

Renibacterium salmoninarum: effect of hypochlorite treatment, and survival in water

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ABSTRACT: The effect of different concentrations of sodium hypochlorite on *Renibacterium salmoninarum* and the survival of the bacterium in autoclaved river water and groundwater were examined. The disinfection trial was performed using *R. salmoninarum* ATCC 33209. The concentrations of free chlorine were 10, 50, 100 and 200 mg l⁻¹, the contact times were 5, 15, and 30 min and 24 h, and the test suspensions were subcultured both on Kidney disease medium (KDM2) agar and in 3 parallel KDM2 broths, which were then subcultured on KDM2 and selective KDM (SKDM) agar. The survival of the bacterium in river water and groundwater was studied using 4 isolates of *R. salmoninarum* including ATCC 33209. Treatment with sodium hypochlorite effectively reduced the number of culturable cells of *R. salmoninarum*, but use of the recovery broth showed that small numbers of cells remained viable at all concentrations of free chlorine. The numbers of *R. salmoninarum* decreased to an undetectable level after 4 wk incubation in the survival trials, but low numbers of colonies were again found in the subculture after 5 wk incubation. Viable cells of *R. salmoninarum* were still detected in subcultures of all strains after 20 wk of incubation in river water.

KEY WORDS: *Renibacterium salmoninarum* · Disinfection · Water · Survival

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INTRODUCTION

Bacterial kidney disease (BKD) is one of the most serious infectious diseases of salmonid fishes, causing health problems worldwide (Fryer & Lannan 1993). A great deal is known about the disease and its causative agent *Renibacterium salmoninarum*, but there are many areas where the epidemiology of the disease is poorly understood (Evenden et al. 1993, Fryer & Lannan 1993). Vertical transmission of the disease through infected eggs has been investigated intensively (Bullock et al. 1978, Evelyn et al. 1984, 1986, Bruno & Munro 1986, Lee & Evelyn 1989, Pascho et al. 1991), and is known to play an important role in the maintenance of *R. salmoninarum* infection in fish populations (Fryer & Lannan 1993), while horizontal transmission through water is known to occur under natural conditions (Pippy 1969, Mitchum & Sherman 1981, Sakai & Kobayashi 1992). The infection has been induced experimentally using immersion and cohabitation

challenges (Murray et al. 1992, Mesa et al. 1998, O'Farrell et al. 2000), by the faecal-oral route (Balfry et al. 1996) and through abrasion of the skin (Wolf & Dunbar 1959), but the mechanisms of transmission and minimal infective dose under natural conditions are not clearly understood (Evenden et al. 1993, Fryer & Lannan 1993). The capacity of *R. salmoninarum* to survive in the environment has been reported to be limited (Austin & Rayment 1985, Evelyn 1988, Balfry et al. 1996).

BKD is difficult to control (Elliott et al. 1989), and the destruction of infected stocks and complete disinfection of holding facilities are recommended for its eradication (Fryer & Sanders 1981). Eradication of BKD is not considered possible in areas where *Renibacterium salmoninarum* is enzootic and wild fishes may act as carriers (European Commission 1999). In Finland, *R. salmoninarum* has never been isolated from wild fish populations in inland waters. One isolation was made once from a wild or feral fish population in sea-

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water, but only by virtue of a large-scale screening programme lasting over 10 yr that focused on migrating brood-fish populations of salmon, trout and whitefish (Rimaila-Pärnänen 2002). BKD is presumably present in Finland only in fish farms, and its eradication from the country's inland waters has been attempted on individual fish farms following the instructions issued by the Scientific Committee of the European Commission (1999). Disinfection is an essential part of the eradication programme, but there are few publications on the efficiency of disinfection, as the current literature comprises 2 papers on the use of hypochlorite (Pascho et al. 1995, Pascho & Ongerth 2000) and 1 on iodophors (Ross & Smith 1972).

In the current study, the effect of sodium hypochlorite on *Renibacterium salmoninarum*, detected either by subculturing test suspensions directly on agar plates or after incubation in a recovery broth, and the survival of the bacteria in river water and groundwater were examined.

MATERIALS AND METHODS

Disinfection trial. A qualitative suspension test was performed as described by Quinn (1987). The bacterial suspension used for inoculation was prepared from a 25 d old culture of *Renibacterium salmoninarum* ATCC 33209. The bacterial population obtained on KDM2 agar (Evelyn 1977) was suspended in sterile distilled, deionized water to an OD of 0.307 (615 nm), corresponding to a bacterial concentration of approximately 10^8 ml⁻¹. Sodium hypochlorite was added to 1.9 ml aliquots of sterile distilled, deionized water or autoclaved tank water to provide concentrations of 0, 10, 50, 100 and 200 mg l⁻¹ free chlorine, and 0.1 ml of the bacterial suspension was added to each mixture. After contact times of 5 min, 15 min, 30 min or 24 h, 0.1 ml of each mixture was subcultured onto KDM2 agar and 0.01 ml into three 5 ml KDM2 broth tubes. Each broth culture was subcultured on KDM2 agar and SKDM agar (Austin et al. 1983), either after observation of turbidity in the tube or after a maximum of 6 wk incubation. Plates were monitored for bacterial growth for at least 5 wk. Bacterial colonies were subcultured on KDM2 agar and trypticase soy agar with 5% bovine blood (TSA). Subcultures that produced good bacterial growth on TSA were discarded. The growth of *R. salmoninarum* was confirmed by a typical pattern of slow growth of white or creamy, smooth, round, raised, entire colonies on KDM2 agar, detection of small Gram-positive rods by Gram-staining, a positive catalase test and a negative oxidase reaction. The incubation temperature for all steps in the trial was 15°C. The tank water was obtained from a tank in which rainbow

trout (age 0+) had been kept for 2 wk under aeration without changing the water. The hardness of the autoclaved tank water was 1.07 mmol l⁻¹ and the pH 9.0, varied between 10.3 (10 mg l⁻¹ free chlorine) and 11.8 (200 mg l⁻¹ free chlorine) after the addition of chlorine, the corresponding pH values for the distilled water being 6.3, 10.6 and 12.0.

Survival in water. I inoculated 4 isolates of *Renibacterium salmoninarum* into separate 5 ml aliquots of river water or groundwater, using 0.1% peptone-saline broth as a control. The strains used were ATCC 33209 and 159, 213 and 516 isolated from rainbow trout *Oncorhynchus mykiss* at fish farms in northern Finland during 1999 and 2002. Isolates 159 and 213 were from the same farm. The inoculations were made in duplicate (Series 1 and 2) from 4 to 7 wk old KDM2 starter cultures. The turbidity in the test tubes was adjusted to 0.5 MacFarland units corresponding to an optical density of approximately 0.065 (492 nm), or a concentration of 10^6 ml⁻¹. The incubation temperature was 16°C ($\pm 1^\circ\text{C}$) throughout. The test tubes for Series 1 were subcultured weekly on KDM2 agar (Evelyn et al. 1990), and the plates were followed for up to 4 or 5 wk for bacterial growth, which was confirmed by the typical appearance of colonies on KDM2 agar, Gram-staining and in some cases also with a fluorescent antibody staining and API ZYM test strips (bioMérieux). To test the reproducibility of the culture method, the river water and groundwater tubes of Isolate 516 in Series 1 were subcultured onto 10 parallel KDM2 agar plates after 14 wk incubation. The optical density of the suspensions was measured at this point (492 nm), and a volume of 2.0 ml from each tube was centrifuged at $2200 \times g$ for 15 min at 4°C, and 50 μl of the sediment was spread onto 2 KDM2 agar plates.

After 20 wk incubation, the test tubes of Series 2 were mixed thoroughly, the optical density was measured (492 nm), and the suspensions were centrifuged at $2200 \times g$ for 15 min at 4°C. A 25 μl aliquot of the sediment was taken for Gram-staining, as well as 25 μl for hanging drop preparation and 100 μl for spreading onto 2 KDM2 agar plates. The hanging drop preparations were examined for bacteria and the degree of aggregation by phase-contrast microscopy (Dialux 22, Leitz Wetzlar), and the KDM2 cultures were examined for bacterial growth over a period of 5 wk.

The river water used was obtained from the mouth of the River Oulujoki and the groundwater from a bored well in NE Finland. Both water samples were autoclaved at 121°C for 15 min before use. The pH, hardness and chemical oxygen demand (COD_{Mn}) of the river water after autoclaving were 7.1, 0.16 mmol l⁻¹ and 13.4 mg l⁻¹, respectively, the corresponding values for the groundwater being 8.6, 0.42 mmol l⁻¹ and 1.1 mg l⁻¹.

RESULTS

Disinfection trial

All the subcultures spread directly from the test mixtures onto KDM2 agar plates were negative, regardless of the concentration of free chlorine and the contact time. After previous incubation in KDM2 broth, occasional growth of *Renibacterium salmoninarum* was detected in nearly all concentrations of free chlorine, indicating that small numbers of bacteria were still viable and able to recover to a culturable state after the disinfection process (Table 1). The white or creamy, smooth, round, raised, entire colonies were confirmed as *R. salmoninarum* if they were catalase-positive, oxidase-negative, unable to grow on TSA, and small Gram-positive rods (0.5 to 1 µm) were revealed by Gram-staining. Several colonies (1 to 6) were subcultured and examined from every plate showing bacterial growth.

The bactericidal effect of chlorine was weaker in the tank water than in the distilled water. Of the total 96 KDM2 broth tubes, 21 (22%) were positive for *Renibacterium salmoninarum* in subculture on either KDM2 or SKDM agar plates, representing 13% of those containing distilled water (6/48) and 31% of those with tank water (15/48). Of the total of 23 positive agar plates, 6 (26%) were subcultured from distilled water and 17 (74%) from tank water. The proportion of positive KDM2 broths in the tank water was approximately the same regardless of the concentration of free chlorine, varying between 4 and 10% (2 to 5/48) per concentration. The colony-forming units (CFUs) of *R. salmoninarum* per agar plate were low,

ranging between 1 and 20 and being 1 to 4 CFUs in most of the subcultures, except for one subculture with 50 colonies on both KDM2 and SKDM agar (Table 1).

The control cultures containing the bacterial suspension in distilled water or tank water had a semiconfluent growth of *Renibacterium salmoninarum* on KDM2 agar plates in all the subcultures performed either directly on KDM2 agar or after previous incubation in KDM2 broth. The growth of *R. salmoninarum* in the control cultures was detectable after 10 d incubation, while after the disinfection process it was delayed and in most cases was not detectable until 21 d after incubation, or not until after 5 wk incubation.

Survival in water

The results of Series 1 are shown in Tables 2 & 3. The number of colonies in subcultures performed from both river water and groundwater remained at the same level for the first 2 wk and then decreased 10-fold during the third week. After 4 wk incubation, all the subcultures were negative, but from 6 wk onwards there was good growth in the subcultures of ATCC 33209 in river water and occasionally also a light growth in subcultures of Isolate 516. Slight growth was also detected in groundwater for Isolate 159 after 5 wk, for ATCC 33209 after 6 wk and for Isolate 516 after 9 wk (Table 2). There were still viable cells of *Renibacterium salmoninarum* in the centrifuged sediments of both groundwater (ATCC 33209 and Isolate 516) and river water (Isolates 213 and 516) after 14 wk incubation (Table 3). All the subcultures of the peptone-saline broths gave abundant growth of *R. salmoninarum* up

Table 1. *Renibacterium salmoninarum*. Colony-forming units in 3 parallel KDM2 recovery broths subcultured on KDM2 and SKDM agar plates after contact with 4 concentrations of free chlorine in distilled water and in tank water. A volume of 0.1 ml of the broth was inoculated on each plate. Negative results are not included

Conc.	Distilled water						Tank water					
	Recovery broth 1		Recovery broth 2		Recovery broth 3		Recovery broth 1		Recovery broth 2		Recovery broth 3	
Contact time	KDM2	SKDM										
10 mg l ⁻¹												
15 min								1		6		
30 min								2		6		
24 h												15
50 mg l ⁻¹												
15 min				2					1	1		1
24 h		2		4			50	50		1		
100 mg l ⁻¹												
15 min								20		1		
200 mg l ⁻¹												
5 min			2									
15 min	1			20							2	
30 min							1		1			
24 h							3					

to 14 wk after incubation, the optical densities of the suspensions varying between 0.064 and 0.096. The density values showed no correlation with the colony counts.

To test the reproducibility of the subcultures, 10 parallel subcultures were performed from both the groundwater and river water tubes of Isolate 516 after 14 wk incubation. All the groundwater subcultures were negative, but growth was detected in river water subcultures on 8 KDM2 agar plates, 1 colony on each, with 80 CFUs ml⁻¹ in the river water tube for Isolate 516, which was consistent with the results of the centrifuged sediment of the same isolate, corresponding to 56 CFUs ml⁻¹. Examination of the centrifuged sediment from groundwater suspensions of Isolate 516

indicated that the number of colony forming units was approximately 1 CFU ml⁻¹.

All the subcultures were shown to be pure cultures of white or creamy, smooth, round, raised, entire colonies similar to *Renibacterium salmoninarum*. Gram-positive rods (0.5 to 1 µm) forming clusters, pairs and short rows were observed in the Gram-stains. Coccoid elements and gram-positive rods with Gram-negatively stained tapered ends could be seen in the subcultures made after 6 wk incubation, whereas after 7 wk incubation in both river water and peptone water, ATCC 33209 resulted in a form resembling coryneform bacteria with lengthened cells (1 to 2 µm) arranged at angles to give Y- and V-shaped formations and small branching rows. The fluorescent antibody test (FA) was used to stain 6 subcultures: ATCC 33209 in river water after 7, 13 and 14 wk incubation and Isolate 516 in groundwater and river water, and Isolate 213 in river water after 14 wk. All the FA smears were positive for *R. salmoninarum*, the bacteria in the stainings of Isolates 516 and 213 being mostly arranged in compact aggregates. Api Zym tests were performed on 5 subcultures: ATCC 33209 in river water after 7 and 14 wk incubation, Isolate 516 in river water and groundwater, and Isolate 213 in river water after 14 wk. The results were consistent with those for *R. salmoninarum*.

The samples in Series 2 were examined only once, after a storage period of 20 wk. Viable cells of *Renibacterium salmoninarum* were still to be found in all the samples representing river water subcultures (Table 3), the colony counts indicating CFUs 1740 ml⁻¹ for Isolate 516, 10 ml⁻¹ for Isolate 159, 40 ml⁻¹ for Isolate 213 and >2000 ml⁻¹ for ATCC 33209. There was no detectable growth in the groundwater subcultures except for ATCC 33209, but microscopically the bacteria appeared to be structurally intact and apparently viable. All the isolates in the peptone-saline subcultures had abundant growth. The bacteria in the hanging drop preparations were mainly arranged in tight aggregates of various sizes, and some preparations contained crystals to which the bacteria had adhered in tightly packed layers. Gram staining of the sediments showed the bacteria to be clumped tightly together.

DISCUSSION

Each concentration of sodium hypochlorite tested effectively reduced the numbers of culturable cells of *Renibacterium salmoninarum*. The direct cultures from the hypochlorite mixtures on

Table 2. *Renibacterium salmoninarum*. Survival of 4 isolates (ATCC 33209, 516, 159, 213) in river water and groundwater at 16°C, assessed in terms of colony-forming units in subcultures on KDM2 agar inoculated with 0.01 ml water. nd: not done

Incubation time in water (wk)	River water				Groundwater			
	ATCC 33209	516	159	213	ATCC 33209	516	159	213
1	70	30	>200	20	18	17	>200	42
2	60	70	>200	20	30	30	>200	3
3	7	13	>100	2	0	3	100	0
4	0	0	0	0	0	0	0	0
5	0	0	0	0	0	0	2	0
6	11	10	0	0	1	0	0	0
7	16	0	0	0	0	0	0	0
8	nd	nd	nd	nd	nd	nd	nd	nd
9	6	1	0	0	0	1	0	0
10	30	0	0	0	0	0	0	0
11	25	3	0	0	0	0	0	0
12	46	0	0	0	0	0	0	0
13	72	0	0	0	0	0	0	0

Table 3. *Renibacterium salmoninarum*. Survival of 4 isolates (ATCC 33209, 516, 159, 213) after 14 wk (Series 1) and 20 wk (Series 2) incubation in river water and groundwater at 16°C, assessed in terms of colony-forming units on 2 parallel KDM2 agar plates inoculated with 0.05 ml of centrifuged water sediment. nd = centrifugation not done; growth of *R. salmoninarum* in weekly subcultures detected for 30 wk

Isolate	Incubation time (wk)	River water		Groundwater	
		KDM2 a	KDM2 b	KDM2 a	KDM2 b
ATCC 33209	14	nd	nd	1	0
	20	>200	>200	>200	>200
516	14	55	58	1	1
	20	165	182	0	0
159	14	0	0	0	0
	20	1	1	0	0
213	14	1	1	0	0
	20	3	4	0	0

KDM2 agar plates showed no growth, but incubation in the recovery broth suggested that there were small numbers of injured but still viable cells of *R. salmoninarum* at all concentrations of free chlorine, even at 200 mg l⁻¹, the highest concentration recommended for eradication of infectious microorganisms at fish farms (Office International des Epizooties 2002). The number of positive subcultures was greater in tank water than in distilled water, presumably due to the lowered activity of free chlorine in water containing organic material, and the length of contact with hypochlorite solution did not seem to influence the recovery rate. Pascho et al. (1995) reported that the chlorine exposure time necessary for a 99% reduction in the *R. salmoninarum* count at 15°C and pH 7.0 does not exceed 18 s even when the concentration of free chlorine is as low as 0.05 mg l⁻¹. On the other hand, they cultured their subsamples directly from the chlorine treatments on charcoal agar plates after neutralising the chlorine by adding sodium thiosulphate, without a recovery period in broth. Their survival curves for *R. salmoninarum* also exhibited a tailing-off effect, which the authors attributed to the formation of bacterial aggregates that may protect the bacteria from contact with chlorine. This arrangement of the bacteria in compact aggregates could be one explanation of the present results. Pascho & Ongerth (2000) also compared the culture method with a 2-colour flow cytometric assay as a means of quantifying the proportions of viable and dead bacterial cells in a *R. salmoninarum* population, and reported the rate of inactivation by chlorine to be much faster based on bacterial culture than based on the results of a flow cytometry assay, concluding that the cellular functions necessary for *R. salmoninarum* to replicate and form colonies on agar plates may be much more sensitive to chlorination than are the biophysiological characteristics associated with the properties used to distinguish between live and dead bacteria in flow cytometry.

According to Pascho et al. (1995), the inactivation of *Renibacterium salmoninarum* was slower at pH 8 (measured before the addition of chlorine) than at pH 6.0 or 7.0, with a shoulder appearing in the survival curve, indicating that 99% inactivation could not be achieved at this pH. The pH of the tank water in the current study was 9.0. The pH values of the distilled water and tank water were similar after adding the chlorine, however, ranging between 8 and 12 depending on the concentration of free chlorine. Hypochlorite is a more active disinfectant at acidic than at alkaline pH (Russell & Hugo 1987), and the high pH values that prevailed here may have reduced the disinfection capacity of chlorine.

The age of the bacterial culture used in the disinfection test affects the susceptibility of the organism to

adverse environmental conditions, bacterial cells at the early exponential growth phase tending to be more susceptible to chemical agents than older ones (Quinn 1987). Pascho et al. (1995) obtained their results using a 7 to 10 d old culture, while we intentionally chose to use older culture of 25 d because we believed that these would correspond better to the state of the bacteria in nature. Several subcultures performed after rehydration of the freeze-dried ATCC strain may also have influenced the results.

Our results showed that *Renibacterium salmoninarum* may survive in water longer than has been reported previously. After the remarkable decrease in the bacterial population during the first 3 wk in water, a long tailing period with a small number of viable bacteria was detected that continued to the end of the trial. The cultures obtained after 14 wk (Series 1) and 20 wk (Series 2) comprised several colonies, indicating an ability to adapt to survival in river water. The general absence of colonies after 4 wk incubation may have been due to the small volume of water used for subcultures. The tendency to form more coccoid cells may indicate starvation of the bacteria (Amy et al. 1983, Thorsen et al. 1992, Magariños et al. 1994).

The significant decrease in the population of *Renibacterium salmoninarum* during the first weeks of incubation in water is consistent with previous reports (Austin & Rayment 1985, Evelyn 1988, Balfry et al. 1996), but the tailing effect has not been detected earlier, probably because of the short duration of the previous experiments. When Austin & Rayment (1985) infected rainbow trout experimentally with *R. salmoninarum* and examined the water and sediments in the fish tank, the faeces and sediment contained low numbers of the bacteria, but there was no evidence of surviving viable bacterial cells beyond 21 d after the last fish had died. They also showed that the number of *R. salmoninarum* ATCC 33209 cells had declined to an undetectable level within 4 d at 15°C in sterile water, and that the bacterial population was completely undetectable between 28 and 35 d of incubation in filter-sterilised river water. Evelyn (1988) examined the survival of *R. salmoninarum* cells in filter-sterilised seawater and hard and soft fresh water for 16 d, while Balfry et al. (1996) examined their survival in seawater, filter-sterilised seawater and physiological saline at 10°C. Viable *R. salmoninarum* bacteria were detected for up to 1 wk in seawater and up to 14 d in filter-sterilised seawater, but only for up to 2 d in saline.

The relevance of the tailing effect in bacterial growth demonstrated here, with a small number of bacterial cells remaining viable in both the disinfection trial and the survival experiments, is difficult to determine with regard to horizontal transmission of the disease, because the minimal infective dose of *Renibac-*

terium salmoninarum is not known (Evenden et al. 1993). In experimental infections, where low doses of *R. salmoninarum* have been used, either no effect on mortality and no pathological changes in the kidney tissue have been detected (O'Farrell et al. 2000), or else clinical signs of BKD have appeared but at a slower rate than after a high dose (Shortt et al. 1988). In some cases mortality and detectable infection have developed and differences in susceptibilities between fish strains have been noted (Olivier et al. 1992). The capacity of *R. salmoninarum* to survive in water for longer periods than suggested previously may be part of its survival strategy in nature, a finding which could offer an explanation for the horizontal transmission of the disease between fish populations through water.

There were differences between the bacterial isolates in their ability to survive in water. Isolates 516 and ATCC 33209 adapted to river water, while Isolates 159 and 213 were not detected in weekly subcultures after 4 wk incubation, even though low numbers were detected after centrifugation of the water. The incubation time of the *Renibacterium salmoninarum* ATCC 33209 culture on KDM2 agar was 7 wk at the time of inoculation, that of Isolate 516 was 5 wk, and those of Isolates 159 and 213 were 4 wk. Austin & Rayment (1985) used a 10 d culture of *R. salmoninarum* ATCC 33209, while Balfry et al. (1996) tested an 18 d culture of a *R. salmoninarum* isolate of their own. It is possible that older cultures of *R. salmoninarum* may have properties that provide them with better resistance to environmental stress factors than younger ones. The cultural history of the bacteria may also affect the results. The *R. salmoninarum* ATCC 33209 used in the present survival trial had been subcultured in the laboratory several times, and it is uncertain whether the results obtained here are relevant to natural conditions. The other isolates had been kept at -70°C , however, and subcultured only once (159 and 213) or twice (516) before the trial. The weaker growth of Isolates 159 and 213 in water could also be explained by insufficient recovery after the stress induced by freezing.

The characteristics of the river water used here were similar to those usually detected in river water in Finland. The pH was 7.1 (average for Finnish rivers is pH 6.7), chemical oxygen demand 13.4 mg l^{-1} (range 10 to 20 mg l^{-1}) and water hardness 0.16 mmol l^{-1} (average 0.18 mmol l^{-1}) (Särkkä 1996). The temperature of river water may be as low as 0.1 to 3°C for long periods in winter, and water temperatures in the River Kemijoki (with one of the largest drainage basins in Finland) are reported to vary between 0.1 and 22.3°C over the year, being higher than 4°C for an average of 154 d yr^{-1} . *Renibacterium salmoninarum* grows very slowly on artificial media at 5°C , well at 15 to 18°C , only very slightly at 22°C and not at all at 37°C (Smith 1964,

Sanders & Fryer 1980). Nevertheless the survival of the bacterium at low temperatures needs more clarification for epidemiological purposes.

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