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Detection and identification of virulent *Yersinia ruckeri*: the causative agent of enteric redmouth disease in rainbow trout (*Oncorhynchus mykiss*) cultured in Fars province, Iran

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Summary

From the winter 2002 to spring 2006, 126 moribund rainbow trout with clinical signs of external body haemorrhages around and within the oral cavity were sampled from 10 rainbow trout farms situated in the northwest and west of Fars province, Iran and examined for the detection and identification of *Yersinia ruckeri*, the causative agent of enteric redmouth disease. Fish kidneys were cultured aseptically on brain heart infusion (BHI) agar plates and incubated at 25ºC for 48 h. Using conventional biochemical tests, *Y. ruckeri* was detected in 7 fish (5.5% of total fish sampled). This was also confirmed using specific polymerase chain reaction (PCR) assay. The 16S rDNA PCR assays produced amplicons of 409 bp when applied to *Y. ruckeri* isolates as well as a reference strain. Results of antibiogram tests on *Y. ruckeri* isolates showed a high susceptibility to enrofloxacin, norfloxacin, ofloxacin, trimethoprim and oxytetracycline. In pathogenicity tests, dilution of 4×10^8 colony forming unit/ml of *Y. ruckeri* by immersion route in challenge experiments showed 70 ± 8.2% mortality during 14 days post-infection. Experimentally infected fish showed typical haemorrhages in mouth, blackening of skin, exophthalmia and a wide haemorrhages on the internal organs.

Key words: *Yersinia ruckeri*, Detection, Rainbow trout, Fars, Iran

Introduction

Rainbow trout culturing has been developed during recent years in different suitable areas in Iran. Fars province, one of the 30 provinces in Iran situated nearly in south with numerous springs provided the expansion of aquaculture industry in the region. Recirculation systems for intensive fish culture are increasing in Fars province. Infectious diseases are being emerged due to imposed stress factors. Enteric redmouth disease (ERM) has been one of the most significant diseases in salmonid aquaculture. Even though the disease has been diagnosed more than 25 years ago and commercial vaccines are available in the European countries (Horne and Barnes, 1999), ERM is being reported from Iran since last few years. Soltani *et al*. (1999) reported a yersiniosis-like infection in farmed rainbow trout. Soltani and Tarahomi, (2002) also reported the isolation of highly virulent *Yersinia ruckeri*-like bacterium from diseased rainbow trout cultured in Tehran province with clinical signs of the disease. Since 2002, several clinical cases of rainbow trout suspected to ERM have been reported from northwest and north rainbow trout farms of Fars province.

This study reports the occurrence of *Y. ruckeri* infection from cultured rainbow trout in Fars province, Iran using conventional microbiological methods and polymerase chain reaction (PCR) assay.

Materials and Methods

Fish

One hundred and twenty six moribund rainbow trout (45-220 g weight) suspected to ERM were sampled from 10 rainbow trout farms situated in the northwest and west of Fars province from winter 2002 to spring 2006 and examined for the presence of the aethiological agent of the disease.

Moribund fish with external body haemorrhages including haemorrhage around eyes and mouth were euthanized with a blow on the head and used immediately for bacteriological examination.

Bacteriological examination

Fish kidneys were cultured aseptically by streaking a loop onto brain heart infusion (BHI) and MacConkey agar plates and incubated at 25°C for 48 h. Bacterial colonies were subcultured onto BHI and identified using conventional biochemical system (Austin and Austin, 1999). Characteristics of our isolated *Y. ruckeri* were compared with the reported biochemical properties of this bacterium in the literature (Austin and Austin, 1999; Horne and Barnes, 1999; Romalde *et al*., 2003). Disk diffusion antibiogram tests (Padtan Teb, Iran) were performed to determine the antibiotic susceptibility of *Y. ruckeri* isolates on mueller-hinton agar (Oraic *et al*., 2002).

Genomic DNA extraction

Yersinia ruckeri DNAs were extracted as described by Holmes and Quiqley (1981) with some modifications. Briefly, a medium-sized bacterial colony was taken from bacterial cultures and suspended in 200 µl of sterile distilled water, incubated in a boiling water bath for 15 min, centrifuged for 5 min at 10,000 g and the supernatant was used as template for the PCR amplification. The DNA extracted by these methods was visualized by gel electrophoresis on a 0.9% agarose gel before being stored at -20°C.

PCR assay

The used PCR assay was previously developed for definitive identification of *Y. ruckeri* by Lejeune and Rurangirwa (2000). Oligonucleotide primers were as ruck1, 5´-CAG CGG AAA GTA GCT TG-3´ and ruck2 5´-TGT TCA GTG CTA TTA ACA CTT AA-3´ (Lejeune and Rurangirwa, 2000). The specificity of these primers was checked on all sequences available from the GenBank database using the blast program. The primers were commercially synthesized by Cinnagen Company (Iran).

The following PCR conditions were applied to each assay; 50 mM KCl, 10 mM Tris-HCl (pH = 9.0), 1.5 mM $MgCl_2$, 200 µM dNTPs, 20 pmol of each primer, and 2 U *Taq* DNA polymerase (Fermentas) per 50 µl reaction using 4 µl of DNA extracted as template. For amplification, samples were cycled in a Bio-Rad thermocycler (Bio-Rad Laboratories Inc., Hercules, CA, USA) with an initial denaturation at 95°C for 5 min, followed by 30 cycles, denaturation at 94°C for 45 sec, annealing at 55.5°C for 1 min and extension at 72°C for 1 min. A final extension at 72°C for 7 min at the end of the amplification cycles was included. Sterile water, DNA of *Y. enterocolitica* O:9 and DNA of *Y. ruckeri* standard strain 90/3988 isolated at Fish Health Unit Department of Primary Industries, Water and Environment, Launceston, Tasmania, Australia (Wilson *et al*., 2002) were used as the negative and positive control, respectively. Each sample was tested at least in duplicate.

The presence of PCR products was determined by electrophoresis of 7 µl of each reaction product in 2% (w/v) agarose gel (Gibco BRL, Karlsruhe, Germany) with tris-borate-EDTA buffer and visualized by staining with ethidium bromide (final concentration of 0.5 µl/ml) under UV light.

Pathogenicity test

A challenge protocol was designed to evaluate the pathogenicity of the isolate of the year 2004 in this study using the challenge experiment procedure described by Rigos and Stevenson (2001) with some modifications. Briefly, six groups of rainbow trout (10 fish in each group with mean weight of 82 g) including a control group were placed into 300 L fiberglass tanks to acclimate for a week before the challenge procedure. Preliminary challenge trials were conducted using rainbow trout of the same stock and similar weight (culture negative from kidneys in random sampling) to verify the optimal bacterial concentration for the challenge in order to cause infection. For this, fish were immersed for 30 min with appropriate aeration in a dilution of 4-8 × 10^8 colony forming unit/ml of the *Y. ruckeri*. Samples that were taken from each of the bacterial dilution used in the challenge and processed for plate count. Fish were kept in aerated tanks with an average water

temperature of 15°C and watched for two weeks. Dead fish due to the ERM were examined and kidney samples were cultured on BHI.

Results

Bacteriological cultures of kidneys of moribund fish showed 5.5% *Y. ruckeri* (7 fish), 15% *Streptococcus* spp. (19 fish) and 10.3% *Aeromonas hydrophila* (13 fish) by conventional biochemical tests. Comparison of the biochemical characteristics of *Y. ruckeri* in this research with those reported in the literature is shown in Table 1. All *Y. ruckeri* isolates with white colonies were motile at 12-30°C and catalase positive.

Antibiogram tests showed that the isolates were highly susceptible to enrofloxacin, norfloxacin, Ofloxacin, trimethoprim and oxytetracycline (Table 2).

In the specific PCR assay, DNAs were successfully extracted from all isolates. As expected, the PCR assays produced amplicons of 409 bp fragment of 16S rDNA sequenses unique to *Y. ruckeri* when applied to *Y. ruckeri* field strains isolated from rainbow trout in Iran and reference strain (Fig. 1). The 409 bp band was not observed when distilled water and DNA obtained from non-*Y. ruckeri* bacteria were used as the template.

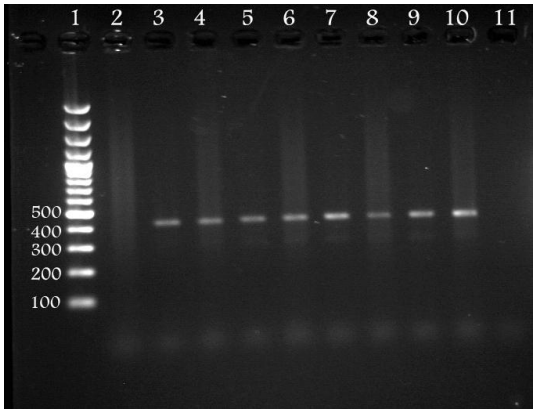

Fig. 1: Electerophoretic analysis (2% agarose gel) of DNA amplified fragments from different *Y. ruckeri* strains that isolated in this study compared with standard strain. Lane 1: Marker 100 base pair; Lane 2: Negative control (*Y. enterocolitica* O:9); Lane 3-9: *Y. ruckeri* field isolates; Lane 10: Positive control (*Y. ruckeri* strain 90/3988); Lane 11: Negative control (distilled water)

In the pathogenicity tests, immersed fish in the bacterial suspension by challenge experiments showed 70 ± 8.16% (mean ± SD) mortality during 14 days post-infection. Experimentally infected fish were reluctant to eat feed, swimming at the water surface and revealed typical haemorrhages, blackening of skin, exophthalmia and wide haemorrhages within the oral cavity and on the internal organs. No mortality was observed in the control group. *Yersinia ruckeri* colonies were detected on BHI plates from all the dead fish kidneys.

Discussion

Since 2002, there have been unpublished reports on the occurrence of the ERM in Chahar-Mahal Bakhtiary province in the center of Iran and then in Fars province. We received moribund rainbow trout of all ages with typical clinical signs of ERM as heavy haemorrhages on lips, in mouth and pharynx as well as skin darkening and a septicemic haemorrhages on the internal organs. The number of mortalities was significantly greater in water recirculating than flow-through system farms. The occurrence of the disease during winter 2002 - spring 2006 in Fars province is notifiable, however, adverse environmental conditions have possibly intensified the severity of the disease. In this research only 39 fish out of 126 sampled moribund fish were infected with bacteria. Other moribund fish might be suffered from viral or non-infectious diseases such as environmental gill damages due to suspended materials after rain, particularly during winter.

Enteric redmouth disease is an acute or chronic fish disease which has caused significant losses in aquaculture associated with intensive culture and poor water quality (Horne and Barnes, 1999).

Diagnosis of the causative agent of disease is important to specify a preventive strategy. In the current study, seven *Y. ruckeri* isolates were diagnosed from 126 moribund fish for ERM. Comparing the reported biochemicals by Austin and Austin (1999), the isolated bacteria in this study found to be *Y. ruckeri*. The reported results of biochemical characteristics of *Y. ruckeri* and *Hafnia alvei* by Austin and Austin

(1999); Horne and Barnes (1999); Romalde *et al*. (2003) were compared with the results of biochemical tests of *Y. ruckeri* isolates in this survey. Not all tests have been tried by the researchers and variation in the results could be seen. *H. alvei* produces acid from arabinose, utilizes sodium citrate, and degrades tween 40 and 80 but it is unable to degrade gelatin while its other properties are similar to *Y. ruckeri*. Our *Y. ruckeri* isolates,

Table 1: Biochemical characteristics of *Yersinia ruckeri* and *Hafnia alvei*

Tests	Bacteria				
	Y. ruckeri (our isolates)	*Y. ruckeri* (A. A. 1999)	*Y. ruckeri* (H.B. 1999)	*Y. ruckeri* Ser. 02 (Romalde *et al*. 2003)	*Hafnia alvei* (A. A., 1999)
Colony Pigmentation	W	W			W
Motility					
12°C	+				
24°C	+				
30°C	+				
37°C	-				
Oxidative-fermentative metabolism of glucose	F	F		F	F
Production of:					
Catalase	+		+	+	
Oxidase	-	-		-	-
H_2S	-	-	-	-	-
Indole	-	-	-	-	-
Lysine decarboxylase	+	+	+ (88%)	+	+
Ornithine decarboxylase	+	+	+	+	+
Methyl red	+	+	+ (79%)		+
Vages Proskauer	-	-		+	-
Nitrate reduction	+		+ (85%)	+	
Degradation of:					
Aesculin	+ (weak)		-		
Gelatin	+ (weak)	+	+ (52%)	-	-
Tween 40		-	+		+
Tween 80		-	+ (86%)		+
Urease production	-	-	-	-	-
Growth in:					
0% NaCl	+	+			+
0-3% NaCl	+				
6.5% NaCl	-				
Growth at:					
4°C	+ (72 h)				
12°C	+				
24°C	+				
30°C	+	+			+
37°C	+	+			+
Utilization of sodium citrate	-	+	+ (3%)	-	+
ONPG	+		+		
Production of acid from:					
Glucose	+	+	+	+	+
Fructose	+				
Raffinose	-	-	-		
Rhamnose	-	-	-	-	-
Sorbitol	-	-	+ (25%)	+	-
Sucrose	-	-	-	-	-
Trehalose	+		+ (97%)		
Arabinose	-	-	-	-	+
Inositol	-	-	-	-	-
Mannitol	+	+	+	+	+
Maltose	+	+	+	+	+
Melibiose	-	-	-	-	-

W: White, A. A: Austin and Austin, and H. B: Horne and Barnes

Table 2: Results of antibiogram test on seven *Y. ruckeri* isolates of rainbow trout farms situated in Fars province, Iran

	Antibiotic	Effective amount in disk (mcg)	Inhibition diameter (cm) (Mean ± SD)
1	Enrofloxacin	10	3.3 ± 0.26
2	Norfloxacin	10	3.2 ± 0.3
3	Ofloxacin	5	3.2 ± 0.23
4	Trimethoprim	5	3 ± 0.32
5	Oxytetracycline	30	2.8 ± 0.13
6	Tetracycline	30	2.6 ± 0.19
7	Chloramphenicol	30	2.6 ± 0.17
8	Streptomycin	10	2 ± 0.2
9	Clarithromycin	15	1.4 ± 0.31
10	Ampicilin	10	1 ± 0.22
11	Erythromycin	15	0.9 ± 0.11
12	Vancomycin	30	0

in comparison with *Y. ruckeri* reported by Austin and Austin (1999) differ in utilization of sodium citrate. Our isolates easily grew on sheep blood agar at 12°C and were motile. These seven isolates did not produce acid from arabinose and did not utilize sodium citrate, but degraded gelatin. Using profiles of fish pathogens provided by Austin and Austin (1999), the isolates were biochemically different with *H. alvei*. Even though *H. alvei* has been reported as a bacterium responsible for epizootic haemorrhagic septicaemia in rainbow trout (Gelev *et al*., 1990), not many case reports have been published on the pathogenicity of this opportunistic bacterium for rainbow trout.

Our isolates were different in vages proskauer negative reaction and inability to produce acid from sorbitol when compared with *Y. ruckeri* serotype O2 reported by Romalde *et al*. (2003). O'Leary *et al*. (1979) reported that the ability to ferment sorbitol is characteristic of serovar II and could be used to distinguish it from serovar I; former serovar I and II are newly named serotype O1 and O2, respectively. Although a presumptive diagnosis of serotype O1 may be made based on the high mortality (70% mortality), high virulence of the isolates and biochemical characteristics (Furones *et al*., 1993), a definitive diagnosis can only be made following serological examination using antisera for serogrouping which were not used in present study. Serotype O1 has a worldwide distribution while serotype O2b is restricted to North America and Baltic countries (Romalde *et al*., 1993).

In the present study, *Yersinia ruckeri* isolates were sensitive to enrofloxacin, norfloxacin, ofloxacin, trimethoprim and oxytetracycline. This is consistent with the finding of reported antibiotic susceptibility of *Y. ruckeri* isolates in Croatia (Oraic *et al*., 2002).

In the pathogenicity tests, the isolate of 2004 showed 70 ± 8.2% mortality in challenge experiments during 14 days post-infection. It can be concluded that the isolate is quite virulent for rainbow trout. A virulent *Y. ruckeri*-like bacterium from diseased rainbow trout cultured in Tehran province has already been reported with 100% mortality when 1.12×10^9 cells/L for 1 h in immersion and 9×10^7 cells/fish in injection were employed (Soltani and Tarahomi, 2002).

Polymerase chain reaction amplification of DNA extracted from all *Y. ruckeri* isolates were confirmed with the specific primers as *Y. ruckeri* by producing the suspected size of 409 bp. Comparison of the conventional method and PCR results from isolated bacteria for the diagnosis of *Y. ruckeri* infection in fish, showed 100% agreement between both tests. The result of PCR on *Y. enterocolitica* O:9 that is phylogenically related to *Y. ruckeri* was negative and no amplification signal was detected when distilled water used as template. The traditional microbiological methods (isolation and identification) usually take 2 to 3 days for the definitive detection of *Y. ruckeri*. In addition several biochemical profiles for *Y. ruckeri* must be interpreted with caution (Furones *et al*., 1993), whereas the results of PCR assay on isolated bacteria as *Y. ruckeri* are available in an 8-h. Results of this study showed that this PCR procedure has high potential as a rapid screening test for definitive diagnosis of *Y. ruckeri* strains from other bacteria isolated in culture media e.g. *Streptococcus* spp. and *Aeromonas hydrophila*, both causing haemrrhagic septicaemia in rainbow trout.

The PCR method was applied as supplementary and complementary test for a definitive identification of the bacteria cultured from clinically suspected samples in this study. In future studies, this PCR method can be used as a direct test for detection of *Y. ruckeri* in tissues of infected trout, but it is necessary to remove PCR

inhibiting components and needs extensive sample preparation (Gibello *et al*., 1999).

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