1 **Isolation of flavobacteria from wild and cultured freshwater fishes**

2 **in Hungary**

3

4 **Zsuzsanna Varga[1], Boglárka Sellyei[1], Petra Paulus[2], Melitta Papp[2], Kálmán Molnár[1]**

5 **and Csaba Székely[1]**

6

7 [1]Fish Pathology and Parasitology Team, Institute for Veterinary Medical Research, Centre for

8 Agricultural Research, Hungarian Academy of Sciences, Budapest, Hungary

9 [2]Laboratory for Fish and Bee Diseases, National Food Chain Safety Office, Veterinary

10 Diagnostic Directorate, Budapest, Hungary

11

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

13

14

15

16

17

18

19

20

21

22

23

24 **Correspondence:** *Zsuzsanna Varga, Institute for Veterinary Medical Research, Centre for*
25 *Agricultural Research of the Hungarian Academy of Sciences, 21 Hungária krt., H-1143*
26 *Budapest, Hungary (e-mail:* varga.zsuzsa@agrar.mta.hu; varga.zsuzsanna.iren@gmail.com;
27 Phone: 0036 (1) 467-4060*)*

28

Abstract

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

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

Megjegyzés [SB1]: max. 5-6 szó

Introduction

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

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

3

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]

97

98

4

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

6

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**

7

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]

193

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

216

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

9

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.

11

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

288

289

290 **References**

291

292 Bader J.A., Shoemaker C.A. & Klesius P.H. (2003): Rapid detection of columnaris disease in

293   channel catfish (*Ictalurus punctatus*) with a new species-specific 16S rRNA gene-based

294   PCR primer for *F. columnare*. *Journal of Microbiological Methods* **52,** 209–220.

295 Bernardet J.F., Segers P., Vancanneyt M., Berthe M., Kerters S.K. & Vandamme P. (1996):

296   Cutting a Gordian Knot: Emended classification and description of the genus

297   *Flavobacterium*, emended description of the family *Flavobacteriaceae*, and proposal of

298   *Flavobacterium hydatis* nom. nov. (basonym, *Cytophaga aquatilis* Strohl and Tait

299   1978). *International Journal of Systematic Bacteriology* **46,** 128–148.

300 Boyacioglu M. & Akar F. (2012): Isolation of *Flavobacterium psychrophilum* causing

301   rainbow trout fry syndrome and determination of an effective antibacterial treatment of

302   rainbow trout (*Oncorhynchus mykiss*) fry. Kafkas University Veterinary Fak. Derg. **18,**

303   197–203.

304 Clinical and Laboratory Standards Institute (2010): Performance Standards for Antimicrobial

305   Susceptibility Testing of Bacteria Isolated From Aquatic Animals; First Informational

306   Supplement, VET03/VET04-S1 Standard (ed.: Miller, R. A. *et al.*), 06/01/2010 000000.

307 Csaba Gy. & Békési L. (1977): *Flexibacter columnaris* előfordulása hazai halállományokban

308   [Occurrence of *Flexibacter columnaris* in Hungarian fish stocks; in Hungarian]. II.

309   Halászati tudományos napok [2nd Scientific Days of Fisheries], Szarvas, HAKI

310   Publication, pp. 100–103.

311 Darwish A.M. & Ismaiel A.A. (2005): Genetic diversity of *F. columnare* examined by

312   restriction fragment length polymorphism and sequencing of the 16S ribosomal RNA

313   gene and the 16S-23S rDNA spacer. *Molecular and Cellular Probes* **19,** 267–274.

314   Darwish A.M., Ismaiel A.A., Newton J.C. & Tang J. (2004): Identification of *F. columnare*

315   by a species specific polymerase chain reaction and renaming of ATCC43622 strain to

316   *F. johnsoniae*. *Molecular and Cellular Probes* **18,** 421–427.

317   Davis H.S. (1922): A new bacterial disease in freshwater fishes. *US Bureau of Fisheries*

318   *Bulletin* **38,** 261–280. US Bureau of Fisheries, Washington D.C.

319   Farmer B. (2004): Improved method for the isolation and characterization of *F. columnare*.

320   MSc Dissertation. Louisiana State University, Agricultural and Mechanical College,

321   Baton Rouge, LA, USA.

322   Shome R. & Shome B.R. (1999): Antibiotic resistance pattern of fish bacteria from freshwater

323   and marine sources in Andamans. *Indian Journal of Fish* **46**, 49–56.

324   Suomalainen L.R., Kunttu H., Valtonen E.T., Hirvela-Koski V. & Tlirola M. (2006):

325   Molecular diversity and growth features of *F. columnare* strains isolated in Finland.

326   *Diseases of Aquatic Organisms* **70,** 55–61.

327

328

329

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)

353

354

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

356

357