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Short communication

Phenotypical and genetic characterization of *Yersinia ruckeri* strains isolated from recent outbreaks in farmed rainbow trout *Oncorhynchus mykiss* (Walbaum) in Peru

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ABSTRACT

A total of 30 strains of *Yersinia ruckeri* causing recent outbreaks in Peruvian trout culture systems, were studied by means of biochemical characteristics, serology, lipopolysaccharide (LPS) and outer membrane protein (OMP) analysis, and ERIC and REP PCR fingerprinting. All the Peruvian isolates were found to be fermentative, oxidase negative and positive for decarboxylation of lysine and ornithine and utilization of glucose and mannitol, allowing their presumptive identification as *Y. ruckeri*. Sequencing of the 16S rRNA gene confirmed that isolates were indeed *Y. ruckeri* (>99.98% identity). Although most of the strains studied were motile and lipase positive corresponding to the biotype 1 of *Y. ruckeri*, 5 of these strains were negative from both tests, being identified as biotype 2. In addition, drug susceptibility tests determined high sensitivity to sulfamethoxazole/trimethoprim, oxytetracycline, ampicillin and enrofloxacin in all the isolates. Serologically, all the Peruvian strains studied were identified as belonging to the serotype O1 subgroup a. Analysis of the lipopolysaccharide (LPS) as well as total and outer membrane proteins (OMPs) profiles and the correspondent inmunoblotting, supported these results. Genotyping performed by means of ERIC- and REP-PCR determined major correlation of the Peruvian isolates with the type strain NCIMB 2194^T regardless of the biotype.

1. Introduction

Yersinia ruckeri the etiological agent of enteric redmouth disease (ERM) is a serious disease causing significant economic losses in the salmonid farming industry. The pathogen *Y. ruckeri* was initially isolated from diseased trout in the Hagerman Valley, Idaho (USA) in the 1950s (Rucker, 1966). Over the last 25 years, the disease also spread rapidly in European countries, Australia, South Africa and South America (Austin and Austin; 2007; Tobback et al. 2007).

The species includes two biotypes where strains positive for motility and lipase activity are grouped into biotype 1, while biotype 2 strains are negative for both tests (Davies and Frerichs, 1989; Evenhuis et al., 2009). In addition, there is a great serological variability within *Y. ruckeri*, with several intraspecies classifications being proposed, comprising four (Romalde et al., 1993) or five (Davies, 1990) different O-serotypes. Biotype 1 strains of serotype O1a (Hagerman strain) and O2b (O'Leary strain) cause most epizootic outbreaks, being the serotype O1a predominant in cultured salmonids (Austin and Austin, 2007; O'Leary et al., 1979; Stevenson and Airdrie, 1984). However, it has been recently proposed that this statement is not the case, describing the existence of new clonal groups also with high virulence (Tinsley et al., 2011).

Although generally well controlled by means of vaccination and antibiotic treatment, outbreaks of this disease have been periodically observed, especially in endemic areas. Formulation of most ERM commercial vaccines is based only on serotype O1a (Hagerman strain) however different degrees of cross-protection among serotypes have been reported (Stevenson and Airdrie, 1984). However, the number of ERM reports in previously vaccinated salmonids in Europe and USA has increased, and some of these outbreaks were attributed to emergent non-motile *Y. ruckeri* isolates (Arias et al., 2007; Fouz et al., 2006).

Rainbow trout is an exotic species in Peru and was introduced from the USA in 1925. The development of rainbow trout culture in Peru during the last three decades, involving a large number of egg and fish importations, mainly from USA, was not accompanied by an evaluation of the sanitary conditions of Peruvian aquaculture facilities. To our knowledge, the only study on this topic was performed by Bravo and Kojagura (2004) who reported the *Y. ruckeri* detection by serological procedures during two surveys of the fish health status of hatcheries, carried out in 1998 and 2000 in Peru. Even though the disease has been diagnosed more than 25 years ago and commercial vaccines are

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available in major salmonid culture countries, vaccination is not used as preventive strategies in Peruvian farms.

In this study, an exhaustive phenotypical, molecular and geneical characterization of 30 *Y. ruckeri* strains isolated from outbreaks occurred during 2008 in different Peruvian farms was performed, allowing to evaluate the genetic variability among strains and to hypothesize whether they represent native strains or are the result of fish imports.

2. Materials and methods

2.1. Bacterial isolates

Peruvian strains were isolated from outbreaks occurred during 2008 in four different fish farms located in the central Andes of Peru at 3283 a 3900 m above sea level. Farms consist of concrete tanks and the freshwater is supplied from three different rivers, with water temperatures ranging from 8.5 to 13 °C and average value of pH = 7. Diseased rainbow trout (n = 154), with variable size (from 0.3 to 45.5 g) showed exophthalmia, dark pigmentation, hemorrhages in the mouth, eyes and around the vent as main external signals as well as petechiae in liver, visceral fat and pyloric ceca in internal organs.

For bacterial isolation, samples were aseptically collected from liver, spleen and kidney and directly streaked onto trypticase soy-agar (TSA; Difco Laboratories, Detroit, USA) and incubated at 25 °C for 24–48 h. Pure cultures were kept frozen at −80 °C in tryptic soy broth (TSB; Difco) supplemented with 15% glycerol. Type strain of the species and representative strains of the different O-serotypes following the scheme of Romalde et al. (1993) were also included in all the studies for comparative purposes (Table 1).

2.2. Biochemical and serological characterization

Bacterial isolates were subjected to morphological, physiological and biochemical tests using classical tube and plate procedures and the taxonomic position of the isolates were determined mainly following the schemes of Austin and Austin (2007). In parallel, all isolates were identified by means of the API 20E, and API ZYM (BioMerieux, France) using saline solution (NaCl 0.85%) for the bacterial suspensions. The study of acid production from carbon sources was also tested with the API 50CH (BioMerieux, France). In addition, susceptibility of the isolates strains to sulfamethoxazole/trimethoprim (25 μg per disc), tetracycline (30), oxytetracycline (30), ampicillin (10) and enrofloxacin (5) was determined by the disc diffusion method.

For the confirmatory serological identification, the isolates were examined for ability to agglutinate cross-absorbed antiserum raised against the reference strains of different serotypes (O1a, O1b, O2a, O2b, O2c, O3 and O4) of *Y. ruckeri*, using both slide agglutination assays and dot blot analysis (Bastardo et al., 2011).

2.3. Lipopolysaccharide (LPS) and outer membrane protein (OMP) analysis

LPS and OMPs of the Peruvian isolates were characterized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) combined with immunoblotting as previously described by Romalde et al. (1993). For immunological analysis, absorbed antisera against the reference strains of serotypes O1a, O1b and O2b were employed.

2.4. 16S rRNA gene sequencing

Total bacterial DNA was extracted from pure bacterial cultures using the Insta-Gene matrix (Bio-Rad, Madrid, Spain). The concentration of DNA was quantified spectrophotometrically and adjusted to a concentration of 100 ng/μl. 16S rRNA gene from DNA templates was amplified and sequenced following the procedures of Bastardo et al. (2011).

2.5. REP and ERIC-PCR genotyping

Repetitive extragenic palindromic (REP) and the enterobacterial repetitive intergenic consensus (ERIC) sequences from all *Y. ruckeri* isolates were amplified by PCR as previously described (Versalovic et al., 1991). The PCR products were electrophoresed in agarose gels (1.5% w/v), stained with ethidium bromide (2 μg/ml), and analyzed with the Diversity Database software (Bio-Rad) as described by Bastardo et al. (2011).

3. Results and discussion

The 30 Peruvian isolates evaluated were Gram-negative fermentative rods, oxidase negative and positive for lysine and ornithine decarboxylase, hydrolysis of gelatin and utilization of glucose and mannitol, which allow their presumptive identification as *Y. ruckeri*. The results from all standard biochemical tests are shown in Table 2. The main differential traits among the Peruvian isolates were motility, Voges–Proskauer reaction, lipase, fermentation of sorbitol and utilization of citrate. Such variation in these biochemical tests has

Table 1
Yersinia ruckeri strains used in this study.

Reference number strain		Source	Collection[a]
Type strain	NCIMB 2194[T]	*Oncorhynchus mykiss* (USA)	NCIMB
O-serotype strains[b]			
O1a	11.4	*O. mykiss* (Norway)	D.P. Anderson
O1b	1533	*Salmo salar* (Norway)	T. Håstein
O2a	RS6	*Salvelinus fontinalis* (USA)	R.M.W. Stevenson
O2b	11.29	*O. tshawytcha* (USA)	D.P. Anderson
O2c	RS2	*O. mykiss* (USA)	R.M.W. Stevenson
O3	11.47	*O. mykiss* (USA)	T. Cook
O4	11.73	*O. mykiss* (USA)	T. Cook
Fresh isolates from			
Farm A	17, 26	*O. mykiss* (Peru)	Laboratory collection
Farm B	23, 28	*O. mykiss* (Peru)	Laboratory collection
Farm C	1, 2, 3, 5, 6, 7, 8, 9, 10, 11, 12, 13, 16, 19, 20, 24, 29, 30, 32.	*O. mykiss* (Peru)	Laboratory collection
Farm D	15, 27, A18, A20, A24, A25, 41II	*O. mykiss* (Peru)	Laboratory collection

[a] NCIMB, National Collection of Marine Bacteria; D.P. Anderson, National Fish Health Research Laboratory, Kearneysville, West Virginia; T. Hånstein, National Veterinary Institute, Oslo; R.M.W. Stevenson, University of Guelph; T. Cook, Dpt. Microbiology, University of Maryland.
[b] Romalde et al. (1993).

Table 2
Differential biochemical characteristics observed for the Peruvian strains.

Test	Motility	Tween 80	Voges–Proskauer	Gelatin	Citrate	Sorbitol
Peruvian isolates	V (25)	V (11)	V (7)	+	V (16)	V (17)
Reference strain						
NCMB 2194[T]	+	+	−	+	−	−
O-serotype reference strains						
11.4(O1a)	+	+	−	−	−	−
1533(O1b)	+	+	−	−	+	−
RS2(O2a)	+	+	+	−	+	+
11.29(O2b)	+	+	−	+	+	+
RS6(O2c)	+	+	+	+	−	+
11.47(O3)	+	+	+	+	+	+
11.73(O4)	+	+	−	−	−	−

[T]: Type strain; +: positive reaction; −: negative reaction; V: variable reaction; (n): number of positive strains.