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

4 Alois Čížek ^{a,*}, Monika Dolejská ^b, Radana Sochorová ^a, Kateřina Strachotová ^a, Veronika
5 Piačková ^c, Tomáš Veselý ^d

6
7 ^a Institute of Microbiology and Immunology, Faculty of Veterinary Medicine, University of
8 Veterinary and Pharmaceutical Sciences, Palackého 1/3, 612 42 Brno, Czech Republic

9 ^b Institute of Biology and Wildlife Diseases, Faculty of Veterinary Hygiene and Ecology,
10 University of Veterinary and Pharmaceutical Sciences, Brno, Czech Republic

11 ^c Research Institute of Fish Culture and Hydrobiology, University of South Bohemia, Vodňany,
12 Czech Republic

13 ^d Veterinary Research Institute, Brno, Czech Republic

14 * Corresponding author:

15 Tel.: +420 41562274; Fax: +420 549248841

16 E-mail address: cizeka@vfu.cz (A. Čížek)

17 **Abstract**

18 The aim of this study was to evaluate antimicrobial susceptibility of *Aeromonas* spp. isolates
19 from common carp and koi carp coming from randomly chosen farms. The isolates were tested
20 for susceptibility to 8 antimicrobial agents using the standard agar dilution susceptibility test. In
21 all isolates, PCR was used to detect the presence of *tet(A-E)* genes, integrase genes, and gene
22 cassettes. From the total 72 isolates of motile aeromonads sampled from koi carp, 36 isolates
23 (50%) were resistant to oxytetracycline, 18 (25%) to ciprofloxacin, 5 (7%) to chloramphenicol,
24 5 (7%) to florfenicol, and 11 (15%) to trimethoprim. Among 49 isolates of motile aeromonads
25 collected from common carp, 20 (41%) were resistant to oxytetracycline, 3 (6%) to

26 chloramphenicol, and 3 (6%) to florfenicol. The resistance of aeromonads isolated from koi
27 carp was significantly higher to ciprofloxacin ($P = 0.00024$). The presence of class 1 integrons
28 was detected in these isolates only ($P = 0.00024$). *Tet* genes were detected in 40% (48/121) of
29 isolates, with *tet*(E) being the most dominant. Our results demonstrated a significant difference
30 in the incidence of resistant isolates collected from koi carp and common carp ($P = 0.00042$).
31 This difference can be ascribed to a distinct antibiotic policy established on consumer fish
32 farms versus ornamental fish farms. The potential risk for resistant bacteria to spread and
33 transmit infection to humans should be considered in cases of technological crossover between
34 the two types of fish farms.

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

37

38 **1. Introduction**

39 The Czech Republic is a traditional producer of common carp (*Cyprinus carpio*). Its annual
40 production is approximately 18,000 tons, half of which is exported. Recently, the development
41 of ornamental (koi) carp (*Cyprinus carpio koi*) farming has appeared in the country, both on
42 small-scale farms and large production farms. Intensive carp farming is associated with risk for
43 the incidence and spread of infectious diseases commonly associated with therapeutic and
44 prophylactic use of antibiotics. In general, two or three agents have been granted marketing
45 authorisations in EU countries (Smith, 2008). In the Czech Republic, for example,
46 oxytetracycline and flumequine are permitted, with the former used extensively on common
47 carp farms. On the other hand, the regulation of antibiotic use is not enforced on such
48 ornamental fish farms as koi carp operations. Therefore, the ornamental fish producers tend to
49 administer antibiotics in a non-systematic and uncontrolled manner, thus making the selection
50 and spread of antibiotic-resistant bacteria possible. The results of existing studies show that
51 administering antibiotics in “aquacultures” leads to higher antibiotic resistance across the entire

52 microbial water ecosystem (Schmidt et al., 2000; Sørum, 2006) and thus also to higher risk for
53 transmitting genetic resistance determinants to bacteria that are pathogenic for fish. Indicator
54 bacteria suitable for studying the incidence and development of antibiotic resistance on fish
55 farms include motile aeromonads. These bacteria are interconnected with the water ecosystem,
56 colonize fish, and can cause various infectious processes in them (Austin and Austin, 2007).
57 Sporadically, aeromonads have been proven to cause human infections (Janda et al., 1994; Ko
58 et al., 2000).

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

65 **2. Material and methods**

66 *2.1 Bacterial cultures*

67 A total of 138 koi and common carp were tested in 2005 (7 localities) and 2006 (7 localities).
68 Fish were subjected to bacteriological examination for differential diagnosis of KHV (koi
69 herpes virus). No increase of morbidity or mortality was observed. Gills and skin swabs from
70 euthanized fish were cultured on Columbia agar (CM331, Oxoid Ltd., Basinstoke, UK),
71 supplemented with 5% sheep blood, in order to isolate *Aeromonas* spp. If the fish had ulcerous
72 changes on skin or gills, samples were taken from these ulcers preferentially. Inoculated agars
73 were incubated at 28 ± 2 °C for 24–48 hours. Primary bacterial cultures thus obtained were
74 pre-identified according to morphology of colonies, Gram reaction, motility, catalase and
75 oxidase production, and resistance to vibriostatic agent O/129 (Rahman et al., 2002).
76 Presumptive cultures of *Aeromonas* spp. were examined using API 20E test (bioMérieux,
77 France). Esculin hydrolysis and growth on triple sugar iron agar slant were used as

78 supplementary tests. Phenotypic identification was done using Aerokey II (Carnahan et al.,
79 1991). Identified cultures were stored in cryoprotective medium at -80°C and tested for
80 susceptibility to antimicrobials and the presence of resistance determinants. For these tests, no
81 more than 1 *Aeromonas* spp. isolate from each fish was used. Quality control was done with
82 reference strains *Escherichia coli* ATCC 25922 and *Aeromonas salmonicida* subsp. *salmocida*
83 ATCC 33658.

84 2.3 Determination of susceptibility to antimicrobials

85 Susceptibility tests were performed by agar dilution method in accordance with Clinical and
86 Laboratory Standards Institute (CLSI; formerly NCCLS) guidelines (CLSI, 2006) and using
87 the following antibacterial substances: chloramphenicol, ciprofloxacin, florfenicol, oxolinic
88 acid, oxytetracycline, trimethoprim, spectinomycin, and streptomycin (Sigma Aldrich, Prague,
89 Czech Republic). Antimicrobial agents were incorporated into Mueller-Hinton agar (Oxoid
90 Ltd., Basingstoke, UK), with each plate containing the agent in log₂ doubling dilutions of the
91 following ranges: chloramphenicol, florfenicol (2–64 $\mu\text{g/ml}$); ciprofloxacin, oxolinic acid and
92 trimethoprim (0.25–16 $\mu\text{g/ml}$); oxytetracycline (1–64 $\mu\text{g/ml}$); streptomycin (0.5–64 $\mu\text{g/ml}$);
93 and spectinomycin (2–1024 $\mu\text{g/ml}$). Frozen *Aeromonas* spp. isolates and control strains were
94 inoculated on nutrient agar (CM3, Oxoid Ltd., Basingstoke, UK) and incubated at $28 \pm 2^{\circ}\text{C}$ for
95 24 hours. Growth was re-suspended in 2 ml sterile PBS using a sterile cotton-tipped swab at a
96 concentration of 1.5×10^8 CFU/ml, in accordance with 0.5 McFarland standard. The turbidity
97 was adjusted with a photometer (Densi-La-Meter, LIAP, Latvia). Plates were inoculated using
98 a multipoint inoculator (Trios, Czech Republic), which delivered 10^5 organisms per spot.
99 Inoculated plates were incubated at $28 \pm 2^{\circ}\text{C}$ for 24 to 28 hours.

100 The cultures were compared with a growth control plate containing no antibiotic, and minimum
101 inhibitory concentration (MIC) for each agent was determined as the lowest concentration of an
102 antimicrobial that inhibited the growth of a given culture. Each isolate was examined three
103 times and each batch of media was checked using the aforementioned control strains. The
104 values obtained were used to calculate MIC_{50} , MIC_{90} and the MIC range. Criteria published in

105 *Standards for susceptibility tests for bacteria isolated from animals* (CLSI, 2008) were used to
106 evaluate susceptibility of isolates to chloramphenicol, ciprofloxacin, florfenicol,
107 oxytetracycline, and trimethoprim. Cut-off values for interpreting MICs for oxolinic acid,
108 streptomycin, and spectinomycin were not determined.

109 2.4 Detection of resistance genes, integrons and integron-associated genes

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

115 2.5 Data analysis

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

118 3. Results

119 3.1 Susceptibility to antimicrobials

120 In total, 121 presumptive isolates were identified as *A. sobria* (60 isolates), *A. hydrophila* (55
121 isolates) and *A. veronii* subsp. *sobria* (6 isolates). MICs obtained for 8 antimicrobial agents are
122 demonstrated in Table 1. Seventy-three of these isolates (60%) were resistant to one or more
123 antibiotics. Significant differences were detected between the prevalence of resistant isolates in
124 koi carp and common carp ($P = 0.0042$). Of 72 isolates of motile aeromonads sampled from koi
125 carp, 36 (50%) were resistant to oxytetracycline, 18 (25%) to ciprofloxacin, 5 (7%) to
126 chloramphenicol, 5 (7%) to florfenicol, and 11 (15%) to trimethoprim. Among 49 isolates of
127 motile aeromonads collected from common carp, 20 (41%) were resistant to oxytetracycline, 3
128 (6%) to chloramphenicol, and 3 (6%) to florfenicol. Comparison of MIC results indicated
129 significant difference in susceptibility to ciprofloxacin ($P = 0.00024$). Significant difference

130 was detected also between the isolates of the two origins in the prevalence of simultaneous
131 resistance to more than one antibiotic ($P = 0.02559$).

132 3.3 *Tet genes, integrons and integron-associated genes*

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

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

148 4. Discussion

149 The aim of this study was to evaluate susceptibility of *Aeromonas* spp. isolates from common
150 and koi carp coming from randomly chosen farms. Standard methods for testing susceptibility
151 of bacteria isolated from fish have been published only recently (CLSI, 2006). However, no
152 criteria for evaluating susceptibility in bacteria isolated from aquatic animals have yet been
153 accepted and published. Moreover, the needs of veterinarians and epidemiologists vary
154 (Bywater et al., 2006; Miller and Reimschuessel, 2006). In this study, criteria published in

155 *Standards for susceptibility tests for bacteria isolated from animals* (CLSI, 2008) were
156 therefore used to evaluate susceptibility of isolates to selected antibiotics.

157 Depending upon their origin, aeromonads examined in this study differed in their
158 susceptibility to antibiotics and the presence of class 1 integrons. Statistically significant
159 differences between two groups of isolates investigated in this study can be explained by
160 wholly different antibiotic policies. While producers of koi carp are not at all restricted in
161 using antimicrobials, common carp farmers must observe *Directive 2001/82/EC of the*
162 *European Parliament and of the Council of 6 November 2001 on the Community code*
163 *relating to veterinary medicinal products*. The *Directive* permits the use of only authorized
164 medicinal products for therapy and prohibits using any other products. In the Czech
165 Republic, antibiotic therapy of infections in common carp is based solely on oxytetracycline
166 that is administered orally in granulated feed at doses dependent on the size of the fish stock
167 in a pond. However, therapeutic use of oxytetracycline leads to contamination of pond
168 sediments with residues of this antibiotic (Svobodová et al., 2006), which can contribute to
169 the selection of bacteria resistant to oxytetracycline. Our results have confirmed that
170 resistance to oxytetracycline and the incidence of *tet* genes among isolates of aeromonads
171 from common carp was rather high. Similar findings were previously described in various
172 species of fish kept on intensive farms (Alcaide et al., 2005; Nawaz et al., 2006; Jacobs and
173 Chenia, 2007). This study proved that in the isolates of motile aeromonads from common
174 carp and koi carp, the resistance to oxytetracycline was most often coded by the *tet(E)* gene
175 or by a combination of the *tet(E)* gene with *tet(A)* and *tet(D)* genes. The 12 oxytetracycline-
176 resistant isolates that were negative for the tested *tet(A-E)* genes could possess one of the
177 less frequent *tet* or *otr* genes that also have been described in *Aeromonas* spp. isolates
178 (Chopra and Roberts, 2001; Gordon et al., 2008). Potential mutation inside the *tet* gene or
179 another antimicrobial resistance mechanism are other explanations.

180 Recently, *tet(E)* has been detected in *Aeromonas* spp. strains on Danish fish farms. The
181 *tet(E)* gene occurs on large plasmids that can be horizontally transmitted to *E. coli* (Agersø

182 et al., 2007). This finding confirms the possibility of spreading tetracycline resistance to
183 other bacterial pathogens in a fish farm ecosystem. In this sense, the possible overlap of
184 farming technologies for koi and common carp (e.g. hatcheries, circulation systems,
185 handling of fish, etc.) should be viewed as a very serious epidemiological problem. This can
186 lead not only to transmission of pathogens (e.g. koi herpes virus) but also of resistant
187 bacterial clones. In this study, resistant aeromonad isolates with class 1 integrons were
188 detected in koi carp produced by 2 out of 4 large farmers who, at the same time, produced
189 also common carp (the results are not presented here).

190 Evaluating the susceptibility of bacteria isolated from fish to fluoroquinolones can be
191 problematic, as no binding breakpoints have yet been approved. In general, one substance
192 should be selected that would function as a “reporter” of susceptibility to other
193 fluoroquinolones. For this purpose, nalidixic acid has been preliminarily accepted (Rodriguez-
194 Avial et al., 2005). MIC results for oxolinic acid did not differ significantly in dependence
195 upon the origin of isolates. Detailed comparison of our results, however, showed a shift of MIC
196 values between the isolates of different origin. These results will need to be evaluated as per
197 epidemiological cut-off values (ECOV) estimated by Miller and Reimschuessel (2006).
198 Unfortunately, the range of oxolinic acid concentrations tested in this study was beyond ECOV
199 for wild-type isolates. This problem may be solved by the proving of quinolone-resistance
200 determinants.

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

206

207

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

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

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); ^a MIC breakpoints for oxolinic acid, streptomycin and spectinomycin were not determined