater et al., 2006; Miller and Reimschuessel, 2006). In this study, criteria published in
Standards for susceptibility tests for bacteria isolated from animals (CLSI, 2008) were therefore used to evaluate susceptibility of isolates to selected antibiotics. Depending upon their origin, aeromonads examined in this study differed in their susceptibility to antibiotics and the presence of class 1 integrons. Statistically significant differences between two groups of isolates investigated in this study can be explained by wholly different antibiotic policies. While producers of koi carp are not at all restricted in using antimicrobials, common carp farmers must observe Directive 2001/82/EC of the European Parliament and of the Council of 6 November 2001 on the Community code relating to veterinary medicinal products. The Directive permits the use of only authorized medicinal products for therapy and prohibits using any other products. In the Czech Republic, antibiotic therapy of infections in common carp is based solely on oxytetracycline that is administered orally in granulated feed at doses dependent on the size of the fish stock in a pond. However, therapeutic use of oxytetracycline leads to contamination of pond sediments with residues of this antibiotic (Svobodová et al., 2006), which can contribute to the selection of bacteria resistant to oxytetracycline. Our results have confirmed that resistance to oxytetracycline and the incidence of tet genes among isolates of aeromonads from common carp was rather high. Similar findings were previously described in various species of fish kept on intensive farms (Alcaide et al., 2005; Nawaz et al., 2006; Jacobs and Chenia, 2007). This study proved that in the isolates of motile aeromonads from common carp and koi carp, the resistance to oxytetracycline was most often coded by the tet(E) gene or by a combination of the tet(E) gene with tet(A) and tet(D) genes. The 12 oxytetracycline-resistant isolates that were negative for the tested tet(A-E) genes could possess one of the less frequent tet or otr genes that also have been described in Aeromonas spp. isolates (Chopra and Roberts, 2001; Gordon et al., 2008). Potential mutation inside the tet gene or another antimicrobial resistance mechanism are other explanations. Recently, tet(E) has been detected in Aeromonas spp. strains on Danish fish farms. The tet(E) gene occurs on large plasmids that can be horizontally transmitted to E. coli (Agersø
et al., 2007). This finding confirms the possibility of spreading tetracycline resistance to other bacterial pathogens in a fish farm ecosystem. In this sense, the possible overlap of farming technologies for koi and common carp (e.g. hatcheries, circulation systems, handling of fish, etc.) should be viewed as a very serious epidemiological problem. This can lead not only to transmission of pathogens (e.g. koi herpes virus) but also of resistant bacterial clones. In this study, resistant aeromonad isolates with class 1 integrons were detected in koi carp produced by 2 out of 4 large farmers who, at the same time, produced also common carp (the results are not presented here).

Evaluating the susceptibility of bacteria isolated from fish to fluoroquinolones can be problematic, as no binding breakpoints have yet been approved. In general, one substance should be selected that would function as a “reporter” of susceptibility to other fluoroquinolones. For this purpose, nalidixic acid has been preliminarily accepted (Rodriguez-Avial et al., 2005). MIC results for oxolinic acid did not differ significantly in dependence upon the origin of isolates. Detailed comparison of our results, however, showed a shift of MIC values between the isolates of different origin. These results will need to be evaluated as per epidemiological cut-off values (ECOV) estimated by Miller and Reimschuessel (2006).

Unfortunately, the range of oxolinic acid concentrations tested in this study was beyond ECOV for wild-type isolates. This problem may be solved by the proving of quinolone-resistance determinants.

The incidence of antibiotic-resistant aeromonads in freshwater fish is generally viewed as a potential risk of infection for human consumers of fish (Alderman and Hastings, 1998). In this study, a high incidence of antibiotic-resistant aeromonads was confirmed in koi carp. Fish of this kind can be a source of contamination for recreational water reservoirs, which can pose a potential risk for human health.
Acknowledgements

This study was supported by Project QH71057 under the National Agency for Agricultural Research (NAZV) and Grant No. MSM 6007665809 of the Ministry of Education, Youth and Sports of the Czech Republic.

5. References


Miller, R.A., Reimsschessel, R., 2006. Epidemiologic cutoff values for antimicrobial agents against *Aeromonas salmonicida* isolates determined by frequency distributions of minimal inhibitory concentration and diameter of zone of inhibition data. AJVR 67, 1837–1843.


Table 1: Distribution of MICs in *Aeromonas* spp. isolates from Koi carp (*n* = 72) and Common carp (*n* = 49)

<table>
<thead>
<tr>
<th>Antimicrobial agent</th>
<th>Origin</th>
<th>Number of isolates with MIC (mg/L) of:</th>
<th>MIC&lt;sub&gt;50&lt;/sub&gt;</th>
<th>MIC&lt;sub&gt;90&lt;/sub&gt;</th>
<th>Range of MIC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>≤ 0.25 0.5 1 2 4 8 16 32 64 128 256 512 ≥1024</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OTC</td>
<td>Koi carp</td>
<td>NT 26 1 1 8 6 18 12 NT</td>
<td>8</td>
<td>≥64</td>
<td>≤1 – ≥64</td>
</tr>
<tr>
<td></td>
<td>Common carp</td>
<td>NT 23 2 3 1 3 15 2 NT</td>
<td>2</td>
<td>32</td>
<td>≤1 – ≥64</td>
</tr>
<tr>
<td>OA&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Koi carp</td>
<td>23 4 1 21 23 0 0 NT</td>
<td>2</td>
<td>4</td>
<td>≤0.25 – 4</td>
</tr>
<tr>
<td></td>
<td>Common carp</td>
<td>13 4 22 6 3 1 0 NT</td>
<td>1</td>
<td>2</td>
<td>≤0.25 – 8</td>
</tr>
<tr>
<td>CIP</td>
<td>Koi carp</td>
<td>28 17 5 4 8 4 6 NT</td>
<td>0.5</td>
<td>8</td>
<td>≤0.25 – ≥16</td>
</tr>
<tr>
<td></td>
<td>Common carp</td>
<td>28 21 0 0 0 0 0 NT</td>
<td>≤0.25</td>
<td>0.5</td>
<td>≤0.25 – 0.5</td>
</tr>
<tr>
<td>CHL</td>
<td>Koi carp</td>
<td>NT 60 1 2 4 2 3 NT</td>
<td>≤2</td>
<td>16</td>
<td>≤2 – ≥64</td>
</tr>
<tr>
<td></td>
<td>Common carp</td>
<td>NT 43 0 2 1 3 0 NT</td>
<td>≤2</td>
<td>8</td>
<td>≤2 – 32</td>
</tr>
<tr>
<td>FFC</td>
<td>Koi carp</td>
<td>NT 67 0 0 1 1 3 NT</td>
<td>≤2</td>
<td>≤2</td>
<td>≤2 – ≥64</td>
</tr>
<tr>
<td></td>
<td>Common carp</td>
<td>NT 46 0 0 0 2 1 NT</td>
<td>≤2</td>
<td>≤2</td>
<td>≤2 – ≥64</td>
</tr>
<tr>
<td>T</td>
<td>Koi carp</td>
<td>48 11 2 0 6 5 0 NT</td>
<td>≤0.25</td>
<td>4</td>
<td>≤0.25 – 8</td>
</tr>
<tr>
<td></td>
<td>Common carp</td>
<td>35 13 1 0 0 0 0 NT</td>
<td>≤0.25</td>
<td>0.5</td>
<td>≤0.25 – 1</td>
</tr>
<tr>
<td>S&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Koi carp</td>
<td>NT 0 3 11 29 15 3 4 7 NT</td>
<td>4</td>
<td>≥64</td>
<td>1 – ≥64</td>
</tr>
<tr>
<td></td>
<td>Common carp</td>
<td>NT 0 4 15 20 10 0 0 0 NT</td>
<td>4</td>
<td>8</td>
<td>1.0 – 8.0</td>
</tr>
<tr>
<td>SPT&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Koi carp</td>
<td>NT 0 3 26 23 6 1 1 1 4 7 16 512</td>
<td>4 – ≥1024</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Common carp</td>
<td>NT 0 3 24 22 0 0 0 0 0 0 0 0 8 16</td>
<td></td>
<td></td>
<td>4 – 16.0</td>
</tr>
</tbody>
</table>

CHL – chloramphenicol; CIP – ciprofloxacin; FFC – florfenicol; OA – oxolinic acid; OTC – oxytetracycline; SPT – spectinomycin; S – streptomycin; T – trimethoprim; NT – not tested; shaded areas denote MIC breakpoints (CLSI, 2008); MIC breakpoints for oxolinic acid, streptomycin and spectinomycin were not determined.