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Characterization and expression of the *gyrA* gene from quinolone resistant *Yersinia ruckeri* strains isolated from Atlantic salmon (*Salmo salar* L.) in Norway

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ABSTRACT

Yersinia ruckeri, the causative agent of yersiniosis has been reported in a number of fish species but the most vulnerable are the salmonids including Atlantic salmon (*Salmo salar* L.). In the present study *Y. ruckeri* isolates were collected from diseased Atlantic salmon juveniles and characterized for resistance against quinolones. Isolates were screened using disk diffusion assays and MIC determination. The QRDR regions of the *gyrA*, *gyrB*, *parC* and *parE* genes were sequenced. Quinolone resistant isolates revealed a single bp mutation which replaced serine by arginine at position 83 in the GyrA protein while no mutations were found in *gyrB*, *parC* and *parE* genes. Isolates were also screened for plasmid encoded *qnrA*, *qnrB* and *qnrS* genes but they were found absent. Cloning of *gyrA* from susceptible and resistant isolates into heterologous *Y. ruckeri* was not successful. The different *gyrA* alleles from susceptible and resistant isolates of *Y. ruckeri* were cloned into *Escherichia coli* TOPO 10 and *E. coli* DH5α. While cloning of the resistant allele into the sensitive host had no effect, cloning of the quinolone susceptible *gyrA* allele into quinolone resistant *E. coli* DH5α increased the inhibition zone diameter from 25 mm to 38 mm and decreased the MIC from 4 μg/ml to 2 μg/ml, suggesting dominance of wild type *gyrA* over the mutant allele. It is assumed that the wild type GyrA protein has more affinity to form the gyrase–DNA complex than mutant GyrA even in the presence of high levels of the mutated enzyme.

1. Introduction

Yersinia ruckeri, the causative agent of yersiniosis or enteric redmouth disease (ERM), has been reported as a cause of infection in a number of fish species. The most susceptible fish are the salmonids, especially rainbow trout (*Oncorhynchus mykiss*, W.) and Atlantic salmon (*Salmo salar* L.) (Bridle et al., 2011; Tobback et al., 2010). Initially isolated in Idaho, USA in the 1950s (Rucker, 1996), the bacterium has now been identified in fish populations throughout North America, South America, Asia, Australia, South Africa and Europe (Furones et al., 1993; Roozbahani et al., 2009). In Norway most outbreaks are associated with Atlantic salmon juveniles farmed in freshwater.

Although *Y. ruckeri* infections are now mostly controlled through vaccination, periodically outbreaks of ERM continue to be reported in Europe and USA (Bastardo et al., 2011). Antimicrobial agents, mainly quinolones, e.g. oxolinic acid or flumequine, are still frequently used in treatment of outbreaks. Persistent *in vitro* exposure to oxolinic acid has previously been shown to increase resistance in *Y. ruckeri* (Raida and Buchmann, 2008). Quinolone resistance is normally introduced by mutations in the quinolone resistance determining region (QRDR) of the DNA gyrase and topoisomerase IV genes; although plasmid encoded resistance by Qnr determinants has also been identified (Ruiz, 2003; Karah et al., 2010; Wang et al., 2004). In Gram negative bacteria, this region normally corresponds to nucleotides 199–318 of the *Escherichia coli gyrA* coding sequence and thus amino acids 67–106 of the *E. coli* GyrA protein (Ruiz, 2003). Single bp mutations in the QRDR, resulting in serine-83 and/or aspartic acid-87 substitutions (Gibello et al., 2004; Izumi et al., 2007) are the most common basis for resistance against quinolones. However, mutations in other positions of *gyrA* have also been reported in some bacteria (Ruiz, 2003). Plasmid mediated quinolone resistance encoded by *qnr* genes has been reported in various bacteria e.g. *Citrobacter* spp., *Enterobacter* spp., *E. coli*, *Klebsiella pneumoniae*, *Proteus* spp., *Salmonella* spp., and *Shigella* spp. etc. (Karah et al., 2010; Van Hoek et al., 2011). The *qnrS* gene has for example been reported from Norway in *Klebsiella pneumoniae* and *E. coli* (Karah et al., 2010),

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but plasmid mediated quinolone resistance has yet to be described in *Y. ruckeri*. With routine vaccination the incidence of yersiniosis has been greatly reduced in Scandinavia and the prevalence of the disease is currently low in Norway (Berger, 2011), although it remains a serious potential threat to the salmonid aquaculture industry.

Reduced quinolone susceptibility was recently revealed in *Y. ruckeri* isolated from a population of freshwater farmed juvenile Atlantic salmon in western Norway. This population had been subjected to repeated treatments with oxolinic acid against a persistent *Y. ruckeri* infection. The aim of the present study was, therefore, to elucidate the quinolone resistance mechanism of the isolated *Y. ruckeri*; to investigate if the resistance is related to mutations in the QRDR regions of DNA gyrase- and topoisomerase IV genes or to any of the less common plasmid encoded *qnr* genes. Further, the functional effect of the likely resistance mechanism was demonstrated by cloning both susceptible and resistance alleles into both quinolone resistant and sensitive host bacteria.

2. Materials and methods

2.1. Materials

We have used a set of strains representing a clinical problem in the same farm. Seven *Y. ruckeri* isolates were cultured from Atlantic salmon smolts kept in a freshwater land-based recycling unit in western Norway. The site had introduced water recirculation in November 2007. Recurrent episodes of increased mortality in different fish groups (parr and smolt) took place between January 2008 and January 2010 and *Y. ruckeri* was isolated on several occasions during diagnostic investigations. Diseased smolts demonstrated reduced appetite and external signs including fin rot, exopthalmus and external bleeding. Internal findings included bleeding in the peritoneal fat, enlarged spleen and bloody ascites. Bacterial isolates were phenotypically identified according to standard methodology including use of the API 20E (BioMérieux) test which was performed according to the manufacturer's instructions, with the exception of incubation temperature and period which were 22 °C for 48 h. Serotyping was performed using the slide agglutination method using polyclonal rabbit antisera raised against *Yersinia ruckeri* O1, O2 and O5.

2.2. Phenotypic antibiotic resistance and MIC determination

Y. ruckeri isolates, recipient *E. coli* TOPO 10, *E. coli* DH5α and all transformants were tested for quinolone susceptibility. Antibiotic susceptibillity was evaluated by the disk (Sigma Chemical) diffusion method (CLSI, 2006) on Müller Hinton agar. Disks containing trimethoprim + sulfamethoxazole 5.2 + 240 μg, flumequine 30 μg, oxolinic acid 10 μg, tetracycline 80 μg, and florfenicol 30 μg (Neo-Sensitabs, Rosco®) were used. The inoculated plates were incubated at 22 °C for 1–2 days. *Aeromonas salmonicida* subsp. *salmonicida* ATCC 14174 was included as quality control.

2.3. MIC determination

MIC determination was performed on Mueller Hinton (MH) plates (Alderman and Smith, 2001) containing dilution series of either oxolinic acid or flumequine respectively. Stock solutions containing 500 mg oxolinic acid and 500 mg flumequine respectively, in 500 ml 0.03 M NaOH were prepared. The solutions were stored chilled (4 °C) in the dark for a maximum of 24 h until use. Mueller Hinton medium containing 0.25 μg/ml to 32 μg/ml of oxolinic acid and 0.003 μg/ml to 16 μg/ml of flumequine were prepared, stored chilled in the dark for a maximum of 2 days before tests were performed. Two isolates of *Y. ruckeri* were streak inoculated onto each plate. MH plates without antibiotics were included as controls for each isolate. All plates were incubated for 24 h at 22 °C. The test was performed in duplicate for each isolate. The MIC of *E. coli* DH5α and transformants was determined by the broth dilution method using our previously published protocol (Shah et al., 2012) however, they were incubated at 22 °C for 12–24 h in Mueller Hinton broth.

2.4. DNA extraction and PCR amplification

Bacterial genomic DNA was extracted using NucliSENS easyMAG (BioMérieux). The quinolone resistance-determining regions of *gyrA*, *gyrB*, *parC*, and *parE* genes were amplified by PCR using primers designed with the PRIMER3 software utilizing the genome sequence of *Y. ruckeri* ATCC 29473 (Table 1). PCR reactions (50 μl) were performed with 1.25 U Taq DNA polymerase and ThermoPol Buffer (New England Biolabs GmbH, Frankfurt, Germany). The thermal program for partial *gyrA*, *gyrB*, *parC* and *parE* gene amplifications consisted of one step at 95 °C for 5 min, then 30 cycles of 95 °C for 30 s, 58 °C for 30 s, and 72 °C for 1 min respectively, sealed by a final step at 72 °C for 4 min.

Full length *gyrA* CDS, including 113 bp upstream and 116 bp downstream of the start and stop codons (totally 2899 bp), were amplified from one susceptible isolate and one resistant isolate using primers YRgyrAf and YRgyrAr (Table 1). PCR was carried out with Invitrogen Platinum Taq DNA Polymerase High Fidelity. The thermal program for amplification of the whole *gyrA* gene including its basal promoter, consisted of one step at 95 °C for 5 min, followed by 35 cycles of 95 °C for 30 s, 52 °C for 30 s, 72 °C for 3 min 30 s respectively, sealed by a final step at 72 °C for 4 min. The plasmid mediated *qnr* screening was carried out as described by Karah et al. (2010) and Wang et al. (2004). Positive and negative controls were included for all amplifications (Shah et al., 2012).

2.5. Cloning, sequencing and sequence analysis

E. coli DH5 α and *Y. ruckeri* YR7212 and YR7143 were made chemically competent using the rubidium chloride method (Hanahan, 1983). YR7212 and YR7143 were also made electro-competent

Table 1
Amplification and sequencing primers for partial *gyrB*, *parC*, *parE*, *qnrA*, *B*, *S* gene sequences as well as both the partial and the full length *gyrA* gene sequence.

Primer name	Gene	Sequence 5′–3′	Size (bp)	References
gyrBf	*gyrB*	TTGAAACGCTGATGAACGAG	647	This study
gyrBr		GGCACCATCTAATGCCAGAG		
gyrAf	*gyrA*	ATGAGCGACCTTGCCAGAG	558	This study
gyrAr		ATTATGTGGCGGAATGTTGG		
parCf	*parC*	GGTGCAGAGCGCCTACC	608	This study
parCr		AATTCTTCCAGCGAGGTTTG		
parEf	*parE*	AAACCAAAGAGCGCCTGTC	652	This study
parEr		TCCAGTACGCCAGCTTTCTC		
YRgyrAf	*gyrA*	GGAAATCGAGGATAAACTTG	2899	This study
YRgyrAr		TGAGCTACCAACAGAATACAC		
qnrAF	*qnrA*	TCAGCAAGAGGATTTCTCA	627	Wang et al. (2004)
qnrAR		GGCAGCACTATTACTCCCA		
qnrBF	*qnrB*	GATCGTGAAAGCCAGAAAGG	469	Karah et al. (2010)
qnrBR		ACGATGCCTGGTAGTTGTCC		
qnrSF	*qnrS1*	ACGACATTCGTCAACTGCAA	417	Karah et al. (2010)
qnrSR		TAAATTGGCACCCTGTAGGC		
YRgyrF1	Full length *gyrA*	GTTATGCCAACCCGCATC		
YRgyrF2	amplicon	CTCTGACCCAACTACAGGTCAC		
YRgyrF3		AACCCAGAGCGTTTGATGG		
YRgyrF4		AAGGGCGTCACGTCTTTATG		
YRgyrF5		ACTCGCGTATCAGAAGTGAGC		This study
YRgyrR1		TCACGCCCTGAGTATTACGAC		
YRgyrR2		ACCATCCGTCAGATCCACAC		
YRgyrR3		CGACGTGCATCATTGTACTG		
YRgyrR4		TGACGTACGAAAGCAACCAG		
YRgyrR5		CTGACGAGCCGTTAACCAG		