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Ornamental fish as a source of plasmid-mediated quinolone resistance genes and antibiotic resistance plasmids

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ABSTRACT

Growing ornamental fish industry is associated with public health concerns including extensive antibiotic use accompanied by increasing antibiotic resistance. The aim of this study was to analyze *Aeromonas* isolates from imported tropical ornamental fish and coldwater koi carps bred in the Czech Republic to assess the potential risk of ornamental fish as a source of plasmid-mediated quinolone resistance genes (PMQR) and antibiotic resistance plasmids. A collection of *Aeromonas* spp. with reduced susceptibility to ciprofloxacin (MIC $\geq$ 0.05 mg/L) was selected for the detection of PMQR genes. Isolates harbouring PMQR genes were further analyzed for the additional antibiotic resistance, integron content, clonality, biofilm production and transferability of PMQR genes by conjugation and transformation.

Comparative analysis of plasmids carrying PMQR genes was performed. Fifteen (19%, $n = 80$) isolates from koi carps and 18 (24%, $n = 76$) isolates from imported ornamental fish were positive for *qnrS2*, *aac(6′)-Ib-cr* or *qnrB17* genes. PMQR-positive isolates from imported ornamental fish showed higher MIC levels to quinolones, multiresistance and diverse content of antibiotic resistance genes and integrons compared to the isolates from the carps. Related IncU plasmids harbouring *qnrS2* and *aac(6′)-Ib-cr* genes were found in *Aeromonas* spp. from imported ornamental fish and koi carps from various geographical areas. Ornamental fish may represent a potential source of multiresistant bacteria and mobile genetic elements for the environment and for humans.

1. Introduction

Intensive fish farming has resulted in increasing problems of bacterial diseases leading to the subsequent heavy use of antibiotics (Cabello et al., 2013). Over 1 billion ornamental fish are traded globally each year (Whittington and Chong, 2007). Several public health concerns have

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been associated with the ornamental fish industry such as the transmission of zoonotic pathogens to humans and terrestrial animals, antimicrobial use practices and antibiotic resistance (Lupo et al., 2012; Weir et al., 2012). The ornamental fish producers tend to administer antibiotics for a prophylactic or preventive treatment in order to decrease the bacterial disease outbreaks while fish are recovering from harvesting and handling and to increase their survival during shipment. Excessive or incorrect use of a prophylactic treatment can increase selection and facilitate the spread of antibiotic-resistant bacteria (Weir et al., 2012). Specific antibiotics as nitrofurans, quinolones and oxytetracycline are commonly used in the ornamental fish trade.

Quinolones are broad-spectrum antimicrobial agents widely used in both human and veterinary medicine and they are among the most widely used antibiotics in the aquacultures (Smith, 2008). Their extensive use has been associated with worldwide increasing quinolone resistance. Resistance to quinolones is mediated by specific chromosomal mutations or by plasmid-mediated quinolone resistance (PMQR) genes that play an important role in dissemination of quinolone resistance *via* horizontal gene transfer (HGT; Strahilevitz et al., 2009). PMQR determinants include Qnr proteins, *aac(6′)-Ib-cr* and efflux pumps and confer high level of resistance to nalidixic acid and low level fluoroquinolone resistance in bacteria according to CLSI breakpoints. However, the presence of PMQR can facilitate the emergence of high level fluoroquinolone resistance *via* mutations in DNA gyrase and topoisomerase IV genes *gyrA* and *parC* (Cattoir et al., 2008; Verner-Jeffreys et al., 2009; Han et al., 2012a,b).

Aeromonads are suitable indicator bacteria for studying the incidence and development of antibiotic resistance in aquacultures (Naviner et al., 2011). These bacteria are interconnected within the water ecosystem, colonize fish and can cause various infections. Antibiotic resistance in *Aeromonas* spp. from imported ornamental fish is of high concern for public health (Verner-Jeffreys et al., 2009; Sreedharan et al., 2012). *Aeromonas* spp. have been proven to cause human infections (Parker and Shaw, 2011).

The greatest potential public health risk linked with extensive antimicrobial use in aquaculture is the development of reservoirs of antibiotic resistance genes in aquatic ecosystems from where such genes can be spread by HGT to other bacteria including human pathogens (Lupo et al., 2012). There is limited information on mobile genetic elements transferring quinolone-resistance genes in *Aeromonas* from ornamental fish (Verner-Jeffreys et al., 2009; Han et al., 2012a,b). The aim of this study was to analyze *Aeromonas* isolates from imported tropical ornamental fish and coldwater koi carps bred in the Czech Republic to assess the potential risk of ornamental fish as a source of plasmid-mediated quinolone resistance genes and antibiotic resistance plasmids.

2. Material and methods

2.1. Bacterial isolates

A collection of 156 *Aeromonas* spp. isolates originating from tropical freshwater ornamental fish (76 isolates) and coldwater ornamental (koi) carps (80 isolates) was analyzed in this study. Koi carp isolates were gained from gills and skin swabs of euthanized healthy fish on 7 farms in 2005 and 2006 (Cizek et al., 2010). Tropical freshwater ornamental fish were sampled in a wholesale distributor (Hodonin, Czech Republic) during their health inspection after they arrived to the Czech Republic from Vietnam, Thailand, China, Slovakia, Brazil and Peru between 2005 and 2007. Fish were kept and transported in their original carriage water in sealed bags in polystyrene boxes. The samples were obtained from skin, gills and body cavity swabs of diseased fish. All samples were cultivated on Columbia agar (CM331; Oxoid Ltd., Basingstoke, UK) supplemented with 5% sheep blood and suspect *Aeromonas* cultures were identified as described elsewhere (Cizek et al., 2010). Susceptibility to ciprofloxacin (0.015–128 mg/L) of all *Aeromonas* isolates was determined by agar dilution method in accordance with Clinical and Laboratory Standard Institute (CLSI, 2006, 2008). Quality control was done using reference strains *Escherichia coli* ATCC 25922 and *Aeromonas salmonicida* spp. *salmonocida* ATCC 33658.

2.2. Detection of PMQR genes and characterization of PMQR-positive isolates

Isolates with minimum inhibitory concentration (MIC) to ciprofloxacin ≥0.05 mg/L were selected for the detection of PMQR genes (*qnrA*, *qnrB*, *qnrC*, *qnrD*, *qnrS*, *aac(6′)-Ib-cr*, *qepA*, *oqxAB*) using PCR followed by sequencing (Literak et al., 2012). Isolates positive for PMQR genes were further characterized. Susceptibility to 8 antibiotics (chloramphenicol, gentamicin, nitrofurantoin, florfenicol, oxytetracycline, sulfonamides cp., sulfamethoxazole/trimethoprim, trimethoprim) was determined by disc diffusion method (CLSI, 2006). The selection of the tested antibiotics is based on their approved common use (e.g. tetracycline) or off-label use (e.g. chloramphenicol) in food or ornamental fish farming. The isolates were tested for tetracycline resistance genes *tet*(A), *tet*(B), *tet*(C), *tet*(D), *tet*(E), chloramphenicol resistance genes *catA1*, *cmlA* and *floR*, sulfonamide resistance genes *sul1*, *sul2*, *sul3*, beta-lactamase gene bla_{OXA-1}, the class 1 and class 2 integrase genes *intI1* and *intI2*, respectively, the variable region of class 1 integrons and gene cassettes inside the integron structure (Wilkerson et al., 2004; Dolejska et al., 2007). Clonality of the isolates was examined by *Xba*I digestion and pulsed-field gel electrophoresis (PFGE). Restriction profiles were analyzed using BioNumerics fingerprinting software (Applied Maths, Belgium). Cluster analysis of the dice similarity indices was done to generate a dendrogram describing the relationships among PFGE profiles. Isolates were considered to be related and to belong to the same PFGE cluster if their Dice similarity indices were ≥85%. Letters were used to discriminate PFGE patterns assigned to the same cluster. Biofilm production was tested using crystal violet biofilm assay (Genevaux et al., 1996) and all tested isolates were assigned to one of the four categories based on their adherence capabilities (non-adherent, weakly, moderately and strongly adherent strains) according to Stepanović et al. (2000). MICs to nalidixic acid (NA), oxolinic acid (OA), flumequine (UB) and enrofloxacin (ENR) were determined by agar dilution method (CLSI, 2006, 2008).