

OCCURRENCE OF ENTERIC REDMOUTH DISEASE IN RAINBOW TROUT (*ONCORHYNCHUS MYKISS*) ON FARMS IN CROATIA

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During the spring of 1996 and autumn of 1997 unusual mortality outbreaks among rainbow trout fry and yearlings occurred at two different trout farms, resulting in mortality of 20 and 10 per cent, respectively. Generally, the affected fish, swimming at the water surface, were reluctant to eat and were dark pigmented with visible haemorrhages around and within the oral cavity. Bacterial isolates from moribund fish from both cases were identified as *Yersinia ruckeri* by standard biochemical tests and API 20E. The isolated strains were found to be sensitive to tetracycline, chloramphenicol, co-trimoxazole, nalidixic acid, flumequine, enrofloxacin, carbenicillin and gentamicin. Microplate agglutination assay confirmed that both isolates belonged to serotype O1. The pathogenicity of the isolated bacteria was confirmed by challenge experiment. Titres of specific antibodies were determined in the sera of survivors. The titre was highest on the 21st day postchallenge and was detectable until the 81st day.

Key words: Rainbow trout, enteric redmouth disease, *Yersinia ruckeri*, serotyping

Enteric redmouth disease (ERM) of rainbow trout (*Oncorhynchus mykiss*) caused by *Yersinia ruckeri* was first described as a separated nosological entity by Ross et al. (1966) and Rucker (1966) in Idaho, USA, although the disease had been clinically observed during several preceding years. Subsequently, Bullock et al. (1978) studied the spread of ERM in the USA, and demonstrated the presence of the disease by bacteriological survey in nineteen states of the country. In the following years the same disease was reported in Australia (Llewellyn, 1980), Canada (Stevenson and Daly, 1982), and several European countries (Richards and Roberts, 1978; Lesel et al., 1983; Fuhrmann et al., 1983; Ghittino et al., 1983; Ocvirk et al., 1988). In addition to salmonids, *Y. ruckeri* has been reported as the pathogen affecting a wide range of both free-living freshwater fish species (McArdle and Dooley-Martyn, 1985; Rintamaki et al., 1986) and in some cultivated marine fish species like turbot and sole (Vigneulle, 1984).

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Infection with *Y. ruckeri* causes the highest losses in rainbow trout under certain stress conditions, where the increased water temperature is clearly recognised as one of the more frequent predisposing factors (Austin and Austin, 1987). Affected fish displayed lethargy, anorexia, occasional darkened pigmentation of skin, and an increased mortality rate ranging from 5 to 30 per cent of the population (Inglis et al., 1993). Reddening of the mouth, throat, jaws, operculum and base of the fins due to haemorrhages were found on autopsy in acute and subacute forms of the disease. In the chronic course, additional signs such as uni- or bilateral exophthalmia were often visible.

The aim of this study is to report the first two recognised outbreaks of ERM in Croatia with the description of the clinical findings and gross pathologic lesions. The properties of isolated bacteria from both cases are given as well as their susceptibility to antimicrobial compounds. In addition, a successful challenge infection experiment was carried out under controlled conditions with prospective monitoring of the specific antibodies by serological tests.

Material and methods

During the spring of 1996 and autumn of 1997 unusual mortality outbreaks occurred among rainbow trout fry and yearlings at two different trout farms. Farm A is located in the northern and Farm B in the central part of Croatia. On Farm A, in the concrete basins overcrowded by fry weighing 30 grams, some specimens swimming at the water surface were reluctant to eat and had visible haemorrhages around and inside the oral cavities. Some fish showed dark discolouration of the skin. At that farm mortality (reaching approximately 20 per cent of the entire population) was higher and lasted shorter than at Farm B.

At Farm B, in the earthen basins for growing the yearlings mortality started after the rainy period which causes the sediment to rise. Generally, fish suffered lower but more chronic mortality in the course of approximately two months, during which period about 10 per cent of the fish was lost. Sick fish were spinning at the water surface and clinically had pronounced reddening around the mouth and in the oral cavities. In addition, haemorrhages were seen in the eyes showing exophthalmia. Dark pigmentation with whitening was more obvious than on the first farm.

Case studies

Thirty moribund rainbow trout fry from Farm A and twenty yearlings from Farm B were subjected to autopsy using widely accepted routine methods described by Ashburner (1989). Samples of kidney, liver, spleen and brain collected during autopsy were plated onto tryptic soy agar (TSA) and blood agar (BA) and incubated for 48 h at 22 °C following standard procedures. Ten initial

bacterial cultures isolated from fry and seven initial cultures from yearlings were transferred onto Shotts-Waltman medium (SW) (Waltman and Shotts, 1984) and ROD medium as recommended by Rodgers (1992). Colonies exhibiting the zone of hydrolysis resulting from degradation of Tween 80 in SW medium and the zone of precipitation of sodium deoxycholate in ROD medium, were subjected to standard biochemical tube tests and commercial API-20E system (Biomérieux, France). Standard biochemical tests performed included oxidase production, indole production by Kovacs reagents, catalase production, oxidation/fermentation, gas production, H₂S production on triple sugar iron agar (TSI), Simmon's citrate reaction (monitored up to 7 days), Voges-Proskauer reaction, methyl red test, gelatine, urea and Tween 80 hydrolysis, tolerance to different concentrations of NaCl in growth media and acid production from glucose. In addition, the motility of isolated bacterial colonies at 22 °C and 37 °C was compared.

All strains isolated from fry were pure and of the same properties. A strain isolated from kidney was chosen as representative (denominated as RY-11) and was used in further experiments. A strain isolated from yearlings' kidney was denominated as RY-12 and was used for further experiments.

Susceptibility to antimicrobial agents

Susceptibility of the isolated strains to various antibiotics was determined by the disc diffusion method according to Ericsson and Sherris (1971) on Mueller-Hinton agar (MHA) using commercially available discs (Becton-Dickinson, USA).

Challenge experiments

Rainbow trout fry (mean weight 6.5 g) obtained from an ERM-free facility were held in 100-litre glass aquaria with flow-through, dechlorinated, aerated tap water at 14 °C and monitored daily for signs of disease for 80 days postinfection. Thirty fish were put into each of 7 experimental aquaria.

Bacterial isolates cultivated from both affected facilities were further grown on tryptone soy broth at 22 °C for 48 h, enumerated by plate count, and added directly to the aquaria filled to contain 20 litres of water (including the volume of fish) with a final concentration of 1×10^8 bacterial cells per ml of aquarium water. After a 60-min contact period, water flow was resumed and the bacterial cells were removed to a disinfecting container. For experimental infection with RY-11 four aquaria while for RY-12 two aquaria were used. Sterile tryptone soy broth was added to the aquarium containing control fish. During the experiment the fish were fed a commercial pelleted food.

Kidney, spleen and liver tissues collected on autopsies of dead fish during the challenge experiment were cultivated on TSA and isolated bacteria were identified by the methods used for identification of the original isolates.

Serological tests

Polyclonal antibodies were prepared by injection of formalin-killed bacterial cultures of *Yersinia ruckeri* (RY-11 and RY-12) four times at 3-day intervals, followed by injection of virulent bacterial cultures three times at 3-day intervals into the ear vein of white rabbits. The rabbits were bled on the 29th and 36th days after the first injection. The sera were prepared according to procedures described by Anderson and Dixon (1981), Stevenson and Daly (1982) and Davies (1990).

Heat-stable O-antigens of isolated strains (RY-11, RY-12), reference bacterial strains of *Y. ruckeri* (RS-4, Type 1; RD-34, Type 2) and O-antigens of control strains of *Aeromonas salmonicida* and *Vibrio anguillarum* were all prepared according to the procedures described by Davies (1990).

Microplate agglutination assay was performed on 96-well microtitre plates in order to evaluate the serological properties of isolated strains of *Y. ruckeri*. The titres of specific antibodies in the sera of survived fish were determined as well (Davies, 1990). The blood samples were pooled from five anaesthetised fish taken immediately before the challenge trial. The same procedure was repeated on the 21st, 51st and 81st days postchallenge on the fish from the experimental group infected by strain RY-11.

Results

Case study

During the disease outbreak in rainbow trout fry from Farm A the mortality started suddenly with nonspecific signs and lasted for five days, climbing up to 5 per cent. Affected fish displayed lethargy and petechiae on the ventral body surface and internal organs. Several specimens showed some haemorrhages around and in the mouth, and yellow discharge from the anus.

At Farm B the mortality rate of yearling rainbow trout was roughly the same as that of fry at Farm A. Diseased fish swam at the water surface showing darkening of the skin, exophthalmus and moderate skin haemorrhages, haemorrhages in the eyes, around the mouth and in the mouth.

The bacteriological examination of all organs listed above yielded pure small bacterial colonies of circular shape and opaque, yellowish colour. All isolated strains plated onto SW medium produced green colonies with halos (Waltman and Shotts, 1984), while plated onto ROD they produced yellow colonies which determined them as type 1 (Rodgers, 1992). They were found to be Gram-negative rods, which were oxidase and indole negative and catalase positive. The results from standard biochemical tests and commercial API 20E system are summarised in Table 1.

Table 1

Comparison of biochemical properties of the *Yersinia ruckeri* isolates from this study with those of the reference strain

Property	Strains of <i>Yersinia ruckeri</i>		
	Farm A* n = 10	Farm B* n = 7	RS 4**
Gram staining	-	-	-
Motility at 22 °C	+	+	+
37 °C	-	-	-
Oxidase	-	-	-
Indole	-	-	-
Catalase	+	+	+
Methyl red (MR)	+	+	+
Voges-Proskauer (API)	- (-)	- (-)	- (+)
Oxidation/fermentation	F	F	F
H ₂ S production (TSI)	-	-	-
Gas production (TSI)	-	-	-
K/A, A/A (TSI)	K/A	K/A	K/A
Sorbitol (API)	K/A (-)	K/A (-)	K/A (-)
Hydrolysis of			
Gelatine (API)	+ (-)	+ (+)	+ (+)
Urea	-	-	-
Lysine	+	+	+
Ornithine	+	+	+
Tween 80	+	+	+
Utilisation of:			
Glucose	+	+	+
Sucrose	-	-	-
Manitol	+	+	+
Rhamnose	-	-	-
Arabinose	-	-	-
Adonitol	-	-	-
Maltose	+	-	+
Aesculin	-	-	-
Simmon's citrate (API)	+ (-)	+ (-)	+ (-)
Tolerance to NaCl			
0%	+	+	+
3%	+	+	+
7%	-	-	-

*Croatian isolates of *Yersinia ruckeri*; **Reference strain of *Yersinia ruckeri*

Susceptibility to antimicrobial agents

Both isolated strains (RY-11, RY-12) were sensitive to tetracycline, chloramphenicol, co-trimoxazole, nalidixic acid, flumequine, enrofloxacin, carbenicillin and gentamicin while being resistant to amoxicillin, sulphamethoxazole, penicillin G and erythromycin.

Challenge experiments

On the 4th day after challenge, mortality started in all experimental groups excluding the control group. Over the following 6 days fish in all infected groups developed signs of disease such as dark pigmentation on the skin, haemorrhages on the vent and around the mouth. Mortality of the fish exposed to isolate RY-12 was 71.7 per cent (43 of 60 fish) while in the group exposed to RY-11 it was 88.33 per cent (106 of 120 fish). Bacteria isolated from all dead experimental fish were morphologically and biochemically identical to the original isolates. Control fish did not develop any signs of disease and no bacteria were recovered from randomly chosen samples.

Serological tests

The results of microplate agglutination assay were positive for both isolated strains (RY-11 and RY-12) and for the reference strain of *Y. ruckeri*, Type 1 (RS-4) with slightly variable degree of reactions. The results for other tested O-antigens were very weak and negative (Table 2). The titres of antibodies in the sera of survived fish exposed to challenge were the highest on the 21st day post-challenge at 1:32 dilution. They decreased towards the 51st day to 1:16, but were still detectable on the 81st day postchallenge at 1:8.

Table 2

Antibody titre in microplate agglutination assay with different heat-stable O-antigens

Polyclonal antiserum	Heat-stable O-antigen					
	<i>Y. ruckeri</i> RY-11	<i>Y. ruckeri</i> RY-12	<i>Y. ruckeri</i> RS-4*	<i>Y. ruckeri</i> RD-34**	<i>V. anguillarum</i>	<i>A. salmonicida</i>
RY-11	256	128	512	< 2	0	4
RY-12	128	512	128	0	0	< 2

*RS-4 – reference strain of *Y. ruckeri* (Serotype O1); **RD-34 – reference strain of *Y. ruckeri* (Serotype O2)

Discussion

Although yersiniosis of rainbow trout has been reported from most European countries (Meier, 1986; Csefay et al., 1989; Toranzo et al., 1990; Rodriguez et al., 1999), the present work is the first report of the disease caused by *Yersinia ruckeri* in the Croatian rainbow trout farms.

Clinical observation, epidemiological data and gross pathological findings supported by microbiological survey allowed the diagnosis of enteric red mouth disease (ERM) caused by *Y. ruckeri*. The results of biochemical tests performed on API 20E showed a high degree of uniformity in these two isolates. However,

there were some minor differences between them and the reference strain as shown by the Voges-Proskauer test which was negative for both Croatian isolates while positive for RS 4. According to the results obtained on API 20E as well as by standard bacteriological tests, our own isolates are similar to 22 per cent of *Y. ruckeri* isolates tested by Davies and Frerichs (1989). Hydrolysis of gelatine for isolates from Farm A on API 20E were also negative in comparison to the other tested isolates. Utilisation of maltose for isolates from Farm B were negative. Generally, the results of bacteriological studies were consistent with those for reference strain RS 4, and *Y. ruckeri* described by Stevenson and Daly (1982), Davies and Frerichs (1989) and Austin and Austin (1993). The isolates from the clinical cases of the disease were of the same morphological, staining and biochemical characteristics as isolates from dead fish from the challenge experiment.

Testing of bacterial susceptibility to antimicrobial agents revealed sensitivity patterns similar to those determined by Ceschia et al. (1987) and Rodriguez et al. (1999), except for the sensitivity of the Croatian isolates to gentamicin.

The signs of enteric redmouth disease and mortality appeared on days 4 to 10 after bath challenge exposure in 71.7 per cent of rainbow trout exposed to isolate RY-12 and in 88.33 per cent of the fish exposed to isolate RY-11. These results, congruent with earlier findings described by Toranzo and Barja (1993), also confirm that the virulence of the two isolated strains is rather similar. In contrast, in the immersion experimental infection of brown trout (*Salmo trutta*) Hietala et al. (1995) could not produce disease or mortality due to *Y. ruckeri*, although they were able to re-isolate bacteria from the organs of half of the challenged fish. However, during the same experiment these authors were able to provoke the disease resulting in mortality by intraperitoneal (i.p.) injection of the same bacteria. In this way, they postulated lower pathogenicity of the Finnish isolate and probably higher resistance of brown trout. Comparing the experimental models of immersion exposure and i.p. inoculation of bacteria it is obvious that the former should be used in all experiments the aim of which is to closely imitate the natural way of infection.

Performing the test of sorbitol fermentation as a biochemical characteristic in order to differentiate the type of *Y. ruckeri* according to the typing given by O'Leary et al. (1979), both our isolates were determined as Type 1. This finding was confirmed by the microplate agglutination assay carried out by means of polyclonal antibodies derived from our two isolates and the heat-stable O-antigen of reference strain RS-4 (Serotype O1). The assay clearly showed high titres indicating homology of serotypes. There were no reactions with reference strain RD-34 (Serotype O2). Therefore, it is obvious that the Croatian *Y. ruckeri* isolates belong to Serotype O1 according to the serotyping scheme proposed by Davies (1990). The same serotype is associated with epizootics and mortality of rainbow trout in Portugal (Sousa et al., 1996), Turkey (Cagirgan and Tanrikul, 1998), and Spain (Romalde et al., 1993).

The specific antibody titres in survivors were monitored for 81 days post-challenge. The highest titres were detected on the 21st day followed by decreasing values until the 81st day, which is comparable with the results of Cossarini-Dunier (1986) and Hietala et al. (1995).

The necessity of further research into immunogenicity is indicated by the fact that, along with the worldwide production, the production of salmonids in Croatia has also been suffering considerable losses due to ERM, especially in stressed fish. Since stress is practically unavoidable in intensive salmonid culture, vaccination should be employed as a measure of successful ERM control. Our future work, therefore, will concentrate on evaluating the possibility of immunoprophylaxis as a routine preventive measure in national rainbow trout production.

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