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Antimicrobial susceptibility pattern of *Flavobacterium columnare* isolates collected worldwide from 17 fish species

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Abstract

Flavobacterium columnare is the causative agent of columnaris disease in diverse fish species worldwide. Although columnaris is an important disease, the antimicrobial susceptibility pattern of *F. columnare* is not well studied. Thus, the purpose of this study was to test the *in vitro* antimicrobial susceptibility of 97 *F. columnare* isolates collected worldwide between 1987 and 2011 from 17 fish species. The broth microdilution technique was utilized for reliable testing of these fastidious organisms. None of the isolates displayed acquired resistance to florfenicol, gentamicin, ormetoprim-sulfadimethoxine and trimethoprim-sulfamethoxazole. Acquired resistance to chloramphenicol was detected in 1%, to nitrofuran in 5%, to oxytetracycline in 11% and to enrofloxacin, flumequine and oxolinic acid in 10%, 16% and 16% of the isolates, respectively, as reflected by a bimodal or trimodal distribution of their minimum inhibitory concentrations (MICs). One isolate showed acquired resistance towards several antimicrobial agents including erythromycin. Another isolate revealed acquired resistance towards – amongst others – ampicillin. The isolates displaying acquired resistance originated from ornamental fish species or Vietnamese catfish, except for two isolates coming from wild channel catfish in which acquired resistance was encountered towards oxytetracycline only. Fifty per cent of the resistant isolates from ornamental fish were shown to have acquired resistance against three classes of antimicrobial agents, assigning these isolates as multiple resistant. These data might indicate less prudent use of antimicrobials especially in ornamental fish species.

Keywords: antimicrobial susceptibility testing, broth microdilution, columnaris, *Flavobacterium columnare*, multiple resistance.

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Introduction

Columnaris disease is a predominant bacterial disease of both cultured and wild freshwater fish. Many commercially important fish species are susceptible to columnaris disease, such as (but not limited to) salmonids, eels, carp, goldfish, tilapia and channel catfish (Řehulka & Minařík 2007; Soto *et al.* 2008; Suomalainen, Bandilla & Valtonen 2009). This disease also poses a problem for many freshwater tropical aquarium fish (Post 1987; Decostere, Haesebrouck & Devriese 1998). *F. columnare* infections may result in skin lesions, fin erosion and gill necrosis, with a high degree of mortality, leading to severe economic losses (Decostere *et al.* 1998). In the United States, *F. columnare* is the second most prevalent bacterium, after *Edwardsiella ictaluri*, to cause disease and mortality (Hawke & Thune 1992; Wagner

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et al. 2002), with yearly losses estimated at 30 million dollars (Shoemaker *et al.* 2011).

Up until now, only a limited number of effective preventive measures against columnaris disease are available, including an attenuated immersion vaccine registered for use in channel catfish in the United States only (Shoemaker *et al.* 2011). A *F. columnare* bacterin was also brought to market in the USA as an aid in the prevention of columnaris disease in healthy salmonids of over 3 g (AFS-FCS 2011). Awaiting further research aimed at developing and validating more precautionary measures, there is still an excessive use of antimicrobial agents in the contemporary treatment of *F. columnare* (Shoemaker *et al.* 2011). It should be noted that significant negative attributes are associated with the use of antimicrobials including the extensive expenditure on these substances and possible allergic reactions elicited in the user after food contact. Potential impacts on human health resulting from the emergence of drug-resistant bacteria and the associated risk of transfer of these resistant traits to the environment and human-associated bacteria are also a major concern (Serrano 2005).

To seize on the aforementioned risks associated with antimicrobial use and to inform veterinary practitioners on the therapeutic value of antimicrobial agents, enabling them to make an informed choice, antimicrobial susceptibility monitoring is a necessary prerequisite. Hitherto, only limited information is available on the antimicrobial susceptibility pattern of *F. columnare*. Antimicrobial susceptibility studies of Thomas-Jinu & Goodwin (2004) using agar disc diffusion revealed susceptibility of two of the four tested strains towards a combination of sulfadimethoxine and ormetoprim and acquired resistance of two isolates towards oxytetracycline. The same assay was adopted to assess the antimicrobial susceptibility of eight Finnish *F. columnare* strains by Suomalainen *et al.* (2006). All strains were susceptible to ampicillin, erythromycin, gentamicin, nitrofurantoin, streptomycin, tetracycline, trimethoprim-sulpha and florfenicol, but displayed acquired resistance to neomycin and polymyxin B. However, agar disc diffusion tests are challenging because of the rhizoid growth of the organism, which tends to make the zones of inhibition ill defined. Moreover, no specific breakpoints are available for *F. columnare*. Additionally, the apparent zones of inhibition may be the result of delayed growth (Hesami *et al.* 2010; Miller *et al.* 2005). The Clinical and Laboratory Standards Institute (CLSI) therefore proposed the use of the broth microdilution technique in 1:7 diluted cation adjusted Mueller Hinton broth (CAMHB) for fastidious growing organisms like *F. columnare* (CLSI 2006). Darwish, Farmer & Hawke (2008) adopted much of the CLSI 2006 recommendations but used 1:5 dilution of CAMHB as an alternative method to the one proposed by Clinical & Laboratory Standards Institute (CLSI) (2006), based on the work of Farmer (2004). They determined the minimum inhibitory concentration (MIC) of 23 *F. columnare* isolates to eight antimicrobial agents. All isolates came from channel catfish except for three isolates originating from a common carp and two type strains isolated from a Chinook salmon and a brown trout. All isolates were procured in the USA except for one (type strain) which was isolated in France. The authors underscored the noted resistance against the combination of metoprim/sulfadimethoxine but did not interpret the other encountered MIC values and acknowledged the relatively low number of tested isolates.

The purpose of this study was to address the above described lack of information on the antimicrobial susceptibility pattern of *F. columnare*. Therefore, 97 isolates of *F. columnare* collected worldwide from 17 different fish species were tested for their susceptibility to 12 antimicrobial agents by means of the broth microdilution technique.

Materials and methods

Bacterial strains

Ninety-seven *F. columnare* isolates originating from 17 fish species collected worldwide between 1987 and 2011 from both cultured and wild fish populations were included in this study (Table 1). The identity of *F. columnare* was confirmed by polymerase chain reaction (PCR). Briefly, genomic DNA was extracted by suspending one colony of a pure bacterial culture in 20 mL lysis buffer (0.25% SDS, 0.05 N NaOH). This suspension was heated at 95 °C for 5 min and centrifuged for 10 s at 16.100 ***g*** for vapour deposition. One hundred and eighty millilitres of sterile distilled water was added and centrifugation was carried out at 16.100 ***g*** for 5 min. Specific primers were

Table 1 *Flavobacterium columnare* isolates included in the antimicrobial susceptibility testing

Isolate of *Flavobacterium columnare*	Fish host	Origin	Year of isolation	Provided by
JIP 44/87 (ATCC 49512)	Brown trout (*Salmo trutta*)	France	1987	Dr J.F. Bernardet
JIP 39/87 (ATCC 49513)	Black bullhead (*Ictalurus melas*)	France	1987	Dr J.F. Bernardet
LVDL 3414/89	European eel (*Anguila anguilla*)	France	1989	Dr J.F. Bernardet
P06/90	Black bullhead	Unknown	1990	Dr J.F. Bernardet
90-106	Channel catfish (*Ictalurus punctatus*)	USA	1990	Dr A. Karsi
L90-629	Channel catfish	USA	1990	Dr A. Karsi
P 11/91	Rainbow trout (*Oncorhynchus mykiss*)	France	1991	Dr J.F. Bernardet
C91-20	Channel catfish	USA	1991	Dr A. Karsi
LVDI 39/I	Unknown	France	1992	Dr J.F. Bernardet
92-002	Channel catfish	USA	1992	Dr A. Karsi
AI 94	Channel catfish	USA	1994	Dr A.E. Goodwin
LVDJ (D7461)	Rainbow trout	France	1994	Dr J.F. Bernardet
94-078	Channel catfish	USA	1994	Dr A. Karsi
BioMed	Channel catfish	USA	1996	Dr C. Arias
LDA 39 H4927	Black bullhead	France	1998	Dr J.F. Bernardet
Au 98-24	Channel catfish	USA	1998	Dr C. Arias
AJS-6	Koi carp (*Cyprinus carpio*)	Belgium	1999	Unknown
JIP14/00	Neon tetra (*Paracheirodon innesi*)	France	2000	Dr J.F. Bernardet
JIP 13/00	Neon tetra	France	2000	Dr J.F. Bernardet
ALG-00-530	Channel catfish	USA	2000	Dr C. Arias
Grizzle	Channel catfish	USA	2000	Dr C. Arias
JIP 17/01	Koi carp	France	2001	Dr J.F. Bernardet
VB2	Guppy (*Pycnostachys reticulata*)	France	2001	Dr J.F. Bernardet
VB1	Guppy	France	2001	Dr J.F. Bernardet
JIP 07/02	Koi carp	France	2002	Dr J.F. Bernardet
#27	Channel catfish	USA	2002	Dr C. Arias
MS-02-475	Channel catfish	USA	2002	Dr C. Arias
H2	Rainbow trout	Finland	2003	Dr L.R. Sundberg
ALG-03-57	Channel catfish	USA	2003	Dr C. Arias
04017018	Koi carp	Netherlands	2004	Dr O.L.M. Haenen
ALM-05-26	Blue catfish (*Ictalurus furcatus*)	USA	2005	Dr C. Arias
ALM-05-28	Blue catfish	USA	2005	Dr C. Arias
ALM-05-29	Blue catfish	USA	2005	Dr C. Arias
ALM-05-30	Channel catfish	USA	2005	Dr C. Arias
ALM-05-35	Freshwater drum (*Aplodinotus grunniens*)	USA	2005	Dr C. Arias
ALM-05-36	Threadfin shad (*Dorosoma petenense*)	USA	2005	Dr C. Arias
ALM-05-39	Threadfin shad	USA	2005	Dr C. Arias
ALM-05-43	Threadfin shad	USA	2005	Dr C. Arias
ALM-05-53	Channel catfish	USA	2005	Dr C. Arias
ALM-05-58	Blue catfish	USA	2005	Dr C. Arias
ALM-05-105	Threadfin shad	USA	2005	Dr C. Arias
ALM-05-106	Threadfin shad	USA	2005	Dr C. Arias
ALM-05-107	Threadfin shad	USA	2005	Dr C. Arias
ALM-05-111	Threadfin shad	USA	2005	Dr C. Arias
BGFS-08	Blue catfish	USA	2005	Dr C. Arias
BGFS-28	Channel catfish	USA	2005	Dr C. Arias
BGFS-29	Channel catfish	USA	2005	Dr C. Arias
S03-579	Channel catfish	USA	2005	Dr A. Karsi
S05-79	Channel catfish	USA	2005	Dr C. Arias
PB 06-113#1	Largemouth bass (*Micropterus salmoides*)	USA	2006	Dr A.E. Goodwin
JIP 02/06	Siamese fighting fish (*Betta splendens*)	France	2006	Dr J.F. Bernardet
C5	Chinese high fin banded shark (*Myxocyprinus asiaticus*)	China	2007	Prof P. Nie
LD 40 07/2489	Siberian sturgeon (*Acipenser baeri*)	France	2007	Dr J.F. Bernardet
B259	Rainbow trout	Finland	2009	Dr L.R. Sundberg
S09-108	Channel catfish	USA	2009	Dr A. Karsi
S09-157	Channel catfish	USA	2009	Dr A. Karsi
S09-162	Channel catfish	USA	2009	Dr A. Karsi

(continued)

Table 1 (*continued*)

Isolate of *Flavobacterium columnare*	Fish host	Origin	Year of isolation	Provided by
S09-177	Channel catfish	USA	2009	Dr A. Karsi
S09-194	Channel catfish	USA	2009	Dr A. Karsi
S09-378	Channel catfish	USA	2009	Dr A. Karsi
S09-382	Channel catfish	USA	2009	Dr A. Karsi
09013931	Koi carp	Netherlands	2009	Dr O.L.M. Haenen
FCVK2 8T2	Rainbow trout	Finland	2010	Dr L.R. Sundberg
10012573-2	Koi carp	Netherlands	2010	Dr O.L.M. Haenen
10009061-1	Koi carp	Netherlands	2010	Dr O.L.M. Haenen
10012931	Koi carp	Netherlands	2010	Dr O.L.M. Haenen
S10-025	Channel catfish	USA	2010	Dr A. Karsi
S10-239	Channel catfish	USA	2010	Dr A. Karsi
C-066	Channel catfish	USA	2010	Dr A. Karsi
C-068	Channel catfish	USA	2010	Dr A. Karsi
C-069	Channel catfish	USA	2010	Dr A. Karsi
C-074	Channel catfish	USA	2010	Dr A. Karsi
S10-302	Channel catfish	USA	2010	Dr A. Karsi
CB10-151	Channel catfish	USA	2010	Dr A. Karsi
DT2	Vietnamese catfish (*Pangasius hypophthalmus*)	Vietnam	2011	Dr T.T. Dung
HG13	Vietnamese catfish	Vietnam	2011	Dr T.T. Dung
CT1	Vietnamese catfish	Vietnam	2011	Dr T.T. Dung
CT4	Vietnamese catfish	Vietnam	2011	Dr T.T. Dung
DT4	Vietnamese catfish	Vietnam	2011	Dr T.T. Dung
HG1	Vietnamese catfish	Vietnam	2011	Dr T.T. Dung
HG9	Vietnamese catfish	Vietnam	2011	Dr T.T. Dung
HG12	Vietnamese catfish	Vietnam	2011	Dr T.T. Dung
HG10	Vietnamese catfish	Vietnam	2011	Dr T.T. Dung
ALU A	Koi carp	Belgium	2011	Prof A. Decostere
1191-B	Channel catfish	USA	Unknown	Dr A. Karsi
97-01	Unknown	USA	Unknown	Dr C. Arias
ALM-05-32	Unknown	USA	Unknown	Dr C. Arias
ALM-05-37	Unknown	USA	Unknown	Dr C. Arias
ALM-05-51	Unknown	USA	Unknown	Dr C. Arias
C56	Unknown	USA	Unknown	Dr A. Karsi
CDI-A	Koi carp	Netherlands	Unknown	Unknown
Coho92	Rainbow trout	USA	Unknown	Dr L. Caslake
Fathead minnow	Fathead minnow (*Pimephales promelas*)	USA	Unknown	Dr L. Caslake
IC(B)E	Unknown	USA	Unknown	Unknown
JIF E	Unknown	Unknown	Unknown	Unknown
RP	Unknown	Unknown	Unknown	Unknown
TAC	Unknown	Unknown	Unknown	Unknown

ATTC, American Type Culture Collection.

synthesized at Integrated DNA Technologies. PCR mixtures and cycle conditions were the same as described before (Panangala, Shoemaker & Klesius 2007). *Escherichia coli* (ATCC 25922) and *Aeromonas salmonicida* subsp. *salmonicida* (ATCC 33658) were included as reference strains in the broth dilution tests.

Antimicrobial agents

The following 12 antimicrobial agents were included: ampicillin, chloramphenicol, enrofloxacin, erythromycin, florfenicol, flumequine, gentamicin, nitrofuran, ormetoprim-sulfadimethoxine, oxolinic acid, oxytetracycline and trimethoprim-sulfadimethoxazole.

These antimicrobials were obtained as laboratory standard powders from Sigma-Aldrich N.V., except for enrofloxacin and ormetoprim which were obtained from Medini and from Alpharma, respectively. They were dissolved in sterile distilled water to make stock solutions of at least ten times the highest concentration to be tested.

Afterwards they were further diluted in twofold dilution series in appropriate solvents according to the macrodilution method as stipulated by the

Clinical and Laboratory Standards Institute (CLSI 2006) with the following ranges of twofold serial dilutions: ampicillin 0.016–16 μg mL^{-1}, chloramphenicol 0.03–32 μg mL^{-1}, enrofloxacin 0.002–2 μg mL^{-1}, erythromycin 0.06-64 μg mL^{-1}, florfenicol 0.016–16 μg mL^{-1}, flumequine 0.008–8 μg mL^{-1}, gentamicin 0.02–16 μg mL^{-1}, nitrofuran 0.03–32 μg mL^{-1}, ormetoprim-sulfadimethoxine 0.03/0.6–32/608 μg mL^{-1}, oxolinic acid 0.025–25 μg mL^{-1}, oxytetracycline 0.008–64 μg mL^{-1} and trimethoprim-sulfadimethoxazole 0.03/0.6–32/608 μg mL^{-1}. Fifty microlitres of these dilutions was added into the wells of a plastic standard 96-well plate (Greiner-Bio One GmbH). Each well of the last column of the tray was filled with 1:7 diluted (3 g L^{-1}) CAMBH (Becton Dickinson) without antimicrobials added and served as a positive control for growth of the isolate tested.

Susceptibility testing

All strains, stored at −70 °C, were defrosted and grown overnight at 28 °C in 1:7 (3 g L^{-1}) diluted CAMHB (CLSI 2006). Consequently, the broth culture was adjusted to a 0.5 McFarland suspension equivalent to 1×10^8 colony-forming units (CFU) per mL and then further diluted 1:100 in 1:7 (3 g L^{-1}) CAMHB. Subsequently, 50 μL of this dilution was added to each well of the 96-well plates. Bacterial growth was assessed after 44–48 h aerobic incubation at 28 °C. The MIC was defined as the lowest concentration of the antimicrobial agent without any visible bacterial growth (CLSI 2006). Following incubation, the positive control of each isolate was inoculated on Columbia agar with 5% sheep blood (Oxoid), which was incubated at 28 °C for 72 h and checked after 24, 36 and 72 h to check purity. *E. coli* (ATCC 25922) and *A. salmonicida* subsp. *salmonicida* (ATCC 33658) were included in the assays.

Results

For the antimicrobial agents included in the CLSI document M49-A, the MIC values of the *E. coli* and *A. salmonicida* subsp. *salmonicida* reference strains fell within acceptable quality ranges (CLSI 2006) (Table 2). The MIC values for chloramphenicol, erythromycin and nitrofuran for *E. coli* were 4–8, 16–32 and 8–32 μg mL^{-1}, respectively. For *A. salmonicida* subsp. *salmonicida*, the MIC value for chloramphenicol was 0.5–1 and for nitrofuran 2–8 μg mL^{-1}. An overview of the MIC values for the 97 tested *F. columnare* isolates is shown in Table 2.

For florfenicol, gentamicin and for the combinations ormetoprim-sulfadimethoxine and trimethoprim-sulfamethoxazole, a monomodal distribution of MICs was noted, indicating absence of acquired resistance. In contrast, for ampicillin, chloramphenicol, erythromycin and nitrofuran, the MICs showed a bimodal distribution. This was also the case for enrofloxacin, but in addition, an extended frequency distribution of MICs (tailing) was present here for isolates belonging to the *F. columnare* population with the lower MIC values. The MIC values for flumequine, oxolinic acid and oxytetracycline displayed a trimodal distribution. According to the microbiological criterion, isolates in the higher range of MICs should be considered to have acquired resistance (Turnidge & Paterson 2007).

Resistance phenotypes of the *F. columnare* isolates are shown in Table 3. Sixty per cent of the resistant isolates procured from ornamental fish had acquired resistance towards two and 50% towards three classes of antimicrobial agents, respectively, assigning these latter isolates as multiple resistant (Schwarz *et al.* 2010). As for the isolates originating from cultured Vietnamese catfish, 100% showed acquired resistance towards at least one of the tested quinolone antimicrobials and one isolate was multiple resistant.

Upon inspecting the blood agar plates inoculated with the positive controls for growth in the MIC assays, small colonies were noted for some *F. columnare* isolates after 72 h of incubation. When these colonies were scraped off from the blood plate and inoculated onto Shieh agar plates (Shieh 1980; Song, Fryer & Rohovec 1988), pure colonies with the typical *F. columnare* morphology appeared after 24- to 36-h incubation of the Shieh plates at 28 °C. Gram staining revealed the typical long and slender *F. columnare* morphology. Upon testing the colonies from the blood agar phenotypically and using PCR, all inocula were confirmed to yield only *F. columnare*.

Discussion

For the interpretation of the MIC results, the microbiological criterion was used as CLSI breakpoints for aquatic organisms are not yet

Table 2 Distribution of minimum inhibitory concentration (MIC) of 12 antimicrobial agents on 100 *Flavobacterium columnare* isolates from 15 different fish species collected worldwide between 1987 and 2011

	Number of isolates with MIC (μg mL^{-1})																	
Antimicrobial agent	0.008	0.016	0.032	<0.06	0.064	0.125	0.25	0.5	1	2	4	8	>8	16	32	>32	64	Total number of resistant isolates out of 100
Ampicillin			1		23	6	69				**1**[a]							1
Chloramphenicol									5	62	31			**1**	**1**			2
Enrofloxacin	4	75	4		2	5[b]		**2**	**5**	**3**								10 (+5 tailing)
Erythromycin				1	2	14	17	49	16		**1**							1
Florfenicol							14	30	51	5								0
Flumequine					17	63	4	**1**	**7**	**1**	**1**	**1**	**5**					16
Gentamicin					1	4	11	33	39	12								0
Nitrofuran						1	1	12	38	7	34	2			**1**	**4**		5
Oxytetracycline			4		66	19				**1**	**3**				**6**		**1**	11
Ormetoprim-sulfadimethoxin (1/19)						1	10	8	41	26	11	1		2				0
Trimethoprim-sulfametoxazole (1/19)					1	5	8	31	28	14	13							0

	Number of isolates with MIC (μg mL^{-1})												
Antimicrobial agent	0.05	0.05	0.1	0.2	0.4	0.8	1.6	3.2	6.3	12.5	25	>25	Total number of resistant isolates out of 100
Oxolinic acid		2	34	48			**1**	**7**	**2**	**1**		**5**	16

[a]*F. columnare* isolates considered to have acquired resistance are represented in bold.
[b]Extended frequency distribution (tailing).
Black background: value was not measured for this antibiotic agent.

Table 3 Resistant phenotypes of *Flavobacterium columnare* isolates displaying acquired resistance.

Isolate	Fish host	Origin	Year of isolation	Resistance phenotypes
CDI-A	Koi carp	Netherlands	Unknown	Enro, flum, oxol
JIP 13/00	Neon tetra	France	2000	Enro, flum, nf, oxol, oxyt
JIP 14/00	Neon tetra	France	2000	Enro, flum, nf, oxol, oxyt
VB1	Guppy	France	2001	Enro, flum, nf, oxol, oxyt
VB2	Guppy	France	2001	Ampi, chlor, nf, oxyt
04017018	Koi carp	Netherlands	2004	Oxyt
ALM-05-28	Blue catfish	USA	2005	Oxyt
BGFS-28	Channel catfish	USA	2005	Oxyt
09013931	Koi carp	Netherlands	2009	Enro, flum, oxol
10009061-1	Koi carp	Netherlands	2010	Enro, flum, nf, oxol, oxyt
10012931	Koi carp	Netherlands	2010	Enro, flum, oxol, oxyt
CT1	Vietnamese catfish	Vietnam	2011	Flum, oxol
CT4	Vietnamese catfish	Vietnam	2011	Enro, flum, oxol
DT2	Vietnamese catfish	Vietnam	2011	Enro, flum, oxol
DT4	Vietnamese catfish	Vietnam	2011	Enro, flum, oxol
HG1	Vietnamese catfish	Vietnam	2011	Flum, oxol (tailing enro)
HG9	Vietnamese catfish	Vietnam	2011	Flum, oxol (tailing enro)
HG10	Vietnamese catfish	Vietnam	2011	Flum, oxol (tailing enro)
HG12	Vietnamese catfish	Vietnam	2011	Flum, oxol (tailing enro)
HG13	Vietnamese catfish	Vietnam	2011	Erythro, flum, oxol, oxyt (tailing enro)

ampi: ampicillin; chlor: chloramphenicol, enro: enrofloxacin; erythro: erythromycin; flum: flumequine; nf: nitrofuran; oxol: oxolinic acid; oxyt: oxytetracycline.

available. This parameter gives a reasonable approximation to distinguish wild-type populations of bacteria from those with acquired resistance (Turnidge & Paterson 2007). This criterion does not necessarily predict how the fish will respond to antimicrobial treatment. However, for most included antimicrobial agents, MIC values were at least ten times higher for isolates designated as acquired resistant. It is therefore highly unlikely that fish infected with these isolates will be successfully treated with these antimicrobial agents. Nonetheless, it will be necessary to carry out experimental challenges adopting isolates with varying MIC values or to have clinical data to be able to draw well-founded conclusions on the *in vivo* efficacies of the antimicrobial agents in question.

In general, in countries where antimicrobial agents are allowed for use in aquatic species, only two or three antimicrobials have been granted a marketing authorization (CLSI 2006; FDA 2011; Rigos & Troisi 2005; Cizek *et al.* 2010). This tight control on antibiotic use applies to fish cultured for human consumption, but is not enforced on ornamental fish. The ornamental fish industry is massive and many ornamental fish are extremely valuable, exceeding values of aquaculture products for human consumption in some countries (Alderman & Hastings 1998; Verner-Jeffreys *et al.* 2009; Weir *et al.* 2012). This, combined with intensive rearing conditions and transport stress rendering the fish more susceptible to bacterial diseases, encourages the use of antimicrobial agents in this sector. Veterinarians might, using the prescription cascade, prescribe many antibiotic agents that may cure the sick fish, as consumption is excluded for ornamental fish (Alderman & Hastings 1998; Cizek *et al.* 2010). Indeed, extra-label drug use occurs in many countries (Bal & Gould 2011; CLSI 2006; Nikaido 2009; Rigos & Troisi 2005; Serrano 2005), which is why the susceptibility tests in the present study included more antimicrobial agents than specifically approved for use in aquatic species.

Nowadays, the use of chloramphenicol is limited to some life-threatening conditions in humans because of the adverse side effects (aplastic anaemia and bone-marrow suppression) and the availability of less toxic antimicrobials (Schwarz *et al.* 2004). Although not allowed for use in aquaculture, chloramphenicol residues have been detected in fishery products from South-East Asia, giving rise to risks for the human health (Serrano 2005). In some studies, *Aeromonas* spp. were shown to have acquired resistance towards chloramphenicol (Weir *et al.* 2012). In the present study, acquired resistance of one strain towards this antimicrobial agent was found, making it the first to describe acquired resistance of *F. columnare* towards chloramphenicol. Florfenicol

is a fluorinated structural analogue of chloramphenicol without the above-mentioned side effects and is increasingly popular in aquaculture (Aoki 2000; Liao 2000; Sapkota *et al.* 2008).

The FDA approved this antibiotic for the treatment of enteric septicaemia of catfish, for coldwater disease in salmonids, for furunculosis in freshwater-reared salmonids and for columnaris disease in catfish (FDA 2011). It is also registered in some European countries for use in aquatic species. In this investigation, no acquired resistance was noted towards florfenicol. Nevertheless, prudent use of this antibiotic remains necessary. In Finland, florfenicol is only accepted as an alternative antibiotic in case resistance towards oxytetracycline occurs (Suomalainen *et al.* 2006).

No acquired resistance was found for the combinations ormetoprim/sulfadimethoxine and trimethoprim/sulfamethoxazole. This is in contrast to Darwish *et al.* (2008) who noted acquired resistance in American *F. columnare* channel catfish isolates for ormetoprim/sulfadimethoxine. In the present study, both the combinations ormetoprim/sulfadimethoxine and trimethoprim/sulfamethoxazole were tested because in the CLSI guidelines (CLSI 2006) it is stated that it is not yet confirmed whether the former combination can be used to predict the susceptibility to the latter at 28 ± 2 °C. Isolates showing high values for ormetoprim/sulfadimethoxine, however, also displayed higher values for trimethoprim/sulfamethoxazole.

For the quinolones, enrofloxacin, flumequine and oxolinic acid were included in this study. Quinolones are mainly broad-spectrum antibacterial agents, commonly used in both human and veterinary medicine. Their extensive or unnecessary use in some countries, which can to some degree be considered as a misuse, or the use of quinolones with poor activity, has resulted in bacteria rapidly developing resistance to these agents (Ruiz 2003). Multiple studies demonstrated resistance of different ornamental fish pathogens (*Aeromonas* spp. and *Vibrio* spp.) towards the quinolones (Verner-Jeffreys *et al.* 2009; Weir *et al.* 2012). The FDA prohibited extra-label use of fluoroquinolones in food animals (Serrano 2005). These second-line antimicrobials are to be reserved for conditions that have responded poorly to other classes of antimicrobial agents and they should not be used for prophylaxis (European Medicines Agency 2011). In some countries including Vietnam, flumequine and/or oxolinic acid are registered for use in consumable aquatic species. In this study, the bimodal or trimodal distribution of MICs indicated acquired resistance to enrofloxacin, flumequine and oxolinic acid in 10%, 16% and 16% of the isolates, respectively. Of the ten isolates displaying acquired resistance to enrofloxacin, seven were sampled from ornamental fish. The remaining three isolates originated from Vietnamese catfish. This is the first time acquired resistance of *F. columnare* towards this antimicrobial class is described.

Resistance to quinolones typically arises as a result of alterations in the target enzymes (mostly gyrase) and of changes in drug entry and efflux. It can also be mediated by plasmids that produce the Qnr protein which protects the quinolone targets from inhibition (Drlica & Zhao 1997; Jacoby 2005). In many Gram-negative bacteria, resistance develops progressively through stepwise mutations. A single mutation in the gyrase gene results in resistance to the first-generation quinolones, such as oxolinic acid and flumequine, and reduced susceptibility to other quinolones. A second mutation in the gyrase gene mediates full resistance to the quinolones (Marien *et al.* 2007). In this study, all isolates resistant to enrofloxacin as indicated by the bimodal distribution of MIC values, also displayed acquired resistance to flumequine and oxolinic acid. Remarkably, the MICs of enrofloxacin for isolates belonging to the *F. columnare* population with the lower MIC values showed an extended frequency distribution, possibly indicating the presence of a mechanism providing decreased susceptibility in some isolates. Indeed, the five isolates with an enrofloxacin MIC of 0.125μg mL^{-1} also demonstrated acquired resistance towards flumequine and oxolinic acid. One isolate displaying a MIC value of 0.5μg mL^{-1} for flumequine was considered resistant to this antimicrobial agent as this same isolate displayed acquired resistance for oxolinic acid (6.3 μg mL^{-1}) and an evaluated MIC value for enrofloxacin (0.12 μg mL^{-1}).

In this study, 10 isolates displayed acquired resistance towards oxytetracycline. Oxytetracycline is one of the most commonly used tetracyclines worldwide for the treatment of bacterial fish diseases (Rigos & Troisi 2005). Multiple studies have revealed resistance of ornamental fish pathogens *Aeromonas* spp. and *Vibrio* spp. towards

tetracyclines (Cizek *et al.* 2010; Jongjareanjai, Assawawongkasem & Chansue 2009; Verner-Jeffreys *et al.* 2009; Weir *et al.* 2012). Previous studies showed no resistance of *F. columnare* to oxytetracycline (Thomas-Jinu & Goodwin 2004; Suomalainen *et al.* 2006) designating this study as the first to report acquired resistance against this antimicrobial agent.

Nitrofuran is not allowed for use in aquaculture. Resistance of *Vibrio* and *Aeromonas* species towards nitrofuran in ornamental fish has been described before (Weir *et al.* 2012). The present study shows acquired resistance of five *F. columnare* isolates coming from ornamental fish. This study is the first to report acquired resistance of *F. columnare* towards nitrofuran.

In this study, except in one case, all isolates displaying multiple resistance originated from ornamental fish. This may reflect the differences in antimicrobial use policy in fish destined for human consumption and in ornamental fish, as was stipulated by other research groups (Verner-Jeffreys *et al.* 2009; Cizek *et al.* 2010) that came across similar findings in aeromonads from ornamentals.

Conclusion

The present study is the first of its kind in terms of the high number and mixed origin of *F. columnare* isolates in respect of fish species, year of isolation and geographical area. Acquired resistance to chloramphenicol, oxytetracycline, flumequine, oxolinic acid, enrofloxacin and nitrofuran is reported for the first time in *F. columnare*. The results obtained in this study might indicate less prudent use of antimicrobials especially in the ornamental fish industry and therefore urges limits to their use and to focus on preventive measures.

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