

1 **Isolation of flavobacteria from wild and cultured freshwater fishes**
2 **in Hungary**

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12 **Short running title:** *Flavobacterium* in Hungarian fishes

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28

29 **Abstract**

30

31 This study surveyed the prevalence of *Flavobacterium columnare* in wild and cultured
32 freshwater fishes in Hungary. This bacterium usually causes disease in waters of more than
33 25°C temperature. However, with the introduction of intensive fish farming systems, infected
34 fish exposed to stress develop disease signs also at lower temperatures; in addition, the
35 temperature of natural waters rises to the critical level due to global warming. Twenty-five
36 isolates from ulcerous fish stocks were identified as *F. columnare* by specific PCR, although
37 both the fragment lengths and the results of PCR-RFLP genotyping with BsuRI (HaeIII) and
38 RsaI restriction enzymes raised doubts regarding this species classification. Sequencing of the
39 16S ribosomal RNA gene revealed that 23 isolates belonged to the species *F. johnsoniae* and
40 2 represented *Chryseobacterium* spp. The isolates were found to have high-level multidrug
41 resistance: all were resistant to ampicillin and polymyxin B, the 23 *F. johnsoniae* strains to
42 cotrimoxazole, 88% of them to gentamicin and 72% to chloramphenicol. The majority of the
43 25 isolates were sensitive to erythromycin (88%), enrofloxacin (84%), furazolidone (76%)
44 and florfenicol (68%).

45

46 **Keywords:** freshwater, *Flavobacterium columnare*, *F. johnsoniae*, PCR-RFLP, multidrug
47 resistance.

Megjegyzés [SB1]: max. 5-6 szó

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49

50 **Introduction**

51

52 The genus *Flavobacterium* comprises more than 100 species
53 (<http://www.bacterio.net/flavobacterium.html>), several of which have been demonstrated to
54 be causative agents of different fish diseases. Ulcerative disease in fish caused by *Bacillus*
55 *columnaris* had first been described by Davis (1922). The name of this bacterium was
56 changed several times because of reclassifications. Ultimately, it was ranked into the genus
57 *Flavobacterium* in 1996 and designated as *F. columnare* on the basis of its 16S rRNA gene
58 sequence (Bernardet et al., 1996). Using the *F. columnare* specific PCR primers of Bader et
59 al. (2003), Darwish et al. (2004) identified some isolates in South Africa whose 16S rRNA
60 gene sequence differs from that of *F. columnare*. These isolates were described as a new
61 species, *F. johnsoniae* (Darwish *et al.*, 2004). Although *F. columnare* and *F. johnsoniae*
62 generally induce diseases in fishes in a water temperature exceeding 25°C, but infected fish
63 may also show clinical signs at lower water temperature under conditions of intensive fish
64 farming (Suomalainen et al., 2006). As a result of global warming, the temperature of natural
65 waters more and more frequently reaches and exceeds the level critical for the manifestation
66 of clinical disease in countries of the temperate zone as well. Infection spreads rapidly and
67 without treatment it can cause severe economic losses.

Megjegyzés [SB2]: nem dőlt, sima

68 The aim of this study was to survey the prevalence of *F. columnare* in both wild and
69 cultured freshwater fishes. The presence of this bacterium had already been documented in
70 Hungary previously (Csaba & Békési, 1977), and its occurrence was presumed to be more
71 frequent due to climate change and rising natural water temperatures. Another objective was
72 to determine the resistance of our strains to antimicrobial agents. Although the general
73 scientific opinion holds that the increasing emergence of bacterial drug resistance in human
74 and veterinary medicine is primarily the result of imprudent antibiotic use, saprophytic and

Megjegyzés [SB3]: and

75 pathogenic multidrug resistant piscine bacteria could be detected also in territories unaffected
76 by human influence (Shome and Shome, 1999). The study of antibiotic-resistant bacteria from
77 treated and untreated fishes provides information about the prevalence of innate resistance.

78

79 **Materials and methods**

80

81 **Fishes examined**

82 The examined fishes [asp (*Leuciscus aspius* L.), roach (*Rutilus rutilus* L.), bighead carp
83 (*Hypophthalmichthys nobilis* Richardson), tench (*Tinca tinca* L.), European perch (*Perca*
84 *fluviatilis* L.), freshwater bream (*Abramis brama* L.), gibel carp (*Carassius gibelio* Bloch),
85 pikeperch (*Sander lucioperca* L.), sichel (*Pelecus cultratus* L.), white bream (*Blicca bjoerkna*
86 L.), Volga pikeperch (*Sander volgensis* Gmelin), bleak (*Alburnus alburnus* L.), common carp
87 (*Cyprinus carpio* L.) and Siberian sturgeon (*Acipenser baerii* Brandt)], healthy and suffering
88 from ulcerative dermal necrosis, originated from Lake Balaton and from two intensive fish
89 farms. Unsatisfactory conditions, intensive stocking and poor water quality were common in
90 both of the fish farms.

91 Forty-eight fish from 13 cases were selected during the period from January to May 2014.
92 Bacterial samples were collected from the skin, eyes, gills and visceral organs (spleen, liver,
93 kidney and intestine) of fish with skin erosions (Figures 1–3) and from the gills of healthy fish
94 from the same source.

95

96 [Figures 1–3]

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99 **Isolation of *Flavobacterium* sp.**

100 The isolation of *Flavobacterium* sp. was performed on Selective Cytophaga Agar (SCA)
101 (Farmer, 2004) and on Tryptic Soy Agar (TSA, Lab M Limited, Lancashire, UK)
102 supplemented with 5% sheep blood. Cytophaga agar was supplemented with neomycin and
103 polymyxin B (Lab M Limited, Lancashire, UK) to suppress flavobacteria other than *F.*
104 *columnare*. Plates were incubated at 25°C for 48 hours. Yellow or yellowish colonies were
105 passaged on Cytophaga Agar without antibiotics (CA) and in brain-heart infusion (BHI) broth
106 (Lab M Limited, Lancashire, UK). After 24 hours, the BHI culture was supplemented with
107 30% glycerol and stored at -70°C. A loopful of defrosted sample was streaked onto CA and
108 the plates were incubated at 25°C for 24 hours before further study. In the preliminary
109 examinations, the morphology, motility and staining characteristics of the bacterial colonies
110 were studied. Native smears were examined by microscopy, and long, thin, rod-shaped
111 bacteria were selected for PCR studies.

112 Morphologically selected colonies grown on TSA were also studied to detect co-
113 infections of the animals examined.

114

115 **Species-specific PCR**

116 The selected colonies were used as a template for a species-specific PCR designed for the 16S
117 rRNA gene by Bader et al. (2003). The amplified products were separated by electrophoresis
118 in 1% agarose gel stained with ethidium bromide and visualised by UV transillumination. The
119 length of PCR fragments was verified by GeneRuler 100bp Plus DNA ladder (Thermo Fisher
120 Scientific, Waltham, MA, USA).

121

122

123 **Genotyping**

124 The genetic diversity of the PCR-positive strains was examined by restriction fragment length
125 polymorphism (RFLP) of the 16S rRNA gene (Darwish and Ismaiel, 2005). Briefly, the PCR
126 product was precipitated by absolute ethanol and resolved in double-distilled water. The DNA
127 concentration was determined by NanoDrop 2000 Spectrophotometer (Thermo Fisher
128 Scientific, Waltham, MA, USA). Approximately 1 µg of DNA was cut with restriction
129 enzymes BsuRI (schizoisomer of HaeIII) and RsaI (Thermo Fisher Scientific, Waltham, MA,
130 USA) according to the manufacturer's protocol. The resulting fragments were separated in
131 2% agarose gel stained with ethidium bromide and visualised by UV transillumination.

132

133 **Sequencing**

134 Six isolates (A8, C2, C3, C9, D1 and D3) of four genotypes (A, B, C and D) with different
135 RFLP patterns were selected for sequencing. Isolate A8 represented genotype A, isolates C9,
136 D1 and D3 belonged to genotype B, isolate C2 to genotype C, and isolate C3 to genotype D.
137 Approximately 1350 bp long fragments of the 16S rRNA gene were sequenced with
138 bidirectional direct sequencing. The sequences were aligned with each other and the reference
139 sequences in the GenBank..

140

141 **Antibiotic resistance**

142 The disc diffusion method was performed on diluted Mueller-Hinton agar (DMHA) plates in
143 harmony with the guidelines of CLSI VET03/VET04-S1 (Clinical and Laboratory Standards
144 Institute 2010).

145 Fresh DMHA plates were supplemented with 5% inactivated horse serum, then 20 ml
146 aliquots were poured into 90 mm plastic Petri dishes and stored at 4°C for up to 2 weeks.

147 A loopful of defrosted bacterium was streaked onto DMHA plates and incubated at 28°C
148 for 24 hours. Fresh bacterial culture was suspended in phosphate-buffered saline solution, the

149 turbidity of the suspension was adjusted to McFarland 1.2 (DEN-1B Densitometer, Biosan,
150 Riga, Latvia) and the suspension was plated onto two DMHA plates. Antibiotic discs were
151 applied onto the dried surface of agar plates and incubated at 28°C for 48 hours. The
152 following antibiotic discs (Abtek Biologicals, Liverpool, UK) were used, in the final
153 concentrations indicated after each: Chloramphenicol 30 µg, Enrofloxacin 5 µg, Florfenicol
154 30 µg, Gentamicin 10 µg, Ampicillin 10 µg, Erythromycin 15 µg, Oxytetracycline 30 µg,
155 Furazolidone 20 µg, Cotrimoxazole 25 µg, Polymyxin B 300 µg. Standards culture of
156 *Escherichia coli* (ATCC 25922) and *Aeromonas salmonicida* subsp. *salmonicida* (ATCC
157 33658) were used as control.

158 After incubation, the antibiotic inhibition zone diameters (IZD) were measured at the
159 lowest diameter from one edge of the zone to the other, and the results were recorded in
160 millimetres. In the absence of a standardised antibiotic inhibition zone breakpoint for
161 *Flavobacterium* sp., in doubtful cases the evaluation was carried out according to the study of
162 Boyacioglu and Akar (2012).

163

164 **Results**

165

166 **Isolation of *Flavobacterium* sp.**

167 Yellow or yellowish colonies (Figs 4–6) cultured from 48 sorted fishes were examined as
168 unstained smears, and long rod-shaped bacteria were selected for examination by species-
169 specific PCR.

170

171 [Figures 4–6]

172

173 **Species-specific PCR**

174 Twenty-five isolates collected from the ulcerative skin, eye, gills and inner organs of diseased
175 and from the gills of healthy fishes belonging to different species (common carp, freshwater
176 bream, gibel carp, European perch, sichel, pikeperch, Siberian sturgeon, tench, Volga
177 pikeperch and white bream) gave positive results in the species-specific PCR.

178

179 [Figure 7]

180

181 **Genotyping**

182 Cleavage of the amplified approx. 1200 bp long PCR fragments with BsuRI (schizoisomer of
183 HaeIII) and RsaI resulted in three RFLP patterns with each restriction enzyme. Twenty (A1,
184 A2, A8, A9, B1, B2, B3, B4, B5, B6, B7, B8, B9, C1, C4, C5, C6, C7, C8 and D2) out of 25
185 strains presented uniform RFLP patterns with both restriction enzymes. This pattern was
186 designated as genotype A. Further three strains (C9, D1 and D3), genotype B, possessed
187 identical HaeIII pattern as the strains of genotype A but their RsaI digestion profiles differed
188 from those of the former. One strain each belonged to the two remaining genotypes. Strain C2
189 (genotype C) had an RsaI pattern similar to that of genotype B and a unique HaeIII profile,
190 while strain C3 (genotype D) exhibited unique RFLP patterns with both restriction enzymes.

191

192 [Figure 8]

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194 Genotypes B and C originated from Lake Balaton, genotype D from a fish farm,
195 whereas strains of genotype A could be detectable from both sources.

196

197 **Sequencing**

198 Based on the genotyping, the 16S rRNA gene of six representative strains (A8, C2, C3, C9,
199 D1 and D3) was sequenced directly in both directions and compared with bacterial sequences
200 available in databanks using the BLASTn algorithm (<http://www.ncbi.nlm.nih.gov/>). Strains
201 A8, C9, D1 and D3 presented high (>94%) sequence similarity to *F. johnsoniae* bacteria,
202 while strains C2 and C3 were related to *Chryseobacterium* sp. (96%). Strain A8 represented
203 genotype A, isolates C9, D1 and D3 belonged to genotype B, strain C2 to genotype C, and
204 strain C3 to genotype D.

205

206 **Antibiotic resistance**

207 The antibiotic drug susceptibility test was carried out for all the isolates on DMHA plates and
208 zones of inhibition were measured by the Kirby-Bauer agar disk diffusion method in
209 accordance with the recommendations of the Clinical and Laboratory Standards Institute
210 (2010). The antibiotic susceptibility pattern of the strains indicates that they have high-level
211 multidrug resistance. All of them were resistant to ampicillin and polymyxin B. The results
212 are shown in Tables 1 and 2.

213

214 Table 1

215 Table 2

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217

218 **Discussion**

219

220 Members of the genus *Flavobacterium* are widespread in freshwater environments. The
221 majority of them are saprophytic bacteria but some are pathogens that cause severe disease in
222 fishes. The best studied species is *F. columnare*, although it is difficult to isolate because it is

223 easily overgrown by other opportunistic bacteria present in the mixed culture. Thus,
224 successful isolation of this bacterium requires special conditions. In this study, the isolation of
225 flavobacteria, with the exception of two strains, was successful only on specific media
226 supplemented with antibiotics, which indicates the necessity of selective media. Bader et al.
227 (2003) developed a species-specific PCR reaction for the rapid detection of *F. columnare*, and
228 the strains were separated into four genetic types using additional PCR-RFLP. During the
229 identification and sequencing of their own strains, Darwish et al. (2004) demonstrated that
230 strain ATCC 43622 represented a new species, designated as *F. johnsoniae*. Our strains
231 studied with the species-specific PCR developed by Bader et al. (2003) differed from the
232 published data both in the amplified fragment size and in the RFLP patterns. On cleavage of
233 the PCR fragments with restriction endonucleases HaeIII and RsaI the majority of the strains
234 (80%) had identical RFLP patterns (genotype A). Further three strains differed from the
235 former only in the RsaI profile (genotype B). Strain C2 strain was similar to genotype B but
236 had a unique HaeIII pattern (genotype C). Strain C3 possessed unique RFLP patterns with
237 both restriction endonuclease enzymes.

238 Sequence analysis of the 1360 bp long 16S rRNA gene fragment identified 23 strains as *F.*
239 *johnsoniae*, a species closely related to *F. columnare*, while two strains proved to be
240 *Chryseobacterium* spp.

241 The most frequently occurring *F. johnsoniae* strains of genotype A (80%) were isolated
242 both from Lake Balaton and fish farms. While the disease manifestation on trap-captured fish
243 from Lake Balaton was not typical, the fish farm animals showed clear ulcerative signs. For
244 lack of experimental infection, it was supposed that clinical disease after infection is
245 manifested only in the presence of some predisposing factor. The detection of flavobacteria
246 by culture requires selective media that suppress the growth of other environmental bacteria.

247 Fish without disease signs yielded only few colonies of flavobacteria on culture, while from
248 animals with ulcerative disease a denser colony growth was obtained.

249 The isolation of *F. columnare* in Hungary was first reported by Csaba and Békési (1977),
250 but unfortunately those strains have not been maintained. Based on our present monitoring,
251 we hypothesise that the strains identified by traditional methods could be *F. johnsoniae*
252 isolates, since the two closely related species had not been distinguished then.

253 The evaluation of concomitant bacteria was performed in order to identify other putative
254 fish-pathogenic bacteria. From carp originating from a fish farm, *Aeromonas* and *Vibrio*
255 species were isolated together with environmental bacteria (*Rhodococcus*, *Acinetobacter* and
256 *Shewanella*). *Aeromonas* was also demonstrated in a common carp originating from Lake
257 Balaton. No other bacteria of pathological significance were detected in the fishes examined.

258 Antibiograms of the strains determined by the disc diffusion method using 10 antimicrobial
259 agents were similar to the international results. The absence of a standardised antibiotic
260 inhibition zone breakpoint for *Flavobacterium* sometimes made it problematic to evaluate the
261 results, but it was clear that all of our strains were multidrug resistant. Thus the lack of
262 standard resistance values was not a real problem, as in most cases no inhibition zones were
263 detectable at all, except for the oxytetracycline data which were close to the breakpoint.

264 Two of our isolates were resistant to all tested antibiotics. All strains possessed resistance
265 to ampicillin and polymyxin B, while 23 strains were resistant to gentamicin and
266 cotrimoxazole as well. A high level of antimicrobial resistance was revealed to
267 chloramphenicol, with a total of 19 resistant strains. Oxytetracycline was the only antibiotic
268 where the results should be treated with caution, as resistance values were close to the
269 'estimated' breakpoint. Prevalences of resistance and sensitivity to furazolidone and
270 cotrimoxazole were similar, with 11 and 13 resistant strains, respectively. Erythromycin,
271 enrofloxacin and florfenicol proved to be effective against the strains tested in this study.

272 These results give cause for concern, since the majority of our strains came from untreated
273 fishes. Considering the prevalence of *Flavobacterium* strains with high-level multidrug
274 resistance in fishes, further studies on their epidemiological role and public health
275 implications are needed.

276 Our results indicate that the genus *Flavobacterium* was represented by the hitherto not
277 identified *F. johnsoniae* and *Chryseobacterium* spp. in both wild and cultured freshwater
278 fishes during the period of this study in Hungary. Fishes collected from Lake Balaton (n = 17)
279 had not been treated with any antibiotic, and still the strains recovered from them possessed
280 multiple drug resistances. This high-level antibiotic resistance calls for more in-depth studies
281 about the transmission of antibiotic resistance and its implications for human infections.

282

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330 **Figures 1 to 3** Ulcerative dermal necrosis of examined *Flavobacterium*-positive cultured
331 Siberian sturgeons (Fig 1) and common carp (Figs 2 and 3) from intensive fish farms
332
333 **Figures 4 and 5** Primary culture and subculture of flavobacteria on Cytophaga agar
334
335 **Figure 6** *Flavobacterium* subculture on Tryptic Soy Agar supplemented with 5% sheep blood
336
337 **Figure 7** Amplification product of *F. columnare* specific PCR assay developed by Bader,
338 Shoemaker and Klesius (2003)
339 Lane 1: Gene Ruler 100bp Plus marker (Thermo Fisher Scientific, Waltham, MA, USA).
340 Lanes 2–13: The *F. columnare* specific PCR products of Hungarian fish bacteria according to
341 Bader’s method (2003). Lane 14: negative control
342
343 **Figure 8** RFLP patterns of genotypes A, B, C and D. Lanes 1, 8 and 15: Gene Ruler 100bp
344 Plus marker (Thermo Fisher Scientific, Waltham, MA, USA). Lanes 2–7: PCR products of
345 strains A8, C2, C3, D1, D3 and C9 cut with restriction enzyme HaeIII (Thermo Fisher
346 Scientific, Waltham, MA, USA). Lanes 9–14: PCR products of strains A8, C2, C3, D1, D3
347 and C9 cut with restriction enzyme RsaI (Thermo Fisher Scientific, Waltham, MA, USA)
348
349
350

351 **Table 1** Individual antibiotic resistance patterns of *Flavobacterium* isolates (diameter of
352 resistance zone in mm)

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354

355 **Table 2** Summary of antibiotic resistance of the studied *Flavobacterium* isolates

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