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Short communication

Disease caused by *Yersinia ruckeri* serotype O2b found in Chilean-farmed coho salmon, *Oncorhynchus kisutch* (Walbaum, 1792)

R Avendaño-Herrera[1,2,3], D Tapia-Cammas[1,2], A Aedo[4], P Saldivia[4], C Ortega[5] and R Irgang[1,2]

1 Laboratorio de Patología de Organismos Acuáticos y Biotecnología Acuícola, Departamento de Ciencias Biológicas, Facultad de Ciencias Biológicas, Universidad Andrés Bello, Viña del Mar, Chile
2 Interdisciplinary Center for Aquaculture Research (INCAR), Concepción, Chile
3 Centro de Investigación Marina Quintay (CIMARQ), Quintay, Chile
4 Laboratorio de Salud, Aquagestion S.A., Villarrica, Chile
5 Centro de Investigación y Estudios Avanzados en Salud Animal (CIESA), Facultad de Medicina Veterinaria y Zootecnia, Universidad Autónoma del Estado de México, Toluca, México

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Yersinia ruckeri is a Gram-negative, rod-shaped, oxidase-negative and catalase-positive bacterium responsible for causing enteric redmouth disease (ERM), or yersiniosis, in economically important salmonids, including rainbow trout (*Oncorhynchus mykiss* [Walbaum, 1792]) and Atlantic salmon (*Salmo salar* [Linnaeus, 1758]) (Tobback *et al.* 2007). Notably, this bacterium is widely distributed across much of the world (Tobback *et al.* 2007; Shaowu *et al.* 2013).

Yersinia ruckeri strains are categorized by biotypes, serotypes and outer membrane protein types. Biotype 1 is comprised of generally motile and lipase activity-positive isolates, whereas biotype 2 isolates are non-motile and lipase activity negative (Davies & Frerichs 1989). *Yersinia ruckeri* can be subdivided into six serotypes (Stevenson & Airdrie 1984), five O-serotypes (Davies 1990) or four O-serotypes with subgroups (Romalde *et al.* 1993) depending on the serotyping system. Strains of serotype O1a (classic serovar I) and O2b (classic serovar II) cause most outbreaks, and the motile serotype O1a is predominant in epizootic salmonid outbreaks (Stevenson & Airdrie 1984; Romalde *et al.* 1993).

Vaccination is used to control ERM, but vaccine breakdowns have occurred in European -farmed rainbow trout (Austin, Robertson & Austin 2003; Fouz, Zarza & Amaro 2006; Calvez *et al.* 2014) and USA-farmed brown trout (*Salmo trutta* [Linnaeus, 1758]) (Arias, Olivares-Fuster & Hayden 2007). These outbreaks are mostly attributed to non-motile, lipase activity-negative *Y. ruckeri* strains (biotype 2), but in Spain, the O2b serotype was found in rainbow trout (Romalde *et al.* 2003).

Y. ruckeri was first reported in Chile at an Atlantic salmon farm (Toledo *et al.* 1993) and was widely spread across the southern regions of the country until a commercial rainbow trout ERM vaccine was developed in 1995 (Bravo & Midtlyng 2007). This vaccine, a bivalent bacterin (O1/O2b), successfully prevented *Y. ruckeri* infection for over a decade, but in 2008, ERM outbreaks occurred in vaccinated Atlantic salmon

Correspondence *R Avendaño-Herrera, Universidad Andrés Bello, Facultad de Ciencias Biológicas, Quillota 980, Piso 4-Torre C, Viña del Mar, Chile. (e-mail: reavendano@yahoo.com or ravendano@unab.cl)*

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weighing between 2 and 60 g, resulting in stock mortalities of up to 10%. These outbreaks were mostly the result of the *Y. ruckeri* O1b/biotype 1 (Bastardo *et al.* 2011a), but an O2b Atlantic salmon isolate was also recovered. During September and October 2015, farmed coho salmon (*Oncorhynchus kisutch* [Walbaum, 1792]) suffered outbreak mortalities. For all outbreaks, cumulative losses of the affected population reached up to 15%. Three representative bacterial isolates were recovered with the objective of antigenically identifying and characterizing the causative microorganism of the ERM outbreaks in Chilean coho salmon. The obtained micro-organisms were characterized as *Y. ruckeri* O2b, making this the first report of a mortality-associated isolate in coho salmon farmed in Chile.

The three epizootic outbreaks occurred in cultured, unvaccinated coho salmon at two freshwater farms located in Bio-Bio and Araucania Regions of southern Chile. In accordance with standard rearing conditions, river water (10 ± 1 °C) was used for culturing fish (1–2 and 3–5 g, respectively) at the Bio-Bio and Araucania farms. These fish were held in fibreglass tanks at densities of 10 and 20 kg m^{-3} for Bio-Bio and Araucania farms, respectively. At both farms, infection was suspected when farm personnel externally detected abdominal distention, pale gills, a pale liver with splenomegaly, a liquid-filled stomach and haemorrhaging in visceral fat and the swim bladder (Fig. 1). Nine fish were sampled during each outbreak ($n = 27$), and complete pathological and bacteriological workups were performed on the same days as sampling, with no more than 5 h between sampling and laboratory analysis (Aquagestión Diagnostics Laboratory).

Tissue samples (kidney, liver and spleen), pooled from three fish for each outbreak, were aseptically collected for presumptive diagnosis of freshwater pathogens, including infectious pancreatic necrosis virus, *Flavobacterium psychrophilum*, and *Y. ruckeri* through PCR (Urdaci *et al.* 1998; Bastardo, Ravelo & Romalde 2012; Avendaño-Herrera *et al.* 2015). The samples tested positive only for *Y. ruckeri*, with Ct values of 22.2 ± 0.8

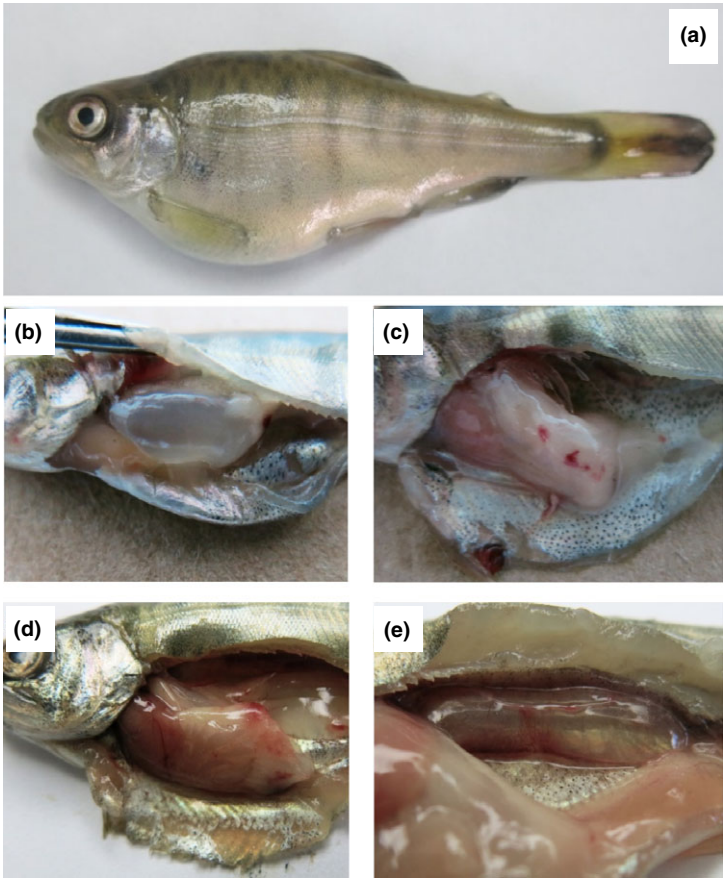

Figure 1 ERM presentation in different coho salmon specimens. Characteristic abdominal distention (a), liquid-filled stomach (b), visceral fat haemorrhaging (c and d) and swim bladder haemorrhaging (e).

and 18.33 ± 0.5 for the Bio-Bio and Araucania farms, respectively. Samples aseptically obtained from infected kidneys ($n = 6$, each outbreak) were streaked onto tryptone soya agar plates (Oxoid) at 25 °C for 24–96 h. Pure bacterial isolates were recovered from all moribund coho salmon, regardless of the farm, but only one kidney-obtained representative isolate was studied from each outbreak. PCR-based analysis (Gibello *et al.* 1999) confirmed all bacterial strains to be *Y. ruckeri*, showing a single 575-bp band identical to *Y. ruckeri* CECT 956 (data not shown). Stock cultures were maintained frozen at −80 °C in Criobille tubes (AES Laboratory).

The three isolates (termed C-1039, C-1040 and C-1045) were characterized using morphological, physiological and biochemical tests, as described by MacFaddin (1980) and Austin & Austin (2007). Biochemical reaction tests included Gram staining; cell morphology and motility; cytochrome oxidase; catalase reaction (3% H_2O_2); oxidation/fermentation reactions; Voges-Proskauer and citrate tests; gelatinase and lipase activities (Tween-80); and the fermentation of sorbitol. Additionally, all isolates were examined with the API 20E Miniaturized System (bioMérieux) according to the manufacturer's instructions, except for incubation temperature (25 °C). The *Y. ruckeri* CECT 955 (i.e. isolate 11.4), CECT 956 (i.e. isolate 11.29) and CECT 4319^T (i.e. ATCC 29473^T) strains, classified as two O-serotypes (O1a or O2b) (Romalde *et al.* 1993), were included for comparative purposes, the sera of which were prepared by our laboratory.

All of the coho salmon isolates were Gram-negative, motile and fermentative rods that positively reacted to the Tween-80, Voges-Proskauer, gelatin and citrate tests and negatively reacted to sorbitol, which was the key difference of these isolates from the O2b serotype strain CECT 956 (Table 1). The inability to ferment this polyalcohol is normally a trait of non-O2 serotype isolates (Romalde *et al.* 2003; Austin & Austin 2007). Interestingly, *Y. rukeri* strains that are motile and present lipase activity are classified as biotype 1, and this biotype includes the most virulent isolates. In contrast, those strains lacking both capacities are classified as biotype 2 (Davies & Frerichs 1989).

All coho salmon isolates rendered the API profile 5307500. Although *Y. ruckeri* is not included in the API Database, which is focused mainly on human pathogens, numerous biochemical profiles (5107100, 5104100, 5105100, 5106100, 5307100 and 5106500) have been previously reported for this pathogen (Fouz *et al.* 2006; Topic, Coz-Rakovac & Strunjak-Perovic 2007; Ström-Bestor *et al.* 2010; Bastardo *et al.* 2011b; Tinsley *et al.* 2011). The coho salmon API pattern was different from those previously reported, including those described by Bastardo *et al.* (2011a) and Tinsley *et al.* (2011) for Chilean Atlantic salmon isolates (5017100, 5307100 and 5107500). Therefore, the coho salmon isolates might have a species-specific pattern, although further studies are needed.

The minimum inhibitory concentrations (MICs) for the *Y. ruckeri* isolates were determined in cation-adjusted Mueller–Hinton broth following the broth microdilution method recommended by CLSI (2014) for Group 1 organisms (non-fastidious bacteria). Oxytetracycline and florfenicol, frequently used in treating ERM, were used in these determinations. All coho salmon isolates had identical antibiotic susceptibilities (1 and 4 mg L^{-1} for oxytetracycline and florfenicol, respectively). The quality control strain, *Aeromonas salmonicida* subsp. *salmonicida* ATCC 33658, was grown in cation-adjusted Mueller–Hinton broth under standard growth conditions and was within

Table 1 Biochemical characterization of the Chilean Coho salmon isolates used in this study, as compared to reference strains

	Source	Motility	Tween 80	Voges-Proskauer	Gelatin	Citrate	Sorbitol	Serotype
Reference strain								
CECT 955	*O. mykiss* (USA)	+	+	–	–	–	–	O1a
CECT 956	*O. tschawytscha* (USA)	+	+	–	+	+	+	O2b
CECT 4319^T	*O. mykiss* (USA)	+	+	–	+	–	–	O1a
Chilean isolates								
C-1039	*O. kisutch* (Araucania)	+	+	+	+	+	–	O2b
C-1040	*O. kisutch* (Bio-Bio)	+	+	+	+	+	–	O2b
C-1045	*O. kisutch* (Araucania)	+	+	+	+	+	–	O2b

+, positive reaction; –, negative reaction.

acceptable limits given in VET03/VET04-S2 (CLSI 2014). Oral oxytetracycline treatments (100 mg kg^{-1} fish for 15 days) were only used at the Araucania farm, with treatment beginning 7 days after the start of the outbreak. This treatment reduced daily mortality from 0.08 to 0.02%.

Antigenic analyses used the thermostable antigens of each strain obtained after heat-killing the bacterial suspensions (10^9 cells mL^{-1}) in phosphate-buffered saline (PBS, pH 7.4) at 100 °C for 60 min as described Avendaño-Herrera *et al.* (2004). Following this, the suspensions were washed once in the same saline solution and maintained at 4 °C until required. For immunological analysis, antisera raised against the reference strains CECT 955 (serotype O1a), CECT 956 (serotype O2b) and CECT 4319^T (serotype O1a) were prepared by intravenous injections of rabbits with formalin-killed cells (10^9 cells mL^{-1}) suspended in PBS according to the methods described by Sørensen & Larsen (1986). Additionally, serological analyses were performed using unabsorbed or absorbed rabbit serum with the heterologous strains (Stevenson & Airdrie 1984). The three Chilean isolates were serologically characterized using dot blot assays as described by Cipriano *et al.* (1985). To evaluate antigenic variability, the isolates were characterized by lipopolysaccharide (LPS) analysis following the procedures of Hitchcock & Brown (1983). The samples were examined by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE; Laemmli 1970) using 12% acrylamide in the resolving gel and 4% acrylamide in the stacking gel. LPS components were transferred onto a nitrocellulose membrane by electrophoretic blotting (Towbin, Staehelin & Gordon 1979) and treated for immunological analysis (Romalde *et al.* 1993).

Unabsorbed serum dot blot assays only showed a strong reaction with the antiserum raised against the CECT 956 reference strain (Table 1). These findings were supported by LPS immunoblotting, which clearly showed a unique O-serotype O2b for the coho salmon isolates. This serotype was distinguished in the coho salmon isolates without the use of absorbed antiserum, just as was found in the dot blot assays. The Chilean *Y. ruckeri* isolates showed a LPS profile similar to the reference serotype O2b strain (Fig. 2).

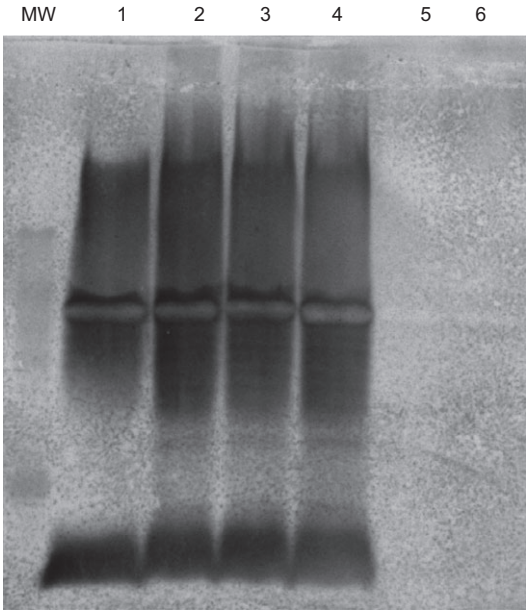

Figure 2 LPS immunoblot of the *Y. ruckeri* isolates using antiserum raised against the strain CECT 956. Lanes: 1, CECT 956; 2, C-1039; 3, C-1040; 4, C-1045; 5, CECT 955; and 6, CECT 4319^T.

Most *Y. ruckeri* isolated from Chilean-farmed salmonids are serotypes O1a and O1b, while only occasionally are serotypes O2b found (Troncoso *et al.* 1994; Bastardo *et al.* 2011a). Interestingly, the predominant serotype found in the three coho salmon isolates was serotype O2b, which is notable considering that, until now, only one previous study in Atlantic salmon reported on a unique Chilean Atlantic salmon O2b isolate (Bastardo *et al.* 2011a). Further investigation is warranted as serologically distinct *Y. ruckeri* strains may be associated with different species-specific outbreaks.

The process of typing is essential for recognizing outbreaks, investigating cross-transmission, studying geographical and host distributions, detecting particularly virulent strains and monitoring vaccination programmes (Olive & Bean 1999). Enterobacterial repetitive intergenic consensus (ERIC-PCR) and repetitive extragenic palindromic (REP-PCR) can molecularly characterize bacterial pathogens in fish (Romalde 2005; Toranzo, Magariños & Romalde 2005). Therefore, amplifications were performed using previously described primer pairs (Versalovic, Koeuth & Lupski 1991) and protocols reported for *Y. ruckeri* (Bastardo *et al.* 2012), with the

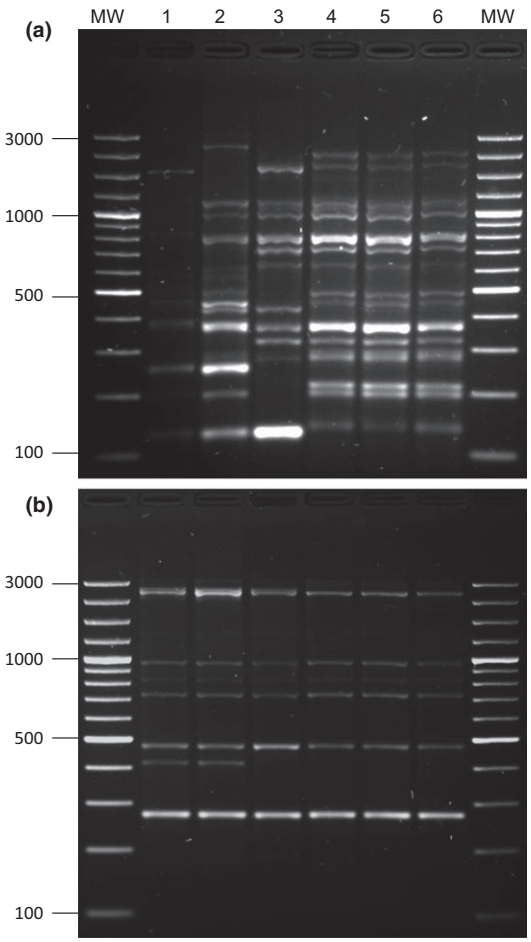

Figure 3 Amplification fingerprints obtained for the *Y. ruckeri* isolates and type strains using ERIC-PCR (a) and REP-PCR (b). Lanes: MW, GeneRuler 100 bp plus ladder (Thermo Scientific); 1, CECT 955; 2, CECT 4319^{T}; 3, CECT 956; 4, C-1039; 5, C-1040; and 6, C-1045. Numbers on the left indicate the position of molecular size markers in bp.

exception that PCRs were performed using the GoTaq Green Master Mix (Promega).

The three Chilean isolates returned identical fingerprinting profiles, regardless of the molecular tool used (i.e. ERIC-PCR or REP-PCR), geographical origin (Bio-Bio or Araucania State) and sampling data (Fig. 3). REP-PCR analysis consisted in nine amplification bands (200–3000 bp) for all Chilean isolates and CECT 956 (O2b), while CECT 955 and 4319^{T} represented other patterns (11 amplification bands). For ERIC-PCR fingerprinting, an identical amplification pattern was detected among the coho salmon isolates (17 amplification patterns, 100–2000 bp), but each reference strain showed a different profile. REP-PCR analysis established a relationship between serotype and a higher discriminatory power of ERIC-PCR, as previously described (Bastardo *et al.* 2011a).

New *Y. ruckeri* strains discovered within the last 10 years have been associated with highly aggressive outbreaks and epizootics in distinct geographical regions worldwide. Resulting from this, emerging *Y. ruckeri* strains in Spain and the USA have been classified as serotype O1 biotype 2 despite lacking motility (Fouz *et al.* 2006; Arias *et al.* 2007). In England, these strains have been classified as a new biogroup (Austin *et al.* 2003). Other outbreaks in Spain have been caused by serotype O2b, but with lower incidence and mortality rates (Romalde *et al.* 2003). Although the three representative *Y. ruckeri* isolates were associated with diseased coho salmon (15% population mortality), challenge experiments are needed to confirm that the Chilean isolates cause ERM and fulfil Koch's postulates and whether pathogenesis is species specific.

This is the first report of *Y. ruckeri* serotype O2b in Chilean-farmed coho salmon, representing a potential risk that could impact salmonid farming. The vaccination of farmed coho salmon against *Y. ruckeri* in Chile should be considered to reduce pathogenic impact, but further studies should verify coho salmon vaccination efficiency, particularly to serotype O2b.

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