Phenotypic and genetic homogeneity of *Yersinia ruckeri* strains isolated from farmed rainbow trout, *Oncorhynchus mykiss* (Walbaum), in Mersin Province, Turkey

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Abstract

Twenty-four *Yersinia ruckeri* strains isolated from farmed rainbow trout, *Oncorhynchus mykiss* (Walbaum), in Mersin Province (Turkey) were investigated for phenotypic and genetic homogeneity by conventional microbiological techniques, the ID32GN system and arbitrarily primed polymerase chain reaction (AP-PCR). All isolates displayed phenotypic and genetic homogeneity by these techniques.

Introduction

Yersinia ruckeri is a Gram-negative enterobacterium and the causative agent of enteric redmouth disease (ERM) of salmonid and non-salmonid fishes reared in both fresh and marine waters. ERM is an infectious disease, which produces high mortalities and severe economic losses in farmed fish, particularly rainbow trout, *Oncorhynchus mykiss* (Walbaum) and Atlantic salmon, *Salmo salar* L. (Austin & Austin, 1993; Stevenson et al., 1993). Since the disease was first detected in the USA in the 1950's, it has been identified worldwide over the last two decades (Alvarez et al., 1992; Bragg & Henton, 1986; Cipriano et al., 1986; Dalsgaard et al., 1984; Davies & Frerichs, 1989; De La Cruz et al., 1986; Fouz et al., 2006; Fuhrmann et al., 1983; McCormick & McLoughlin, 1993; Petrie et al., 1996; Rodriguez et al., 1999; Savvidis, 1990; Soltani et al., 1999; Sousa et al., 1994; Sparboe et al., 1986; Valtonen et al., 1992). Furthermore, *Yersinia ruckeri* was also recovered from wild fish, birds and mammals (Willumsen, 1989).

Several techniques have been applied to identify *Y. ruckeri* and to investigate relatedness among isolated strains. Traditional phenotypic tests and their miniaturized forms for automation have been used as 'gold standard'. However, many isolates are still poorly identified by these methods and supplementary tests are often required for a complete identification (Ibrahim et al., 1993; Linde et al., 1999). Molecular methods such as PCR have been

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successfully used for *Yersinia ruckeri* identification and characterisation (LeJeune & Rurangirwa, 2000; Lucangeli et al., 2000; Romalde et al., 1993; Sousa et al., 2001; Temprano et al., 2001; Yugueros et al., 2001). Arbitrarily primed polymerase chain reaction (AP-PCR), also known as random amplified polymorphic DNA (RAPD), devised by Welsh and McClelland (1990) and by Williams et al. (1990), has previously been applied for genetic characterization studies of various bacteria (Aguilera-Arreola et al., 2005; Balboa et al., 2007; Emekdas et al., 2006; Grayson et al., 1999; Inglis et al., 1996; Thomas-Jinu & Goodwin, 2004).

In Turkey, following the first identification of ERM in rainbow trout in 1991 (Cagirgan & Yüreklitürk, 1991; Timur & Timur, 1991) the disease has been reported several times (Atesoglu, 1999; Diler et al., 2000; Kirkan et al., 2000). However, this is the first investigation reporting ERM at commercial rainbow trout farms in the Eastern Mediterranean part of Turkey and using AP-PCR for genetic homogeneity of *Yersinia ruckeri* in Turkey.

Therefore, our aim was to detect phenotypic and genetic homogeneity of *Y. ruckeri* strains isolated from seven different rainbow trout farms by conventional cultivation, biochemical characterization, the ID32GN system and AP-PCR methods, in our region.

Materials and methods

Source of Yersinia ruckeri strains

Totally 259 fish were investigated at seven rainbow trout farms in Mersin province in a one year period in 2005. From each farm 5-10 fish were sampled seasonally. For bacterial isolation, samples from both clinically diseased and apparently healthy fish has been taken from gill, liver, kidney and brain and directly streaked onto tryptic soy agar (TSA, Merck) supplemented with 5 % sheep blood. Plates were incubated at 20°C for 48 h.

Characterization of the isolates

Pure cultures were obtained on TSA. Bacterial isolates were biochemical characterized by the following selected criteria: Gram staining, cell morphology, oxidase, catalase, motility, oxidative-fermentative metabolism of glucose, lysine decarboxylase, methyl red, Voges-Proskauer, production of acid and gas from glucose, H_2S, indole, fermentation of sucrose, lactose and sorbitol and utilization of Simmon's citrate. Biochemical characterization was performed according to standardized methods (Austin & Austin, 1993; Cowan, 1975; Frerichs, 1993; Plumb & Bowser, 1983). In addition, the isolates were identified in ID32GN automatic identification system for Gram-negative rods (BioMérieux, France) according to the manufacturer's instructions, except that incubation was at 30°C for 24-48 h.

Genetical homogeneity of Y. ruckeri

Detection of genetic homogeneity of *Y. ruckeri* isolates was done by AP-PCR. As exemplary of 24 *Y. ruckeri* isolates 12 isolates has been used. *Yersinia ruckeri* reference strain (Italian Rainbow trout isolate, 18-83, Serotype O1) (Kubilay & Diler, 1999) and our previous isolates *Yersinia intermedia* and *Hafnia alvei* have been used as control microorganisms.

Bacterial DNA preparation

Each isolate was cultivated on one Mueller Hinton agar plate and bacterial colonies were harvested with sterile saline. Three ml of bacteria suspension was collected and washed 3 times with sterilized saline by centrifugation at 5000x*g* for 5 min for each spin. After the last centrifugation, the supernatant was discarded and the pellet was resuspended with 3 ml sterilized saline. Finally, 100 µl of this fluid was taken and mixed with 300 µl of lysis buffer (13.3 mmol/l Tris–HCl [pH 8.0], 6.7 mmol/l ethylenediaminetetraacetic acid, 0.67 % sodium dodecyl sulfate, 133µg/ml proteinase-K) and incubated overnight at 56ºC. Two phenol–chloroform extractions were followed by one chloroform extraction, and DNA was precipitated with ethanol. The precipitate was dissolved in 50 µl of TE-buffer (10 mmol/l Tris–HCl [pH 8.0], 1 mmol/l ethylenediaminetetraacetic acid).

AP-PCR amplification

The oligonucleotide primer (M13) was chosen from a method book of Durmaz & Ayan (2001). Two µl of DNA solution were amplified in a 50 µl reaction containing 75 mM Tris–HCl [pH 8.8], 0.2 mM dNTPs (Promega, U1330), 1.5 mM MgCl2 (Promega, A3513), 0.5 µM of universal primer M13 (5V-GAGGGTGGCGGTTCT-3V), and 1 U Taq polymerase (Promega M1665). The PCR conditions were 2 cycles of 5 min at 94°C, 5 min at 40°C and 72°C, followed by 40 cycles of 60 s at 94°C, 60 s at 40°C and 60 s at 72°C. PCR products were separated on a 1.8 % agarose gel and stained with ethidium bromide.

Results

Source of Yersinia ruckeri *strains*

Twenty-four *Y. ruckeri* strains were isolated from 24 (9.3%) of 259 rainbow trout samples. *Yersinia ruckeri* was recovered from all seven farms.

Biochemical characterization of the isolates

Pure bacterial cultures have been used for biochemical characterization. The isolates demonstrated a great phenotypic homogeneity. All Gram negative, oxydase negative, catalase positive and fermentative rods showed positive reaction of motility, lysine decarboxylase, methyl red, and production of acid from glucose. The isolates were negative in production of gas from glucose, acid from sucrose, lactose and sorbitol, H_2S, indole, Voges-Proskauer and utilisation of Simmon's citrate.

These data were also confirmed with the ID32GN system. Whereas 23 isolates were

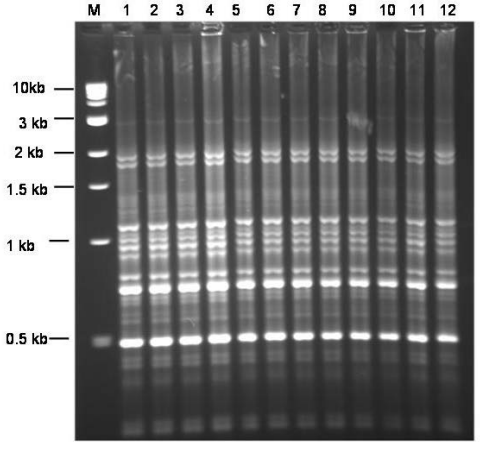

Figure 1. Genotypical discrimination of 12 *Yersinia ruckeri* isolates: Lane M: 1 kb DNA ladder (BioLabs, N3232S), lane 1-lane 12; example isolates form 7 farms.

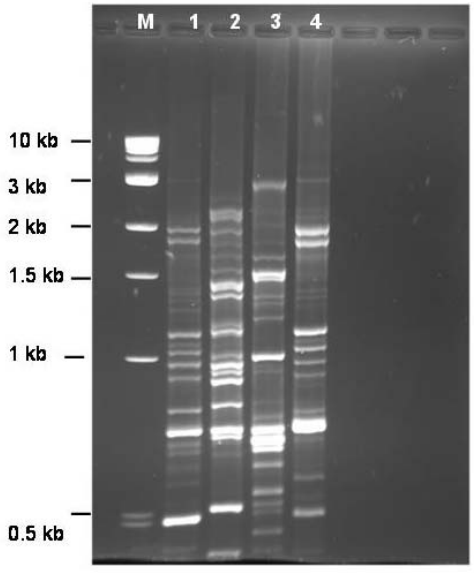

Figure 2. Comparison of *Yersinia ruckeri* isolates with other bacterial isolates and *Yersinia ruckeri* reference strain: Lane M: 1 kb DNA ladder (BioLabs, N3232S), lane 1: *Yersinia ruckeri* from the present study, lane 2: *Yersinia intermedia*, lane 3: *Hafnia alvei*, lane 4: *Yersinia ruckeri* reference strain.

identified as *Y. ruckeri* with good or very good votes, one isolate showed low discrimination.

Genetic homogeneity of Yersinia ruckeri

The AP-PCR results indicated that all isolates were genetically homogenous. Figure 1 demonstrates the band patterns of amplification products from 12 different isolates obtained from seven farms (Figure 1, lane 1-lane 12). All these patterns were clearly the same as seen in Figure 1. We have also compared these band patterns with AP-PCR products of *Y. ruckeri* reference strain (Italian Rainbow trout isolate) and 2 of our previous isolates *Yersinia intermedia* and *Hafnia alvei* (Figure 2). In lane 1, a selected sample reflecting all our 24 isolates was compared with band patterns of *Y. intermedia* (lane 2), *H. alvei* (lane 3) and *Y. ruckeri* reference strain (lane 4) (Figure 2). It is clearly seen that our sample isolate is very similar to, but not identical with reference *Y. ruckeri* isolate and quite different than *Y. intermedia* and *H. alvei*.

Discussion

In the present study, 24 *Yersinia ruckeri* strains have been isolated in 24 (9.3%) of 259 rainbow trout samples obtained from seven commercial rainbow trout farms in Mersin province. These findings demonstrated that the widespread bacteria also threats the health of rainbow trout reared in the eastern Mediterranean part of Turkey.

While many isolates are still poorly identified by traditional biochemical techniques and some commercial kits based on phenotypic properties (Ibrahim et al., 1993; Linde et al., 1999), we have found high agreement between classical biochemical techniques, ID32GN system and AP-PCR methods. All 24 *Y. ruckeri* isolates obtained in the present study demonstrated phenotypic and genetic homogeneity.

Various PCR techniques have been performed to identify *Yersinia ruckeri*. In a study for definitive identification of *Yersinia ruckeri* LeJeune & Rurangirwa (2000) used selected primer pairs to amplify a 409 bp fragment of 16S rDNA and reported the serovar specificity of the bacteria. They propose that this assay provides a rapid, definitive identification of *Y. ruckeri*. Yugueros et al. (2001) used *Alu*I restriction fragment length polymorphism (RFLP) of the *Yersinia ruckeri aroA* gene to differentiate between *Y. ruckeri* and other bacteria. They suggested that the specificity and sensitivity make this PCR assay a useful

method for rapid identification and diagnosis of *Y. ruckeri* infections. Temprano et al. (2001) reported that detection of *Yersinia ruckeri* by PCR amplification of *yruR*/*yruI* genes has great potential for rapid identification of this fish pathogen because of the high specificity of the test.

On the other hand, molecular characterization studies of *Y. ruckeri* strains have been carried out by several researchers. Lucangeli et al. (2000) have studied the ribotypes, patterns of pulsed-field gel electrophoresis (PFGE) and interspersed repeated sequences (IRS)-PCR of 30 *Yersinia ruckeri* O1 strains. They have reported that all of the *Y. ruckeri* O1 strains presents an extremely high level of genetic homogeneity. Sousa et al. (2001) have comparatively studied by means of lipopolysaccharide (LPS) and outer membrane protein (OMP) analysis, plasmid profiling and ribotyping in order to investigate the heterogeneity among *Y. ruckeri* isolates. They have considered that ribotyping is the best candidate for epidemiological studies, because it is easier to perform and offers a slightly better discriminative power. However, AP-PCR has been used for genetic characterization of fish pathogenic bacteria like Aeromonas species (Aguilera-Arreola et al., 2005; Inglis et al., 1996), *Renibacterium salmoninarum* (Grayson et al., 1999), *Flavobacterium columnare* (Thomas-Jinu & Goodwin, 2004) and *Pseudomonas anguilliseptica* (Balboa et al., 2007). There was a concurrence that AP-PCR promises to be useful in epidemiological studies for rapid identification of bacteria. Argenton et al. (1996) have used RAPD to generate specific molecular probes for hybridization tests and PCR-based diagnosis of *Yersinia ruckeri*.

Our observations indicated that all 24 *Y. ruckeri* isolates examined in the present study demonstrated genetic homogeneity by means of the AP-PCR method. This is the first field study using AP-PCR method to determine genetic homogeneity of *Y. ruckeri*. We suggest that this method is quite useful for genetic characterization of *Y. ruckeri*. It is also easily implemented in laboratories working in this area.

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