Acta Veterinaria Hungarica 64 (1), pp. 13–25 (2016)
DOI: 10.1556/004.2016.002

ISOLATION AND CHARACTERISATION OF FLAVOBACTERIA FROM WILD AND CULTURED FRESHWATER FISH SPECIES IN HUNGARY

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(Received 14 July 2015; accepted 28 October 2015)

The objective of this study was to survey the incidence of *Flavobacterium columnare* in wild and cultured freshwater fish species 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 wild and cultured freshwater fishes 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 two 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%), furazolidone (76%), and florfenicol (68%).

Key words: Freshwater, *Flavobacterium columnare*, *F. johnsoniae*, PCR-RFLP, multidrug resistance

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. colum-*

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nare 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 fish at water temperatures exceeding 25 °C, infected fish may also show clinical signs at lower water temperatures 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 wild and cultured freshwater fish species. The presence of this bacterium had already been documented in Hungary previously (Csaba and Békési, 1977), and its occurrence was presumed to be more frequent due to the 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 is that the increasing emergence of bacterial drug resistance in human and veterinary medicine is primarily the result of imprudent antibiotic use, saprophytic and pathogenic multidrug resistant piscine bacteria could be detected also in territories unaffected by human influence (Shome and Shome, 1999). The study of antibiotic-resistant bacteria from treated and untreated fish provides information about the prevalence of innate resistance.

Materials and methods

Fish species examined

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

Forty-eight fish from 11 samplings of fish parasitological monitoring from Lake Balaton and two samplings from farmed fish with ulcers were selected during the period from January to May 2014. Bacterial samples were collected from

the skin, eyes, gills and visceral organs (spleen, liver, kidney and intestine) of fish with skin erosions (Figs 1–3) and from the gills of healthy fish from the same source.

Isolation of Flavobacterium *sp.*

The isolation of *Flavobacterium* sp. was performed on Selective Cytophaga Agar (SCA) (Farmer, 2004) and on Tryptic Soy Agar (TSA, Lab M Limited, Lancashire, UK) supplemented with 5% sheep blood. Cytophaga agar was supplemented with neomycin and polymyxin B (Lab M Limited, Lancashire, UK) to suppress sensitive bacteria, and select only bacteria with low nutrient requirement. Plates were incubated at 25 °C for 48 h. Yellow or yellowish colonies were passaged on Cytophaga Agar without antibiotics (CA) and in brain-heart infusion (BHI) broth (Lab M Limited, Lancashire, UK). After 24 h, the BHI culture was supplemented with 30% glycerol and stored at –70 °C. A loopful of defrosted sample was streaked onto CA and the plates were incubated at 25 °C for 48 h before further study. In the preliminary examinations, the morphology, motility and pigment production of the bacterial colonies were studied. Native smears were examined by microscopy, and long, thin, rod-shaped bacteria were selected for PCR studies.

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

Species-specific PCR

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

Genotyping

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

1

2

Figs 1–3. Ulcerative dermal necrosis of *Flavobacterium*-positive cultured Siberian sturgeons (Fig. 1) and common carp (Figs 2 and 3) from intensive fish farms

Sequencing

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

Antibiotic resistance

The disc diffusion method was performed in harmony with the guidelines of CLSI VET03/VET04-S1 (Clinical and Laboratory Standards Institute, 2010). The antibiotic drug susceptibility test was carried out for all the isolates on DMHA plates, and zones of inhibition were estimated by the Kirby-Bauer agar disk diffusion method in accordance with the recommendations of the CLSI (2010).

The MHA plates were prepared from dehydrated culture medium (dMHA) that was supplemented with 5% inactivated horse serum after dissolution and sterilisation, and then were poured into 90-mm plastic Petri dishes in 20-ml aliquots and stored at 4 °C for up to 2 weeks.

A loopful of defrosted bacterium was streaked onto DMHA plates and incubated at 28 °C for 24 h. Fresh bacterial culture was suspended in phosphate-buffered saline solution, the turbidity of the suspension was adjusted to McFarland 1.2 (DEN-1B Densitometer, Biosan, Riga, Latvia), and the suspension was plated onto two DMHA plates. Optimal bacterium density was adjusted according to the results of preliminary experiments. Antibiotic discs were applied onto the dried surface of agar plates and incubated at 28 °C for 48 h. The following antibiotic discs (Abtek Biologicals, Liverpool, UK) were used, in the final concentrations indicated after each: Chloramphenicol 30 µg, Florfenicol 30 µg, Gentamicin 10 µg, Ampicillin 10 µg, Erythromycin 15 µg, Oxytetracycline 30 µg, Furazolidone 20 µg, Cotrimoxazole 25 µg, Polymyxin B 300 µg. In the absence of recommendations for a set of antimicrobial agents to be used in the Disk Diffusion Susceptibility Test for *F. johnsoniae*, the antibiotic selection was based on the data published about *F. columnare* considering the feasible treatment. Since *Flavobacterium* spp. possess intrinsic resistance to polymyxins, polymyxin was applied as positive control. Standard cultures of *Escherichia coli* (ATCC 25922) and *Aeromonas salmonicida* subsp. *salmonicida* (ATCC 33658) were used as control.

After incubation, the antibiotic inhibition zone diameters (IZD) were measured at the lowest diameter from one edge of the zone to the other, and the results were recorded in millimetres. In the absence of a standardised antibiotic inhibition zone breakpoint for *Flavobacterium* spp., in doubtful cases the evaluation was carried out according to the study of Boyacioglu and Akar (2012), and the category of moderate sensitivity or 'intermediate resistance' to particular antibiotics was not specified.

Results

Isolation of Flavobacterium *sp.*

Yellow or yellowish colonies (Fig. 4) cultured from 48 sorted fish were examined as unstained smears, and long rod-shaped bacteria were selected for examination by species-specific PCR.

Bacterial isolation from fish without any signs of illness yielded only a few colonies of flavobacteria on culturing, while from (farmed) animals with ulcerative disease there was a more dense growth of bacteria on the plates but only from samples from the outside of the fish (including ulcers) and not from internal organs. Yellow bacteria were dominant on the plates of ulcer samples.

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

Fig. 4. Flavobacterium subculture on Cytophaga agar

Species-specific PCR

Twenty-five isolates collected from the ulcerative skin, eye, gills and inner organs of diseased fish and from the gills of healthy fish belonging to different species (common carp, freshwater bream, gibel carp, European perch, sichel, pike-perch, Siberian sturgeon, tench, Volga pikeperch, and white bream) gave positive results with the 1350 bp fragment in the species-specific PCR (Table 1, Fig. 5).

Genotyping

Cleavage of the amplified approx. 1200 bp long PCR fragments with BsuRI (schizoisomer of HaeIII) and RsaI resulted in three RFLP patterns with each restriction enzyme. Twenty (A1, A2, A8, B1, B2, B3, B4, B5, B6, B7, B8, B9, C1, C4, C5, C6, C7, C8, D2 and D4) out of 25 strains presented uniform RFLP patterns with both restriction enzymes. This pattern was designated as genotype A. Further three strains (C9, D1 and D3), genotype B, possessed identical HaeIII pattern as the strains of genotype A but their RsaI digestion profiles differed from those of the former. One strain each belonged to the two remaining genotypes. Strain C2 (genotype C) had an RsaI pattern similar to that of genotype B and a unique HaeIII profile, while strain C3 (genotype D) exhibited unique RFLP patterns with both restriction enzymes (Fig. 6).

Table 1

Isolates used in this study (place, fish species, organ, diagnosis, genotype)

ID	Fish	Organ	Diagnosis	Origin	Genotype
A1	Siberian sturgeon *(Acipenser baerii)*	gill	ulcerous skin	FF	*F. johnsoniae* A
A2	Siberian sturgeon *(Acipenser baerii)*	gill	ulcerous skin	FF	*F. johnsoniae* A
A8*	Freshwater bream *(Abramis brama)*	gill	trap captured	LB	*F. johnsoniae* A
B1	Sichel *(Pelecus cultratus)*	kidney	trap captured	LB	*F. johnsoniae* A
B2	Sander *(Sander lucioperca)*	fin	trap captured	LB	*F. johnsoniae* A
B3	Volga pikeperch *(Sander volgensis)*	gill	trap captured	LB	*F. johnsoniae* A
B4	Common carp *(Cyprinus carpio)*	gill	ulcerous skin	LB	*F. johnsoniae* A
B5	Common carp *(Cyprinus carpio)*	eye	ulcerous skin	LB	*F. johnsoniae* A
B6	Common carp *(Cyprinus carpio)*	ulcer	ulcerous skin	LB	*F. johnsoniae* A
B7	Gibel carp *(Carassius gibelio)*	kidney	trap captured	LB	*F. johnsoniae* A
B8	Tench *(Tinca tinca)*	spleen	trap captured	LB	*F. johnsoniae* A
B9	Tench *(Tinca tinca)*	kidney	trap captured	LB	*F. johnsoniae* A
C1	Tench *(Tinca tinca)*	liver	trap captured	LB	*F. johnsoniae* A
C4	Common carp *(Cyprinus carpio)*	gill	ulcerous skin	FF	*F. johnsoniae* A
C5	Common carp *(Cyprinus carpio)*	ulcer	ulcerous skin	FF	*F. johnsoniae* A
C6	Common carp *(Cyprinus carpio)*	gill	ulcerous skin	FF	*F. johnsoniae* A
C7	Common carp *(Cyprinus carpio)*	ulcer	ulcerous skin	FF	*F. johnsoniae* A
C8	Common carp *(Cyprinus carpio)*	gill	ulcerous skin	FF	*F. johnsoniae* A
D2	White bream *(Abramis bjoerkna)*	gill	trap captured	LB	*F. johnsoniae* A
D4	Sichel *(Pelecus cultratus)*	gill	trap captured	LB	*F. johnsoniae* A
C9*	Perch *(Perca fluviatilis)*	gill	trap captured	LB	*F. johnsoniae* B
D1*	White bream *(Abramis bjoerkna)*	fin	trap captured	LB	*F. johnsoniae* B
D3*	Sichel *(Pelecus cultratus)*	fin	trap captured	LB	*F. johnsoniae* B
C2*	White bream *(Abramis bjoerkna)*	gill	trap captured	LB	*Chryseobacterium* sp.
C3*	Common carp *(Cyprinus carpio)*	ulcer	ulcerous skin	FF	*Chryseobacterium* sp.

FF = farmed freshwater fish; LB = fish from Lake Balaton; *uncut PCR fragments from PCR-RFLP (Darwish and Ismaiel, 2005) were sequenced

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

Sequencing

Based on the genotyping, the 16S rRNA gene of six representative strains (A8, C2, C3, C9, D1 and D3) was sequenced directly in both directions and compared with bacterial sequences available in databanks using the BLASTn al-

gorithm (http://www.ncbi.nlm.nih.gov/). Strains A8, C9, D1 and D3 presented high (> 94%) sequence similarity to *F. johnsoniae* bacteria, while strains C2 and C3 were related to *Chryseobacterium* sp. (96%). Strain A8 represented genotype A, isolates C9, D1 and D3 belonged to genotype B, strain C2 to genotype C, and strain C3 to genotype D.

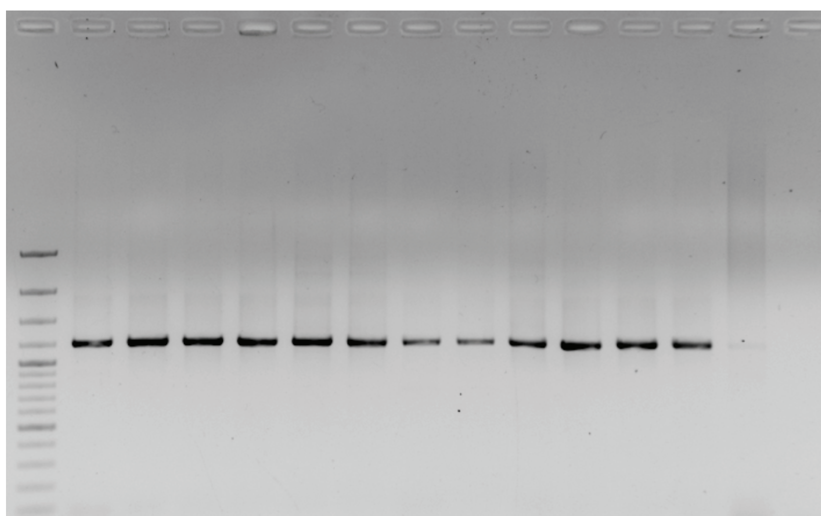

Fig. 5. Amplification product of *Flavobacterium columnare* specific PCR assay developed by Bader et al. (2003). Lane 1: Gene Ruler 100bp Plus marker (Thermo Fisher Scientific, Waltham, MA, USA). Lanes 2–13: *F. columnare* specific PCR products of Hungarian fish bacteria according to Bader's method (2003). Lane 14: negative control

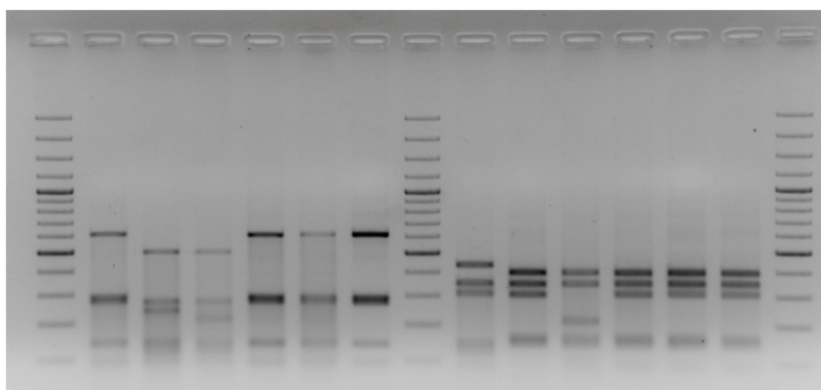

Fig. 6. RFLP patterns of genotypes A, B, C and D. Lanes 1, 8 and 15: Gene Ruler 100bp Plus marker (Thermo Fisher Scientific, Waltham, MA, USA). Lanes 2–7: PCR products of strains A8, C2, C3, D1, D3 and C9 cut with restriction enzyme HaeIII (Thermo Fisher Scientific, Waltham, MA, USA). Lanes 9–14: PCR products of strains A8, C2, C3, D1, D3 and C9 cut with restriction enzyme RsaI (Thermo Fisher Scientific, Waltham, MA, USA)

Table 2

Individual antibiotic resistance patterns of *Flavobacterium* isolates (diameter of inhibition zone in mm)

Isolate	A1	A2	A8	B1	B2	B3	B4	B5	B6	B7	B8	B9	C1	C4	C5	C6	C7	C8	D2	D4	C9	D1	D3	C2	C3
CHL30	8 R	8 R	16 S	16 S	6 R	6 R	6 R	6 R	10 R	6 R	16 S	6 R	8 R	10 R	6 R	6 R	8 R	9 R	6 R	6 R	20 S	21 S	6 R	23 S	8 R
FFL30	24 S	22 S	16 S	28 S	12 R	15 S	18 S	15 S	11 R	12 R	28 S	17 S	15 S	27 S	9 R	12 R	17 S	13 R	19 S	11 R	30 S	30 S	30 S	38 S	10 R
GEN10	8 R	10 R	6 R	6 R	6 R	6 R	6 R	6 R	6 R	6 R	15 S	6 R	8 R	6 R	6 R	6 R	6 R	6 R	6 R	6 R	9 R	11 R	10 R	8 R	13 S
AMP10	6 R	6 R	6 R	6 R	6 R	6 R	6 R	6 R	6 R	6 R	6 R	6 R	6 R	6 R	6 R	6 R	6 R	6 R	6 R	6 R	6 R	6 R	6 R	6 R	6 R
ERY15	14 S	26 S	6 R	24 S	6 R	19 S	24 S	20 S	18 S	15 S	25 S	20 S	24 S	22 S	21 S	20 S	20 S	6 R	22 S	24 S	27 S	28 S	26 S	28 S	15 S
OXT30	12 R	12 R	15 S	19 S	12 R	13 R	10 R	12 R	12 R	11 R	23 S	13 R	11 R	15 S	10 R	12 R	12 R	6 R	12 R	12 R	25 S	18 S	22 S	15 S	14 R
FUR20	22 S	17 S	6 R	26 S	6 R	18 S	16 S	16 S	18 S	14 S	24 S	23 S	22 S	15 S	15 S	18 S	16 S	6 R	18 S	18 S	28 S	26 S	6 R	6 R	6 R
COT25	6 R	6 R	6 R	6 R	6 R	6 R	6 R	10 R	6 R	6 R	6 R	6 R	6 R	6 R	6 R	6 R	6 R	6 R	6 R	6 R	6 R	6 R	6 R	23 S	26 S
POL300	6 R	6 R	6 R	6 R	6 R	6 R	6 R	6 R	6 R	6 R	7 R	6 R	6 R	6 R	6 R	6 R	6 R	6 R	6 R	6 R	7 R	6 R	8 R	6 R	6 R

Table 3

Summary of antibiotic resistance of the *Flavobacterium* isolates studied

	F. johnsoniae A (n = 20)		*F. johnsoniae B* (n = 3)		*Chryseobacterium* sp. (n = 2)		Sum (n = 25)	
	No. of S	No. of R	No. of S	No. of R	No. of S	No. of R	No. of S	No. of R
Chloramphenicol 30	3	17	2	1	1	1	6	19
Florfenicol 30	13	7	3		1	1	17	8
Gentamicin 10	1	19		3	1	1	2	23
Ampicillin 10		20		3		2		25
Erythromycin 15	17	3	3		2		22	3
Oxytetracycline 30	4	16	3		1	1	8	17
Furazolidone 20	17	3	2	1		2	19	6
Cotrimoxazole 25		20		3	2		2	23
Polymyxin B 300		20		3		2		25

The colours indicate the dominance of susceptible (light grey) or resistant (dark grey) strains in the case of the different antibiotics

Antibiotic susceptibility

The antibiotic susceptibility pattern of the strains indicates that they have high-level multidrug resistance. All of them were resistant to ampicillin and polymyxin B. The results are shown in Tables 2 and 3.

Discussion

Members of the genus *Flavobacterium* are widespread in freshwater environments. The majority of them are saprophytic bacteria but some are pathogens that cause severe disease in fish. The best studied species is *F. columnare,* although its isolation is difficult as it is easily overgrown by other opportunistic bacteria present in the mixed culture. Thus, successful isolation of this bacterium requires special conditions. In this study, the isolation of flavobacteria, with the exception of two strains, was successful only on specific media supplemented with antibiotics, which indicates the necessity of using selective media. Bader et al. (2003) developed a species-specific PCR reaction for the rapid detection of *F. columnare*, and the strains were separated into four genetic types using additional PCR-RFLP. During the identification and sequencing of their own strains, Darwish et al. (2004) demonstrated that strain ATCC 43622 represented a new species, designated as *F. johnsoniae*. Our strains studied with the species-specific PCR developed by Bader et al. (2003) differed from the published data both in the amplified fragment size and in the RFLP patterns. On cleavage of the PCR fragments with restriction endonucleases HaeIII and RsaI, the majority (80%) of the strains had identical RFLP patterns (genotype A). Further three strains differed from the former only in the RsaI profile (genotype B). Strain C2 was similar to genotype B but had a unique HaeIII pattern (genotype C). Strain C3 possessed unique RFLP patterns with both restriction endonuclease enzymes.

Sequence analysis of the 1350 bp long 16S rRNA gene fragment identified 23 strains as *F. johnsoniae*, a species closely related to *F. columnare*, while two strains proved to be *Chryseobacterium* spp.

The most frequently occurring *F. johnsoniae* strains of genotype A (80%) were isolated both from Lake Balaton and from fish farms but with different density. While the disease manifestation on trap-captured fish from Lake Balaton was not typical, the animals from fish farms showed clear ulcerative signs and higher colony density with the dominance of yellow bacteria. In the absence of experimental infection, it was supposed that clinical disease after infection is manifested only in the presence of some predisposing factor. The detection of flavobacteria by culture requires selective media that suppress the growth of other environmental bacteria. On the basis of the present results it is difficult to evaluate the pathogenicity status (primary or secondary) of flavobacteria; for this, further studies would be needed.

Based on the monitoring presented here, we hypothesise that the Hungarian *F. columnare* strains identified by traditional methods (Csaba and Békési, 1977) could be *F. johnsoniae* isolates, since the two closely related species had not been distinguished at that time.

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

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

These results give cause for concern, since the majority of our strains came from untreated fish. Only two isolates originated from a stock that had been treated with doxycyclin 6 months prior to the study. Out of the antibiotics tested, oxytetracyclin, sulphamethoxazole-trimethoprim (Cosumix), and florfenicol are authorised for use in the medication of fish. Considering the prevalence of *Flavobacterium* strains with high-level multidrug resistance in fish, further studies on their epidemiological role and public health implications are needed. The origin of the detected antibiotic resistances is unknown; they can be both innate (primary) or acquired (secondary) features of these bacteria.

Our results indicate that the genus *Flavobacterium* was represented by the hitherto not identified *F. johnsoniae* and *Chryseobacterium* spp. in both wild and cultured freshwater fish species during the period of this study in Hungary. Fish collected from Lake Balaton (n = 17) and from freshwater farms (n = 6) had not been treated with any antibiotic, and still the strains recovered from them possessed multiple drug resistances. This high-level antibiotic resistance calls for more in-depth studies on the transmission of antibiotic resistance and its implications for human infections.

Acknowledgements

We thank Dr. Lone Madsen for her critical review and suggestions to improve the quality of this manuscript. The financial support received from projects KTIA-AIK-12-1-2013-0017, OTKA K 100132, OTKA PD 101091 and the 'János Bolyai' Research Scholarship of the Hungarian Academy of Sciences to B. Sellyei is gratefully acknowledged.

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