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Characterisation of a serotype O1 *Yersinia ruckeri* isolate from the Isle of Man: further evidence that O antigen serotype is not a reliable indicator of virulence

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Abstract

As part of a routine disease surveillance exercise, a culture of the Gram negative bacterial pathogen *Yersinia ruckeri* was obtained from one of 150 largely asymptomatic rainbow trout from a farm on the Isle of Man, an island off the North West coast of Great Britain. This is the first reported isolation of *Y. ruckeri* from the Isle of Man. The isolate was phenotypically and serologically indistinguishable from serotype O1 *Y. ruckeri* isolates, which have been the cause of the disease enteric redmouth (ERM) in Europe, the UK and the US for more than 30 years. However, the isolate was relatively avirulent, when tested by bath immersion challenge, in rainbow trout and Atlantic salmon, compared to a positive control ERM disease-causing rainbow trout isolate. Detailed molecular subtyping of the isolate using Pulsed Field Gel Electrophoresis (PFGE) also showed the isolate had a different pulsotype to the isolates known to typically circulate in Europe and the mainland UK. Overall, the results support the suggestion that the O1 serogroup contains a heterogeneous assembly of types with respect to pathogenicity and host.

Introduction

The Isle of Man is an island off the North West coast of Great Britain. The island has a number of rainbow trout farms (including broodstock facilities and hatcheries). It enjoys a high fish health status as a result of health controls and a testing programme over many years and is recognised as free from Viral haemorrhagic septicaemia (VHS), Infectious haematopoietic necrosis (IHN), Infectious pancreatic necrosis (IPN), Bacterial Kidney Disease (*Renibacterium salmoninarum*) and *Gyrodactylus salaris*. There are also no previous reports of the isolation of the Enterobacterium *Yersinia ruckeri*, causative agent of Enteric Redmouth Disease (ERM), (Horne and Barnes, 1999) in the Isle of Man. This is in contrast to mainland UK, Ireland and most of mainland Europe where ERM is endemic (Davies, 1991a; Wheeler et al., 2009).

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In December 2008, the Cefas Fish Health Inspectorate, while undertaking routine disease surveillance on behalf of the Isle of Man government, recovered a pure culture of *Y. ruckeri* from an apparently healthy 200g rainbow trout from an Isle of Man fish farm. Herein we report the characterisation of the first isolation of *Y. ruckeri* at an Isle of Man aquaculture facility.

Materials and methods

A sample of 150 fish from an Isle of Man rainbow trout farm were collected by Cefas FHI personnel in November 2008. For bacteriological investigations, head kidney samples were inoculated onto Tryptone Soya Agar and SKDM for the isolation of *Renibacterium salmoninarum*. Cultures were transported to the laboratory and analysed for the presence of notifiable disease agents (VHS, IHN and *Gyrodactylus salaris*), as recommended by the Office International des Epizootics (OIE 2006). Presumptive *Y. ruckeri* isolates were initially characterised on the basis of colony morphology, primary test results (Gram stain, cell morphology, motility, cytochrome oxidase, and catalase activity) and API 20 E testing (Biomerieux), as described by Buller (2004). For confirmatory species identification, isolates were tested using latex agglutination testing (BioNor Mono Yr). A partial 529 bp sequence (8-563) of the 16S rRNA gene was also obtained as described by Pond et al. (2006). They were then serotyped for the heat stable O antigen as described by Davies (1990). Molecular subtyping using Pulsed Field Gel Electrophoresis (PFGE), as described by Wheeler et al. (2009), was undertaken. The sensitivity of the isolate to florfenicol (30 μg), oxytetracycline (20 μg), amoxicillin (10 μg), oxolinic acid (4 μg) and cotrimoxazole (1.25/23.5 μg) was also determined using a disc diffusion method (disc content indicated in brackets), in compliance with guidelines from the Clinical and Laboratory Standards Institute (CLSI 2004). The isolate was also tested for both pooled Atlantic salmon and rainbow trout naïve serum killing sensitivity, as described by Haig et al. (2011).

To determine whether the *Y. ruckeri* isolate from the Isle of Man posed a risk equivalent to that of typical ERM-causing *Y. ruckeri* strains, rainbow trout fry, 150-250g rainbow trout and Atlantic salmon fry were challenged with the isolate, in conjunction with a range of other isolates that were tested in another study (Haig et al., 2011). For the first stage of testing, one group of ten rainbow trout (150-250g) were anaesthetised, then each injected into the peritoneal cavity with a 0.1 mL dose containing $2.3 \times 10^7 \pm 1.4 \times 10^7$ c.f.u., For the second stage of testing, groups of rainbow trout fry (0.5-1.0g), Atlantic salmon fry (0.5-1.0g) and 150-250 g rainbow trout, were all challenged by bath exposure to for 4 hours in duplicate. Rainbow trout fry and salmon fry were held in separate 30L tanks and the larger rainbow trout in 300 L tanks. For fish held in 30L tanks, volume was reduced to 5L and the fish challenged *in situ* for 4h, before returning the volume to 30L. For the larger rainbow trout, two groups of 12 fish were each transferred to 50L buckets for exposure, before being returned to their 300L holding tanks at the end of the 4h exposure period. For the Atlantic salmon and rainbow trout fry experiments, two tanks of 25 fish for each species were tested. For the larger rainbow trout, two tanks of 12 fish were exposed to each isolate. For the bath exposures, bacterial suspensions were prepared, based on their optical densities, to give predicted approximate dose of 1.0×10^7 c.f.u ml^{-1}. The duplicate tanks for each treatment were tested on separate oc-

casions with independently prepared challenge inocula. All experiments were performed in fresh water flow-through systems, with a test temperature of 16°C ± 1°C. Further details of challenge dose preparation and the challenge procedures used, for both intrapertoneal injection and bath exposures, were as described in Haig et al. (2011).

Results and discussion

The inspector taking samples reported no adverse conditions observed on the farm, with water temperature (6°C) and other factors typical for the time of year (November). Signs of proliferative kidney disease were observed in approximately 20% of the smaller fish (less than 100g) examined, but the larger fish sampled (most of which were more than 1kg) were all seemingly healthy on both external and internal examination. With the exception of *Y. ruckeri* (see below), no notifiable disease agents were recovered from any of the 150 fish sampled.

After incubation at 22°C for 48h, a dense culture of Gram negative motile bacterial rods was observed on a set of TSA plates obtained from the kidney of one of the larger (approximately 2kg) rainbow trout sampled. Three of these isolates were initially identified as *Y. ruckeri*, based on colony morphology, lack of cytochrome oxidase activity and typical API 20E results (5307100). All demonstrated a positive latex agglutination test result for *Y. ruckeri* and 100% nucleotide sequence identity to the partial 16S rRNA gene sequence (Accession no. FJ518718) for the type strain of *Y. ruckeri* (Souza et al., 2010), thus confirming the initial identification. One of these isolates, designated Cefas culture collection number 09003, was characterised in greater depth. The isolate was apparently at least partially or fully sensitive to all five antimicrobials tested (zone sizes indicated in brackets), florfenicol (31mm), oxytetracycline (20.3 mm), amoxicillin (22mm), oxolinic acid (39mm) and cotrimoxazole (37mm). In common with ERM-causing UK and European isolates, 09003 was shown to be serotype O1 (Davies, 1991a; Wheeler et al., 2009). However, it was also determined that the organism was a motile, Tween 80 (phospholipase) degrading biotype 1 isolate (Davies and Freirichs, 1989). This is unusual, as biotype 2 (non-motile, not able to hydrolyse Tween 80), *Y. ruckeri* isolates are typically recovered from UK rainbow, trout (Wheeler et al., 2009). This has been the case, ever since the disease first emerged in the 1980's (Davies, 1991a; Wheeler et al., 2009). PFGE showed the isolate had a *Not*1 pulsotype that was distinct from other previously characterised *Y. ruckeri* isolates (Figure 1). These included typical biotype 1 and biotype 2 serotype O1 ERM-causing isolates that affect rainbow trout in UK and mainland Europe (Wheeler et al., 2009). In particular, it was noted that this isolate, in common with the Type strain ATCC 29473 (Figure 1, Lanes 1, 3 and 14), did not have the approx 350kb band that all the other isolates examined possessed. Isolate 09003 could, in turn, be differentiated from ATCC 29473, particularly with regards the relative mobility of fragments between 138 and 310 kb (Figure 1 lanes 1, 3 and 14, and further comparison of data from Wheeler et al., 2009).

Intraperitoneal (i.p.) injection of rainbow trout with a dose of $2.8 \times 10^7 \pm 1 \times 10^6$ c.f.u. fish^{-1} isolate 09003 resulted in seven out of the ten injected fish being dead by day 4 post injection. Affected fish showed ascites, severe haemorrhaging around the fin bases and internal organs,

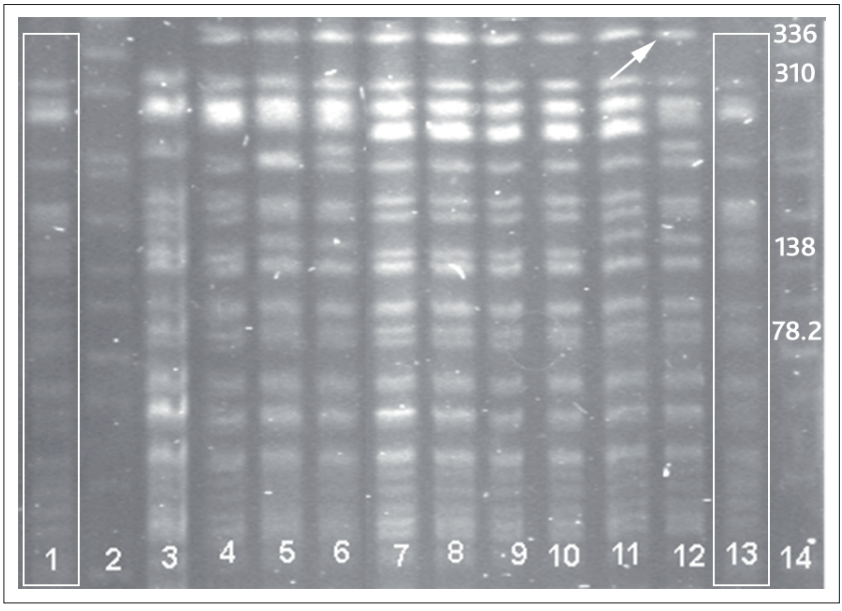

Figure 1. Pulsed-field gel electrophoretogram (1% agarose) of a selection of *Not*1 digests of biotype 1 and biotype 2 serotype O1 *Y. ruckeri* isolates recovered from rainbow trout. Lanes 1 and 13 Isle of Man isolate 09003 (boxed), lane 3 'Hagerman' type strain (pulsotype (pt) 39) ATCC 29473; lane 4, US biotype 1 pt33 isolate RD40 ; lane 5 UK biotype 2 pt 31 isolate 06042, lane 6 US biotype 2 pt32 strain YRNC10; lane 7 , Danish biotype 1 pt35 motile strain RD88; lane 8, Spanish biotype 1 pt35 strain 06077 ; lane 9, Danish biotype 2 pt35 isolate 07073 970611/-2/2 , lane 10, Danish biotype 2 pt 35 isolate 07090030522-2/1; lane 11, Spanish biotype 2 pt36 isolate 06076; lane 12, UK biotype 2 pt32 isolate 18887. Lanes 2 and 14 *Salmonella braenderup* molecular standard, prepared by the same method and restricted in situ with *Xba*I (Hunter et al. 2005). Arrow in lane 12 indicates position of approx 350kb band not present in 09003 profile. Positions of 336.5, 310, 138.9 and 78.2kb bands indicated in lane 14. Pulsotype (pt) as assigned in Wheeler et al. 2009.

darkening and bilateral exophthalmia. However no rainbow trout and only 3 out of 50 (6%) Atlantic salmon fry were killed by isolate 09003 by bath immersion challenge. One of the 24 larger rainbow trout fish challenged with isolate 09003 developed bilateral exophthalmia resulting in blindness in one eye, ascites, darkening, haemorrhaging from internal organs and disoriented swimming. An apparently pure culture of the challenge isolate was recovered from the head kidney of this fish (based on colony morphology and a positive latex agglutination test result). All the remaining fish were apparently healthy when examined 24 days after challenge when the trial was terminated.

Retrospective plate counts of the bacterial suspensions used to prepare the challenge doses confirmed that the two tanks of Atlantic salmon fry were exposed to 1.2 $x10^7$ c.f.u. ml^{-1} and 2.1 $x10^8$ c.f.u ml^{-1} respectively, while the challenge concentrations in rainbow trout fry tanks were 6.4 x 10^7 and 4.3 x 10^7 c.f.u ml^{-1}. The larger rainbow trout were exposed to 5.5 x 10^8 and 8.25 x 10^6 c.f.u ml^{-1}. The individual that developed severe symptoms was from the tank of fish exposed to the higher of the two doses.

In a separate study carried out in parallel (Haig et al., 2011), these same stocks of rainbow trout and salmon were shown to be highly susceptible

to bath immersion exposure to the ERM-causing UK serotype O1 isolate 06041/RD6. The limited mortality induced in fry of Atlantic salmon, but not in rainbow trout, also supports the suggestion from that study (Haig et al., 2011) that this species is more sensitive than rainbow trout to serotype O1 and other *Y. ruckeri* O antigen serotype isolates that are not part of the closely related 'Hagerman'-like group of serotype O1 isolates (Romalde et al., 1993; Wheeler et al., 2009).

The isolate was killed by naive sera from both salmonid species (a greater than 95%, or -1.5 average $\log_{10}$ reduction in bacterial concentration (c.f.u. ml^{-1}) after 3h incubation in both sera). This may explain the low virulence observed in this serotype O1 strain. ERM-disease causing isolates are typically normal (non immune) rainbow trout serum resistant. In contrast, serotype O1 isolates that are not virulent in rainbow trout are usually killed by normal rainbow trout serum, likely via alternative complement pathway mediated killing (Davies, 1991b).

It is unclear whether the *Y. ruckeri* isolate recovered was recently introduced into the Isle of Man from another country, or instead represents organisms that are long established in farmed trout there. In particular, the data is not consistent with the suggestion that the organism was recently introduced via undetected live rainbow trout movements between the mainland UK and Isle of Man , as the organism recovered was different to the strains known to circulate in farmed UK rainbow trout (Wheeler et al. 2009).

These data overall suggest that the risks of isolate 09003 to farmed rainbow trout were not as high as those posed by other serotype O1 strains. Davies (1991b) and Haig et al. (2011) have also shown that serotype was not necessarily a good indicator of *Y. ruckeri* pathogenicity in rainbow trout and Atlantic salmon.

In Australia, the O1 serotype of .*Y. ruckeri* is differentiated as O1a and O1b (Romalde et al. 1993) to distinguish the O1b serotype, which is enzootic (Carson and Wilson, 2009), from the O1a 'Hagerman' serotype, which is exotic and on Australia's National List of Reportable Diseases of Aquatic Animals (Anon 2010). Of note, neither Yersiniosis nor ERM disease occurs in rainbow trout in Australia, although in hatchery raised Atlantic salmon significant and recurrent outbreaks of Yersiniosis occur, caused by serotype O1b. (Dr J. Carson. Personal Communication). The results of this study would generally support this approach to *Y. ruckeri* detection and control in farmed rainbow trout and indicates that the O1 serogroup contains a heterogeneous assembly of types with respect to pathogenicity and host.

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