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Yersiniosis in Atlantic cod, *Gadus morhua* (L.), characterization of the infective strain and host reactions

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Abstract

A disease outbreak in farmed Atlantic cod caused by *Yersinia ruckeri* is reported. Mortality started following vaccination of cod reared in two tanks (A and B). The accumulated mortality reached 1.9% in A and 4.8% in B in the following 30 days when treatment with oxytetracycline was applied. Biochemical and molecular analysis of *Y. ruckeri* isolates from the cod and other fish species from fresh and marine waters in Iceland revealed a high salinity-tolerant subgroup of *Y. ruckeri* serotype O1. Infected fish showed clinical signs comparable with those of *Y. ruckeri* -infected salmonids, with the exception of granuloma formations in infected cod tissues, which is a known response of cod to bacterial infections. Immunohistological examination showed *Y. ruckeri* antigens in the core of granulomas and the involvement of immune parameters that indicates a strong association between complement and lysozyme killing of bacteria. Experimental infection of cod with a cod isolate induced disease, and the calculated LD_{50} was 1.7×10^4 CFU per fish. The results suggest that yersiniosis can be spread between populations of freshwater and marine fish. Treatment of infected cod with antibiotic did not eliminate the infection, which can be explained by the immune response of cod producing prolonged granulomatous infection.

Keywords: Atlantic cod, disease, *Gadus morhua* (L.), granuloma, *Yersinia ruckeri*, yersiniosis.

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Introduction

Atlantic cod, *Gadus morhua* L., is currently being developed for commercial culture, with activities concentrated around the North Atlantic (Paisly *et al.* 2010). Bacterial infections are significant factors contributing to reduced production and profits in cod farming, especially in larval and juvenile cod. There are various species of bacteria that infect cod, and the list is expected to increase concurrently with increased cod production. Classical vibriosis is causing significant problems at temperatures above 12 °C, while atypical furunculosis, winter ulcer disease and coldwater vibriosis are causing problems at lower sea temperatures (Magnadottir *et al.* 2002; Colquhoun *et al.* 2004; Samuelsen *et al.* 2006). Infection by *Francisella noatunensis* has induced high mortality in cultivated cod in Norway. The sea temperature was 14.5 °C when the first outbreak was detected, and the mortality reached 40% in 5 months (Samuelsen *et al.* 2006).

Studies have shown that antibody response of cod to protein antigens is poor, unspecific and non protective (Magnadottir *et al.* 2001; Gudmundsdottir & Bjornsdottir 2007). Granuloma formation in cod following bacterial infection is well known, and recently, it has been shown that cod does not contain the major histocompatibility complex class II (MHC class II), which is considered essential for classical humoral antibody response (Star *et al.* 2011). Cod may therefore depend on cellular and innate humoral parameters to combat infections.

Yersiniosis in fish is a significant bacterial septicaemia caused by the Gram-negative bacterium *Yersinia ruckeri*. This pathogen has a wide geographical distribution and has mainly been a

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problem in intensive rearing of salmonids, but infections in other species have also been reported (Toranzo, Magarinos & Romalde 2005). Several serotypes with different subgroups are recognized among *Y. ruckeri* strains (Stevenson & Airdrie 1984; Romalde & Toranzo 1993; Bastardo, Ravelo & Romalde 2012). Infections have largely been associated with serotype O1, 'Hagerman' forms (Austin & Austin 2007), but two biotypes, bt 1 and bt 2, are reported with phenotypic and antigenic differences. The O antigen is regarded as the dominant immunogenic molecule of *Y. ruckeri* (Davies & Frerichs 1989; Tinsley, Lyndon & Austin 2011).

The objectives of this study are to describe the pathology of yersiniosis in naturally infected farmed Atlantic cod and furthermore, to characterize the isolated bacterium and compare with a *Y. ruckeri* type strain and isolates from different fish and locations in Iceland.

Materials and methods

Naturally infected cod

Mortality was detected simultaneously in two separate tanks (A and B) following experimental injection vaccination (AJ-1200, Pharmaq Ltd) of farmed Atlantic cod (31 g ± 7 SD) at the Mariculture Laboratory of the Icelandic Marine Research Institute. Infection trials were not performed at the Mariculture Laboratory, but wild fish were often taken into the farm. Tank A contained 1200 fish and tank B 3000 fish. The tanks were 3-m circular fibreglass tanks with water depth of 0.8 m, supplied with continuously flowing aerated borehole sea water (9 ± 1 °C) salinity 32 ppt. Natural photoperiod (latitude 64°N) was provided, and the fish were fed standard commercial feed (Laxá hf.). Oxygen concentration, temperature and mortality were monitored daily in each tank. All dead and moribund fish were sampled for bacteriological examination, gross pathological changes were reported and organs were collected for histopathological examination during a period of 30 days, when treatment with oxytetracycline (Aquatet®) 60 mg kg^{-1} fish/day was started. The treatment was given for 4 days in a row, followed by 2 days without treatment and then 3 days with treatment. Mortality was then reported for 5 additional days.

Identification of bacteria

For bacteriological examination, smears from the head kidney were cultured on blood agar (BA, blood agar base (Oxoid) supplemented with 5% sheep blood) or BA with 2% NaCl (BA-S). Single colonies from a virtually pure culture were subcultured twice on tryptic soy agar (TSA, Oxoid) to obtain pure isolates. A whole cell agglutination test was performed to identify *Y. ruckeri*, using the MONO-Yr kit (BIONOR AS), which consisted of antibody-coated latex beads (antisera against the classical serotypes 01, 02 and 03) and was designed for specific identification of *Y. ruckeri*. Identified isolates were stored in TSB containing 15% (v/v) glycerol at −80 °C. *Y. ruckeri* isolates from farmed and wild fish of various species in Iceland and a type strain for the species were included in bacterial examinations for comparison. Isolates examined in the study and their origins are listed in Table 1.

Bacteriological tests (Austin & Austin 2007), listed in Table S1, were performed using overnight cultures in TSB for incubation of test material and were incubated at 15 °C. Examination was performed following 48 h incubation and after a further 5 days of incubation. The cultures were tested in API 20E (BioMérieux, Groco ehf.). Motility was tested by microscopical observation

Table 1 *Yersinia ruckeri* isolates used in this study, their hosts and pulsed field gel electrophoresis (PFGE) type

Arctic charr, *Salvelinus alpinus* (L.)	
F73-00	
F17-01	
F61-05	
F175-05	
F204-05	
Atlantic cod, *Gadus morhua* L.	PAGE type 1
F171-05	19 (NotI) and
F205-05	20 (AscI) bands
F310-05	
F311-05	
Atlantic salmon, *Salmo salar* L.	
F45-03	
European eel, *Anguilla anguilla* (L.)	
F181-01	
Oncorhynchus mykiss (Walbaum)	PAGE type 2
NCTC 10476 2^T	17 (NotI) and
	18 (AscI) bands
	19 (NotI) and
	20 (AscI) bands

NCTC, National Collection of Type Cultures, Colindale, London. All isolates, except the type strain, were obtained from the kidney of diseased fish at the Fish Disease Laboratory, Institute for Experimental Pathology, University of Iceland. The type strain was originally isolated in USA.

of wet mounts prepared from 24-h TSB cultures and by incubation in semisolid TSA (0.4%). The salt tolerance of growth was tested in 1% bacto peptone (Difco, The Icelandic American Trading Company) in distilled water supplemented with 0, 0.5, 1.0, 3.0, 5.0 and 7.0% NaCl (Sigma-Aldrich, Groco). Antibiotic susceptibility was assayed by antimicrobial susceptibility test discs (Oxoid) on TSA. Detection of siderophore production was performed on siderophore-indicative medium modified from the study by Schwyn & Neilands (1987) (CAS, pH 6.8), as described by Lauzon *et al.* (2008). Three *Y. ruckeri* strains (NCTC 10476^T, F175-05 and F171-05) were serotyped by Schering Plough Animal Health Aquaculture.

For the genetic identification of isolates, partial 16S rRNA gene sequencing was performed using Archaea sequential primer, 805.R (Barns *et al.* 1994) with an ABI 377 DNA sequencer. The BigDye terminator cycle sequencing ready reaction kit was used according to the instructions of the manufacturer (PE Applied Biosystems) and sequences were grouped with 98% limits in the Sequencer program (work performed by Matis-Prokaria Ltd). The obtained sequences were then compared with the BLAST alignment program (Altschul *et al.* 1990) for the identification of the isolates.

Characterization by pulsed field gel electrophoresis (PFGE)

The 12 isolates and the reference strain (Table 1) were characterized by PFGE. The DNA preparation and isolation were performed according to Niskanen, Fredriksson-Ahomaa & Korkeala (2002). Ten U *Not*I and *Apa*I (New England Biolabs) were used for restriction endonuclease digestion according to the manufacturer's instructions. The digested samples were electrophoresed through 1.0% (w/v) agarose (SeaKem GTG, FMC Bioproducts) in 0.5 x Tris-borate EDTA (45 mM Tris, 4.5 mM boric acid and 1 mM sodium EDTA) at 200 V, at 12 °C in a CHEF DRIII system (BioRad Laboratories). The pulse times ramped from 1 s to 18 s for 20 h for both restriction enzymes. The gels were stained with ethidium bromide (ICN Biomedicals Inc.) and photographed under UV transillumination using a GelDoc 2000 documentation system (BioRad Laboratories). Mid Range PFG marker I (New England Biolabs) was used for fragment size determination.

Experimental infection

Atlantic cod juveniles (26 g ± 8 SD) free of infections, according to standard routine diagnostic procedures performed at the Icelandic Fish-Disease Reference Laboratory, were used for experimental infection. The fish were kept in 150-L tanks in aerated sea water at 7–8 °C. Prior to treatment, the fish were anaesthetized with 0.3 mL L^{-1} phenoxyethanol (Lifsgledi ehf.) and marked along fin margins with visible implant fluorescent elastomer dye (Northwest Marine Technology).

Challenges were performed by intraperitoneal (i.p.) injections of *Y. ruckeri*, isolate F171-05 (Table 1), using cell suspensions in phosphate-buffered saline(PBS), pH 7.2 with concentrations ranging from 5×10^3 to 5×10^8 CFU mL^{-1}. Control fish received PBS only. Ten fish were in each injection group. The head kidney from all dead and surviving fish were sampled, inoculated onto blood agar and incubated for 7 days at 15 °C. *Y. ruckeri* isolated from dead fish was identified serologically using the MONO-Yr kit. The LD_{50} was calculated according to the method described by Reed & Muench (1938).

Fish experiments were approved and performed according to the Icelandic Animal Research Authority (approval no. 1112–3201).

Pathological examination

A complete necropsy was performed on infected and healthy fish, and gross pathological changes were recorded. The organs collected for microscopical examination were skin and underlying muscle, kidney, liver, spleen, gut, heart and gills. Organs were fixed in 10% buffered formalin, subsequently embedded in paraffin and the blocks were then stored at room temperature until cut into 4 μm sections and stained with Giemsa, haematoxylin and eosin (HE), or immunostained.

Immunostaining was performed with the ABComplex/AP solution kit (Avidin–biotin complex alkaline phosphates, DAKO, Groco) as previously described (Lange *et al.* 2004) with the modification that haematoxylin was used instead of methylene green for counterstaining. The primary antibodies (diluted 1:200) used were rabbit anti-*Y. ruckeri* serotype O1 (Jansson *et al.* 1991); rabbit anti-cod g-lysozyme (Inami *et al.* 2010) and the previously described antibodies prepared at our laboratory (Magnadottir, Gudmundsdottir & Groman

2013); rabbit anti-cod C3; mouse anti-IgM; mouse anti-CRP-PI, mouse anti-CRP-PII and mouse anti-ApoLP A1. A preimmune rabbit serum, normal mouse ascites and the conjugated antibody served as the relevant controls and also cod that were free of infection.

Results

Characterization of bacterium isolated from naturally infected cod and comparison with *Y. ruckeri* isolated from other fish species in Iceland and type strains

Cultures of round, white and shiny colonies were obtained from the head kidney of dead and moribund fish from tanks A and B. The bacterium was identified as *Y. ruckeri*, using the MONO-Yr kit. Serotyping of the isolated strain (F171-05), NCTC 10476^T (Ewing *et al.* 1978), and *Y. ruckeri* isolated in the same week from Arctic charr (F175-05) on a neighbour farm, showed that they all belonged to serotype O1 'Hagerman' forms. Sequencing of 611 bp of the 16S rRNA gene of isolates F171-05, F175-05, F45-03 and F181-01, revealed that sequences were identical and had 100% sequence identity to *Y. ruckeri* strain NBRC102019 and 99% sequence identity with *Y. ruckeri* type strain ATCC 29473^T, which also belongs to serotype O1 'Hagerman' forms (Romalde & Toranzo 1993).

Results from standard bacteriological analysis performed on strain NCTC 10476^T and the isolates listed in Table 1 are shown in Table S1. The 12 Icelandic isolates were identical in all tests performed and only differed from the type strain in producing gelatinase and in the ability to grow in 50 ppt and 70 ppt salinity.

Characterization by PFGE revealed that all 12 Icelandic isolates shared the same pattern with 19 (NotI) and 20 (AscI) bands assigned to PAGE group 1, while the reference strain NCTC 10476^T revealed a different pattern (PAGE group 2) with fewer bands of 17 and 18, respectively (Table 1).

Mortality and pathology of cod naturally infected with *Y. ruckeri*

Naturally infected Atlantic cod showed clinical signs of a systemic disease. *Y. ruckeri* was isolated from the head kidney of 95% of the fish that died during a 2-week period. The accumulated mortality in the two tanks (Fig. 1) had reached 1.9% in A and 4.8% in B when treatment with oxytetracycline were started on day 31. No mortality was observed in tank A after day 37, but fish in tank B, which had higher fish density, continued to die owing to *Y. ruckeri* infection after treatment. The accumulated mortality was 2.5% and 6.3%, respectively, in the two tanks on day 44, when the fish were transported to another experimental wet laboratory located in Sandgerdi, Iceland. Following transport, mortalities due to yersiniosis continued in fish originating from both tanks (data not shown).

Gross pathological signs of infected cod are shown in Fig. 2a–c. The changes involved diffuse haemorrhage on the head, including extensive haemorrhage and oedema on the jaw and palate. Haemorrhages were detected on the body surface primarily on the ventral part of the body, at gill tips, fin bases and tail. Loss of scales was frequently detected on infected fish. Disseminated haemorrhages in liver, serous ascites and passive congestion of the spleen were also signs of the disease.

Microscopical examination of cod tissues (Fig. 2d–i) revealed bacterial colonies and inflammatory changes in heart, brain, eyes, spleen, liver and intestine. The dominant histological features of yersiniosis were focal and multiple granulomas in all investigated organs. Granulomas were detected in different development stages from an initial stage to the chronic fibrous granulomas. Bacterial colonies were evident within many granuloma formations.

These granulomas were characterized by bacterial colonies and cellular debris, surrounded by epithelioid

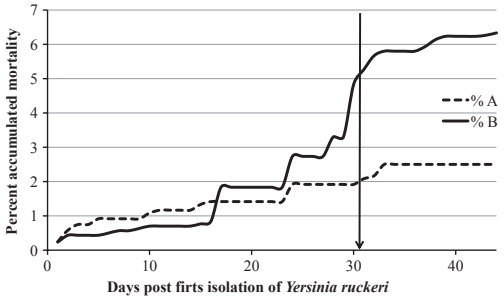

Figure 1 Percent accumulated mortality of Atlantic cod (31 g ± 7 SD) in tanks A (1200 fish) and B (3000 fish) reported for 44 days after the first isolation of *Yersinia ruckeri* from fish that died the same day in the two tanks, respectively. Treatment with oxytetracycline (Aquatet®) 60 mg kg^{-1} fish/day was started on day 31 (vertical arrow). The treatment was given for 4 days in a row followed by 2 days without treatment and then 3 days with treatment. Mortality was recorded for 5-additional days post-treatment.

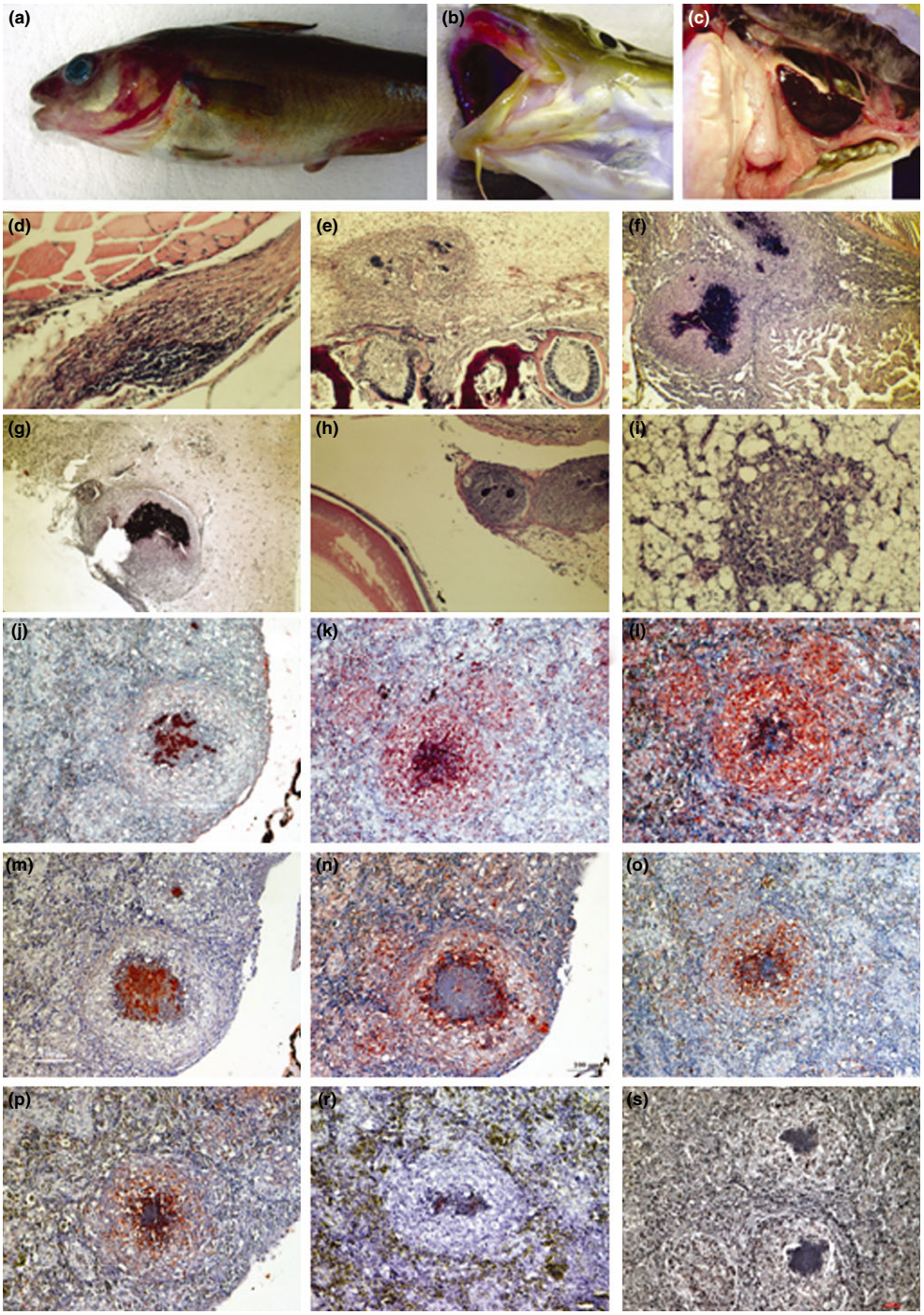

Figure 2 Pathological changes of Atlantic cod naturally infected with *Y. ruckeri*. Gross pathological changes showing congestion on the head, body, at gill tips and fin bases (a–b); haemorrhage in the visceral organs and congestion of the spleen (c). Histopathological changes: in dermis (magnification × 10) (d); dermal maxilla (magnification x 10) (e); in the heart ventricle (magnification × 10) (f); in the brain (magnification × 6.3) (g); in the eye (magnification × 10) (h); and in the liver (magnification × 40) (i). Granuloma in the spleen (magnification × 20) immunostained with the following: rabbit anti-*Y. ruckeri* antibody (j); mouse anti-cod IgM antibody (k); mouse anti-cod ApoLP A1 antibody (l); rabbit anti-cod C3 antibody (m); rabbit anti-cod g-lysozyme antibody (n); mouse anti-cod pentraxin CRP-PI antibody (o); mouse anti-cod pentraxin CRP-PII antibody (p); control rabbit serum (r); and control mouse ascitic fluid (s).

cells, macrophages and lymphocytes and an outer zone of connective tissue. Immunostaining of granulomas in sections from spleen is shown in Fig. 2j–s. The anti-*Y. ruckeri* antibody (Fig. 2j) stained bacterial colonies that were mixed with necrotic debris at the core of the granuloma. Anti-IgM-positive staining was the most prominent in the core of the granuloma, but the outer zone of the granuloma also showed clear positive staining. Scattered positive anti-IgM reactions were also visible in the surrounding parenchyma (Fig. 2k). The anti-ApoLP A1 antibody induced a strong reaction in all layers of the granuloma but to a lesser extent in the core compared with anti-IgM staining (Fig. 2l). The anti-C3 antibody reaction was very strong and confined to the core of the granuloma where bacteria were detected (Fig. 2m). Staining with anti-g-lysozyme revealed a strong reaction within the zone of macrophages and epithelioid cells surrounding the necrotic core of the granuloma. Scattered staining was observed in the core and at the periphery of the granuloma and also in the surrounding parenchyma (Fig. 2n). The anti-pentraxin CRP-PI and anti-pentraxin CRP-PII antibodies reacted likewise with the zone of macrophages and epithelioid cells surrounding the necrotic core of the granuloma, and occasional staining was detected in the surrounding parenchyma (Fig. 2o,p). Neither the control rabbit serum nor the control mouse ascitic fluid showed any positive reaction within the cod spleen (Fig. 2r,s). Granuloma was not detected in the control cod that were free of infection.

Experimental infection of Atlantic cod with *Y. ruckeri*

Injection challenge of cod was performed with isolate F171-05 originating from naturally infected cod. Mortality rates in all groups of challenged fish were dose dependent and the calculated LD_{50}, 14 days post-infection, was 1.7×10^4 CFU per fish. Gross pathological signs were detected on dead and moribund fish, and the infecting bacterium was isolated in pure cultures.

Discussion

An outbreak of yersiniosis was identified in a population of Atlantic cod reared in seawater tanks at the Mariculture Laboratory of the Icelandic Marine Research Institute. The isolated *Y. ruckeri* bacterium was found to fulfil Koch's postulate and to be biochemically and genetically identical to *Y. ruckeri* isolates originating from diseased farmed Arctic charr and Atlantic salmon and wild European eel in Iceland during a 5-year period.

The Mariculture Laboratory is located within 1 km distance from a farm cultivating Arctic charr with a continuous and recent history of yersiniosis outbreaks. The two farms have a common water reservoir. The yersiniosis outbreak in cod occurred simultaneously in two separate tanks following an experimental injection vaccination, which is a stressful operation. Furthermore, mortalities of cod due to *Y. ruckeri* infection were confirmed several weeks later on the same farm in cod that was experimentally grown at elevated temperatures (data not shown). This might indicate that *Y. ruckeri* is dormant in the residing microbiota and can induce an infection when stressful conditions depress the immune defence or other physical host factors.

Biochemical and genetic comparison of 12 *Y. ruckeri* isolates from naturally infected fish in Iceland (five from cod, five from charr, one from salmon and one from eel) in this study revealed that they are a homogeneous group, indicating their clonal origin.

Serotyping of *Y. ruckeri* isolated from infected cod and charr showed that the isolates belong to serotype O1 (Hagerman), as does the type strain, NCTC 10476^T. Bacteriological examination revealed that the strains are motile and hydrolyse Tween, which groups them as biotype 1 (Davies & Frerichs 1989; Tinsley *et al.* 2011). Some genetic variations were identified between the Icelandic *Y. ruckeri* isolates and strain NCTC 10476^T, as the PFGE patterns differed and the sequence of 611 bp of the 16S rRNA gene was 99% similar. None of the tested isolates fermented sorbitol, which is a reaction reported to distinguish serotype O1 from O2 (O'Leary 1977). All isolates were positive for the Voges Proskauer and gelatinase reactions that are characteristic of a group of non-motile *Y. ruckeri* serotype O1 that have caused disease problems in rainbow trout (Austin, Robertson & Austin 2003). The biochemical differences between the Icelandic isolates and the type strain were that they produce active gelatinase and grow at salinity as high as 70 ppt, whereas the type strain was able to grow in 30 ppt, and not in 50 ppt salinity. *Y. ruckeri* has mainly been regarded a disease of fish in fresh or brackish water, and survival of the bacterium in 35 ppt salinity has been reported to be significantly reduced (Barnes 2011).

The finding that the Icelandic isolates were growing well in salinity as high as 50 ppt indicates that these isolates form a new clonal group within serotype O1 with high salinity tolerance, which needs further examination.

Disease signs in cod were comparable with those of infected salmonids (Tobback *et al.* 2007), with the exception that granuloma formations were seen in infected cod tissues, which is a characteristic response of cod to infections with other Gram-negative bacteria, such as *Aeromonas salmonicida* subsp. *achromogenes* (Magnadottir *et al.* 2002), *Moritella viscosa* (Gudmundsdottir *et al.* 2006) and *Francisella* (Olsen *et al.* 2006). Although both *A. salmonicida* subsp. *achromogenes* and *M. viscosa* infect other fish species, granuloma formations have only been described in cod, which reflects the difference observed in the immune system of cod compared with the other teleost fish species studied (Star *et al.* 2011).

Immunohistopathological characterization of granuloma in spleen showed many features in common with those that we have recently described for cod infected with *A. salmonicida* subsp. *achromogenes*, using the same set of antibodies and methods for vimmunostaining, with the addition of anti-cod g-lysozyme in this study and the replacement of the anti-*A. salmonicida* antibody by the anti-*Y. ruckeri* antibody (Magnadottir *et al.* 2013). As previously described for *A. salmonicida*, the *Y. ruckeri* colonies were mainly detected in the mixture with necrotic debris and encased in the granuloma by a strong inflammatory reaction. The main difference was that a stronger association was found with complement killing of bacteria in *Y. ruckeri* -infected cod compared with *A. salmonicida*-infected cod.

Lysozymes are cationic enzymes with antibacterial activity that are mainly produced by macrophages of higher vertebrates and salmonid fish (Gordon, Todd & Cohn 1974; Paulsen, Engstad & Robertsen 2001). Vertebrates have two different kinds of lysozymes called c-type and g-type. A previous study has shown g-type lysozyme in haematopoietic tissues of cod, including the red pulp of spleen (Inami *et al.* 2010). In the present study, g-lysozyme was strongly associated with a zone of macrophages surrounding the necrotic core of granulomas and was also detected within the core. This indicates that cod g-lysozyme might also be produced by macrophages, which is a new finding as cellular expression of g-lysozyme has not previously been identified in cod tissue. The detection of g-lysozyme within the core in association with bacteria and C3 indicates that it is involved in bacterial killing in the core of cod granuloma, which also is a new finding.

The three known complement pathways: the classical complement activation pathway (CCP), the alternative complement pathway (ACP) and the lectin complement pathway (LCP) have been identified in teleosts. Complement-mediated bacterial killing is either induced directly by microorganism activation (CCP, ACP and LCP) or by antibody–antigen complexes (CCP) (Holland & Lambris 2002). Complement factor 3 (C3) plays a central role in the activation of all three pathways. In this study, the antibody staining of C3 was very strong and confined to the core of the granuloma without detectable reactions outside the core.

Staining of IgM was the strongest in association with bacterial colonies in the core of the granuloma, indicating a role of CCP in bacterial eradiation. ApoLP A1 has been associated with the binding of bacterial LPS and with a regulation of complement activity. In cod, a strong binding of ApoLP A1 to C3 has been discovered and a regulatory role in the complement system of cod is suggested (Magnadottir & Lange 2004). Cod pentraxins (CRP-PI, CRP-PII) are pattern recognition acute-phase proteins that can activate CCP (Bayne & Gerwick 2001; Gisladottir *et al.* 2009). Staining with antibodies against the two cod pentraxins and ApoLP A1 in this study showed a moderate reaction within the core of the granulomas plus a strong reaction in all layers of the granuloma. The association of C3, IgM, g-lysozyme, ApoLP A1 and the pentraxins with bacterial colonies shown in this study suggests that activation of CCP may play a principal role in cod defence against *Y. ruckeri* infections.

Experimental infection of cod with a cod isolate of *Y. ruckeri* produced a disease with signs comparable with those of naturally infected cod and the bacterium was re-isolated in pure cultures from diseased fish, showing that the pathogen follows the Koch's four criteria to identify the causative microbe of a disease (Fredericks & Relman 1996). *Y. ruckeri* was found to be highly virulent in cod, as the LD_{50} calculated 2 weeks post-infection was 1.7×10^4 CFU per fish.

Mortality was not observed in cod reared in tank A during a week following treatment with oxytetracycline, but in tank B, which had significantly higher fish density, treated fish continued to die, suggesting the importance of

environmental factors in the development of the infection. The fish were then transported to tanks in another experimental laboratory and then mortalities continued in fish originating from both tanks (data not shown). This indicates the survival of *Y. ruckeri* in the antibiotic-treated cod.

According to the yearly reports of the Icelandic Veterinary Officer for Fish Diseases 2005–12 (in Icelandic), outbreaks of yersiniosis have occasionally been detected in cultivated cod, since first reported, but mortality has not been a significant problem, but the disease has been reported in two other fish species cultivated in sea water. Thus, the bacterium has been isolated from diseased Atlantic halibut, *Hippoglossus hippoglossus* (L.), at the Mariculture Laboratory, but mortality was not significant. On the other hand, repeated outbreaks have occurred in cultivated Atlantic turbot, *Scophthalmus maximus* (L.). The first outbreak was detected at the Mariculture Laboratory, and the disease was distributed with transported turbot to a farm in North Iceland. Despite vaccination of turbot with an injection vaccine produced for salmonids (AquaVac ERM vet., MSD Animal Health), mortalities continued but were reduced.

The results of this study have shown that *Y. ruckeri* infections are a risk factor to farming of marine fish as well as fish cultivated in low salinity and fresh water. Furthermore, a new subgroup with high salinity tolerance was identified within *Y. ruckeri* serotype O1. As in other bacterial diseases described in cod, typical granuloma formations enclosing the pathogen were observed. The involvement of the classical complement pathway and enzymatic degradation of the bacteria was demonstrated within the granuloma. Treatment of infected cod with antibiotic did not eliminate the infection, which can be explained by the immune response of cod producing prolonged granulomatous infection.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Physiological and biochemical characterization of Icelandic *Yersinia ruckeri* isolates studied and comparison with a reference strain.

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