Detecting *Renibacterium salmoninarum* in wild brown trout by use of multiple organ samples and diagnostic methods

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Abstract
Renibacterium salmoninarum, the causative agent of salmonid bacterial kidney disease (BKD), is endemic in many wild trout species in northerly regions. The aim of the present study was to determine the optimal *R. salmoninarum* sampling/testing strategy for wild brown trout (*Salmo trutta* L.) populations in Iceland. Fish were netted in a lake and multiple organs—kidney, spleen, gills, oesophagus and mid-gut—were sampled and subjected to five detection tests i.e. culture, polyclonal enzyme-linked immunosorbent assay (pELISA) and three different PCR tests. The results showed that each fish had encountered *R. salmoninarum* but there were marked differences between results obtained depending on organ and test. The bacterium was not cultured from any kidney sample while all kidney samples were positive by pELISA. At least one organ from 92.9% of the fish tested positive by PCR. The results demonstrated that the choice of tissue and diagnostic method can dramatically influence the outcome of *R. salmoninarum* surveys.

Introduction

Renibacterium salmoninarum is a Gram-positive facultative intracellular pathogen that can cause bacterial kidney disease (BKD) in susceptible fish (Fryer and Sanders, 1981). Susceptible species are members of three subfamilies of *Salmonidae*, i.e. *Thymallinae* and *Coregoninae* (Wiens and Kaattari, 2006). The bacterium is transmitted horizontally and vertically and has been reported in wild and cultured fish in many parts of the world (Wiens and Kaattari, 2006). In the wild, the bacterium is recognized as an integral component of the ecosystem, and its presence in fish may be influenced by a number of biological and ecological processes (Souter et al., 1987; Jónsdóttir et al., 1998; Chambers et al., 2008).

The three native species of salmonids in Iceland are Atlantic salmon, *Salmo salar* (L.), Arctic charr, *Salvelinus alpinus* (L.) and brown trout, *Salmo trutta* (L.). The latter two species occur as anadromous and non-anadromous populations. BKD was first reported in Iceland in 1968 (Helgason, 1985) and screening for *R. salmoninarum* in kidney samples of salmonids has been carried out since 1986 (Guðmundsdóttir et al., 2000;

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Kristmundsson et al., 2009). This applies to every wild female brood fish used for restocking and eggs from positive females are destroyed. Testing of subsamples from cultured fish is also mandatory. Surveys in wild populations have also been conducted (Jónsdóttir et al., 1998; Kristmundsson et al., 2016).

R. salmoninarum is endemic in wild salmonid populations in Iceland as demonstrated in a wide-ranging survey (1993-1995) where Arctic charr and brown trout were sampled in 22 lakes and tested for presence of *R. salmoninarum* antigens by a pELISA test (Jónsdóttir et al., 1998). Prevalence within the lakes ranged from 3 to 100% for Arctic charr and 6 to 81% for brown trout. Most positive fish had low antigen levels in their kidneys. The wide distribution and high prevalence suggest that the bacterium has been endemic in Iceland for a long time. Similar results have been obtained elsewhere in wild salmonid populations (Souter et al., 1987; Elliott et al., 1997; Meyers et al., 1999, 2003; Chambers et al., 2008; Faisal et al., 2012). Clinical signs of BKD have never been reported in wild salmonids in Iceland but a few wild Atlantic salmon brood fish have developed signs after being held in captivity (Kristmundsson et al., 2009).

Lake Ellidavatn, a shallow lake in SW-Iceland, is sustained by two inflowing rivers as well as spring water; there is one outflowing river, River Ellidaár (Malmquist et al., 2009; Kristmundsson et al., 2016). This lake was included in the 1993-1995 survey and overall prevalence obtained using pELISA was 13% for brown trout and 16% for Arctic charr (Jónsdóttir et al., 1998). A pronounced rise in the percentage of *R. salmoninarum*-positive Atlantic salmon brood fish in the outflowing river was observed in autumn 2006 (Kristmundsson et al., 2009). This finding prompted additional surveys in the water system including Lake Ellidavatn. The prevalence for 2008 was 98% for brown trout and 89% for Arctic charr in the lake by pELISA (Kristmundsson et al., 2016). The kidney samples were later tested using a nested PCR (Chase and Pascho, 1998) and only 5 % of the brown trout samples and 11% of Arctic charr samples were positive (Árnason, 2010). This discrepancy between results for different detection methods motivated the current study.

The main aim was to determine the most revealing sampling/testing strategy for a wild population. In autumn 2009, brown trout were netted in the lake and samples gathered from five organs, i.e. kidney, spleen, gills, oesophagus and mid-gut. All samples were tested using three PCR methods. Kidney samples were also tested by pELISA and culture.

Materials and methods

Capture and sampling

In August 2009, brown trout were sampled using a series of gillnets made of monofilament nylon with mesh sizes 12 to 50 mm (knot-to-knot). After 15 h the fish were harvested and placed on ice. They were immediately weighed, length measured, a macroscopic assessment was made and otoliths were sampled for age determination. Necropsy was done to look for pathological changes and the organs were sampled.

Head- and mid-kidney samples for pELISA and culture were placed in sterile stomacher bags. The former were frozen at -20°C but the latter processed immediately (see below). Organ samples for DNA isolation were placed

in RNAlater (Ambion, Texas, USA), kept at 4°C overnight and stored at -20°C until processing a few weeks later.

Culture

Samples were homogenised, washed and re-suspended before inoculation as described by Evelyn (Evelyn, 1977) with slight modification (Benediktsdóttir et al., 1991). After inoculation the selective kidney disease medium (SKDM) agar plates were incubated at 16°C, and assessed weekly for 12 weeks.

Polyclonal ELISA

The pELISA detects *R. salmoninarum* antigens in supernatants of heat-treated kidney samples (Gudmundsdottir et al., 1993). The cut-off value for determination of positive samples was 2.3 times the average OD_{492} value of three negative control samples.

DNA extraction

For DNA extraction from head-kidney, spleen, oesophagus, gut and gills, a DNeasy kit (Qiagen Inc.) was used as instructed by the manufacturer for Gram-positive bacteria. DNA was eluted from a 25 mg sample with 400 µL of the assigned buffer. Negative samples were created for each batch by processing reagents only. The same amount of DNA template, 5 µL, was used in all three PCR tests.

Nested PCR

The protocol for nested PCR (nPCR) (Chase and Pascho, 1998) was used with some modifications (Elliott et al., 2013). Briefly, the final concentration of 20 pM was used for all four primers. The DNA template was 5 µL in the first reaction and 1 µL in the second. For both reactions, thermal cycling started with a denaturation step at 94°C for 2 min, followed by 30 cycles for the first, and 20 cycles for the second round reactions. In each cycle there were three steps, denaturation at 94°C for 30 sec, annealing at 60°C for 30 sec and extension at 72°C for 1 min.

Real-time quantitative PCR #1

This test detects a 69-bp region from the *R. salmoninarum msa* gene (Chase et al., 2006). The primers and probe were selected from a different region of the *msa* gene than the primers in the nPCR test described above. In the current study a modified probe was used (Elliott et al., 2013). The total volume of the reaction mixture was 12 µl, with 0.9 µM of forward and reverse primers, 0.25 µM of the probe and 5 µl of DNA template. Thermal cycling and analysis were done in an ABI 7900HT instrument (Applied Biosystems Inc.).

Real-time quantitative PCR #2

This assay detects a 95-bp sequence of the *msa* gene that is located between the sequences of the primers used in the first-round of the nPCR described above (Powell et al., 2005).

Statistics

A general linear model was used to test the relationship between pELISA OD (dependent variable) and number of organs PCR-positive (explanatory factor) (PASW V.18, IBM). Each PCR assay was considered separately but the final analysis combined results for all three PCR tests (e.g. number of organs positive by any PCR test). $P < 0.05$ was considered significant.

Results

Otolith examination of the 11 females and 17 males showed an age range of 5-8 years, a mean weight of 297 g (range 100-1175 g) and mean

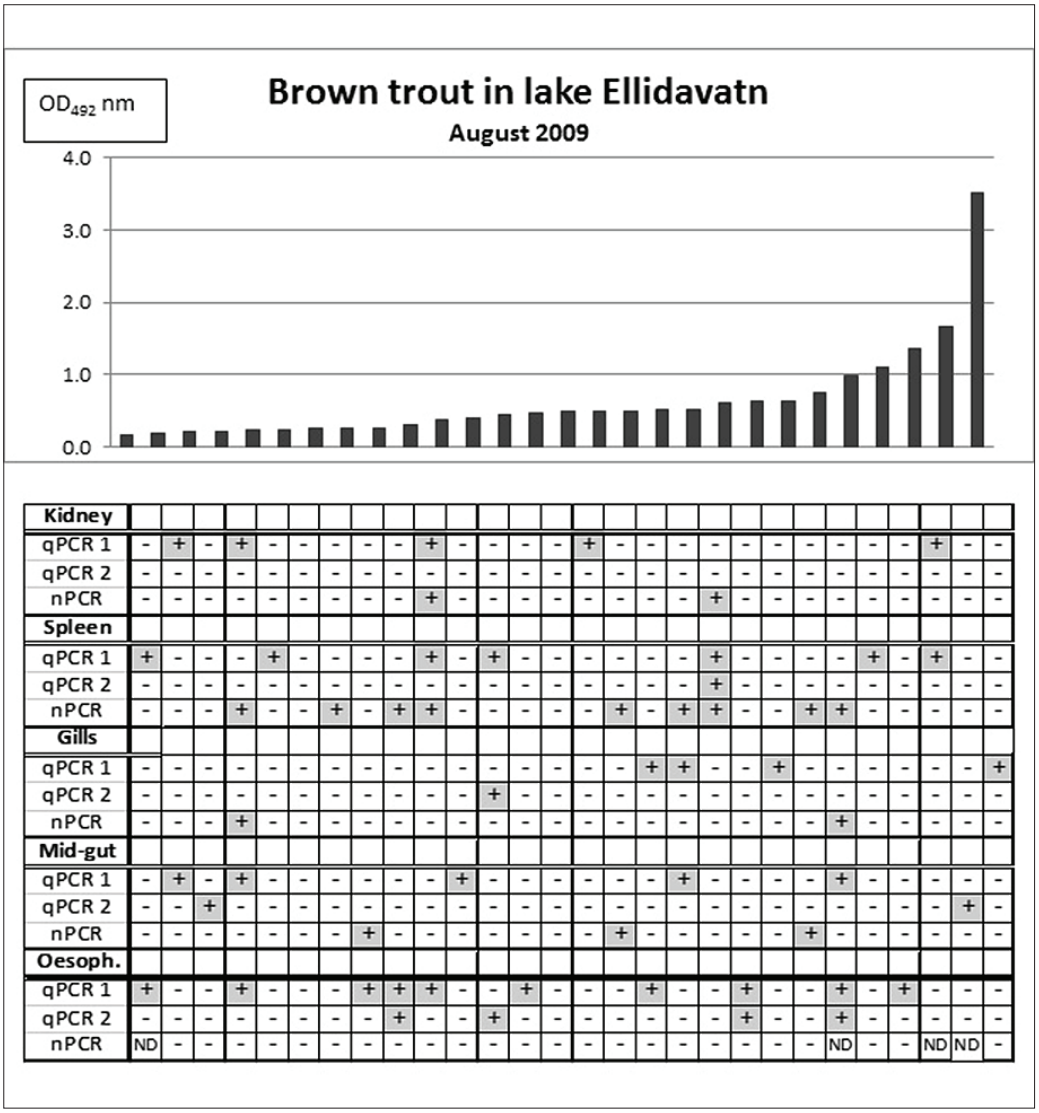
length of 27 cm (range 18-45 cm). External and internal clinical signs of BKD were not observed. Two fish had slightly swollen kidneys and one fish had a small whitish nodule in the spleen.

The combined results revealed a complex picture. The bacterium was not cultivated from any kidney sample but all kidney samples showed positive pELISA OD readings distributed over a wide range (Figure 1). The lowest reading obtained was 0.167, the highest 3.515 and the mean was 0.632. Twenty-six fish (92.9%) tested PCR-positive in at least one organ by one or more PCR tests, one fish had all organs test positive and two fish tested negative in all organs (Figure 1). The highest number of positive results, when all PCR tests for a single organ were included, was 14 (50%) for spleen samples, followed by 11 (39.3%) for oesophagus, 10 (35.7%) for mid-gut, 7 (25.0%) for gills and 6 (21.4%) for head-kidney. There was no significant relation between the number of tissues positive by any PCR test and pELISA OD values (P = 0.72); similar non-significant results were obtained when each PCR assay was compared separately to pELISA OD (P > 0.05 for all comparisons). The highest number of positives for a single PCR test for a given organ was 10 (35.7%) for oesophagus samples tested by qPCR #1, followed by 9 (32.1%) spleen samples positive by nPCR and 4 (21.4%) oesophagus samples positive by qPCR #2 (Table 1). All samples positive by the qPCR methods displayed high cycle threshold (C_T) values, i.e. 32.21-37.99 for qPCR #1, which equates to 617 (kidney) to 59,036 (mid-gut) bacteria g^{-1} tissue. For qPCR #2, C_T ranged from 33.80-36.23 equating to 16,300 (gill) to 88,931 (mid-gut) bacteria g^{-1} tissue (Table 1).

Discussion

The test results indicated that all fish in the study had encountered *R. salmoninarum* but different methods and samples gave dissimilar pictures regarding the status of the infection. Definitive clinical BKD signs were not observed; slightly swollen kidneys in two fish may have been due to proliferative kidney disease (PKD), demonstrated for the first time in Lake Ellidavatn a few years earlier (Kristmundsson et al., 2010). The original intent of the current study was to include Arctic charr, but the sampling yielded nearly all brown trout. In fact, the Arctic charr population in Lake Ellidavatn has declined substantially over the last 15-20 years, a situation that most probably is linked to a rise in temperature (Malmquist et al., 2009) followed by an escalating PKD epidemic. It is suspected that the PKD epidemic has increased the prevalence of *R. salmoninarum* infections in both Arctic charr and brown trout populations in the lake but so far the dual-infections do not seem to affect the survival of brown trout (Kristmundsson et al., 2016).

Negative culture results may have several explanations: the bacterium is not present, it is present in very low numbers, it is unevenly distributed in the sample or it is in a non-culturable state (Faisal and Eissa, 2009; Nance et al., 2010). All of these explanations may be applicable to the current study findings. In this context it is noteworthy that *R. salmoninarum* DNA was detected in kidney samples from 6 of 28 fish and that qPCR results indicated low numbers of bacteria in these samples. While culture was used for standardised quantification of *R. salmoninarum* to evaluate analytical performance of non-culture assays in laboratory studies (Elliott et al., 2013), reliable culture of the bacterium from sub-clinically infected fish in field surveys



Kidney																											
qPCR 1	-	+	-	+	-	-	-	-	-	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+	-	-
qPCR 2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
nPCR	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
Spleen																											
qPCR 1	+	-	-	-	+	-	-	-	-	+	-	+	-	-	-	-	-	-	+	-	-	-	-	+	+	-	-
qPCR 2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-
nPCR	-	-	-	+	-	-	+	-	+	+	-	-	-	-	-	+	-	+	+	-	-	+	+	-	-	-	-
Gills																											
qPCR 1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	+	-	-	-	-	-	-	+
qPCR 2	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
nPCR	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
Mid-gut																											
qPCR 1	-	+	-	+	-	-	-	-	-	-	+	-	-	-	-	-	-	+	-	-	-	-	+	-	-	-	-
qPCR 2	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
nPCR	-	-	-	-	-	-	-	+	-	-	-	-	-	-	+	-	-	-	-	+	-	-	-	-	-	-	-
Oesoph.																											
qPCR 1	+	-	-	+	-	-	-	+	+	+	-	-	+	-	-	-	+	-	-	+	-	-	+	-	+	-	-
qPCR 2	-	-	-	-	-	-	-	-	+	-	-	+	-	-	-	-	-	-	+	-	-	-	+	-	-	-	-
nPCR	ND	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	ND	-	-	ND	ND	-	

Figure 1. The columns on the chart represent OD_{492} nm values in pELISA for kidney tissue samples where each column represents one fish. All kidney samples were above the positive – negative cut-off value defined as 2.3 times the average OD_{492} value of three negative control samples. PCR results, i.e. from two qPCRs and an nPCR for every individual (N=28) are shown in the table below. Positive samples are marked with "+", negative with "-" and ND means not done.

can be difficult to achieve (Pascho et al., 2002). Using culture as the sole survey method in the present study would have led to the conclusion that *R. salmoninarum* prevalence in the brown trout population was nil.

Measuring *R. salmoninarum* antigens in kidney samples is the method most widely used in Iceland to assess infection status in a population. Comparison of current results to pELISA results obtained 1993-1995 using the same method (Jónsdóttir et al., 1998) shows a pronounced change in *R. salmoninarum* antigen prevalence and levels in the brown trout population in Lake Ellidavatn. In the 1993-1995 study the prevalence was 13%, mean OD was 0.029 and the highest reading was 0.245 while in the current study (2009), prevalence was 100%, mean ELISA OD was 0.632 and the highest reading was 3.515. It is unknown whether this

Table 1. Prevalence, calculated from results of different detection methods applied to samples from five different organs of 28 fish. SKDM-2: culture on selective agar; pELISA: polyclonal ELISA that detects antigens of the bacterium; qPCR: quantitative PCR; nPCR: nested PCR. N=28 fish tested[1].

Organ	Method	No. Positive	Prevalence %	Range of DNA copies g^{-1} tissue[2]
Kidney	SKDM-2	0	0.0	
	pELISA	28	100.0	
	qPCR#1	5	17.9	617 - 20491
	qPCR#2	0	0.0	
	nPCR	2	7.1	
Spleen	qPCR#1	7	25.0	5154 - 36810
	qPCR#2	1	3.6	35096
	nPCR	9	32.1	
Gills	qPCR#1	4	14.3	7195 - 36468
	qPCR#2	1	3.6	16300
	nPCR	2	7.1	
Mid-gut	qPCR#1	5	17.9	5171 - 59036
	qPCR#2	2	7.1	63456 - 88931
	nPCR	3	10.7	
Oesophagus	qPCR#1	10	35.7	2252 - 19109
	qPCR#2	4	14.3	30529 - 51957
	nPCR[1]	0	0.0	

[1] Only 24 of the 28 oesophagus DNA samples were tested by nPCR.
[2] Copy number of positive samples only; range represents minimum and maximum values.

change was sudden or gradual as there are no data for the lake between these time points except for 2008 when results of screening with ELISA were similar to the results obtained in the present study (Kristmundsson et al., 2016). The wide range of ELISA readings observed may mirror different stages of infection and/or the length of time passing since the infection was initiated (Faisal and Eissa, 2009, Nance et al., 2010). Similar findings were reported from a study including eight nonanadromous trout species in Alaskan watersheds where the annual prevalence values obtained in 1988-2000 ranged from 18.1% to 100.0% (Meyers et al., 2003).

The combined PCR and pELISA results confirmed that more than 90% of the fish harboured both antigen and DNA suggesting either a recent exposure to *R. salmoninarum* or presence of intact (and perhaps viable) bacteria.

While studies have demonstrated that antigen can persist in host tissues for months in the absence of viable *R. salmoninarum* infections, DNA likely only persists on the order of days to weeks after the bacterium is killed (Pascho et al., 2002).

An association between PCR results and ELISA readings was not deduced. These results differ from those recently described for farmed Atlantic salmon brood fish sampled during an escalating infection where the number of positive PCR results in head-kidney increased with rising ELISA OD readings (Arnason et al., 2013). The differences observed may reflect higher BKD susceptibility of Atlantic salmon compared to brown trout (Elliott et al., 2014) resulting in higher bacterial loads in kidney tissues of the Atlantic salmon. The qPCR results from the current study indicated that most positive samples contained *R. salmoninarum* DNA concentrations near the lower limits of detection for the assays, suggesting that stochastic amplification of bacterial DNA contributed to lack of agreement among PCR tests (Elliott et al., 2013). The ability of ELISA to detect *R. salmoninarum* antigens that circulate into the kidney from infections in non-sampled tissues can also contribute to lack of concordance between ELISA and PCR results (Elliott et al., 2013).

Screening for *R. salmoninarum* is used to select negative or low positive parents in breeding and restocking programs. Brood stock screening programs have been very successful for controlling vertical transmission (Elliott, in press). It seems reasonable that detection of bacterial DNA in samples such as kidney and spleen, where blood-borne infection is evident, reflect the status of infection more accurately than results from oesophagus, mid-gut or gills. Based on the results presented here for brown trout in Iceland, the recommendation would be to sample spleen for PCR testing.

Oesophagus and mid-gut were included in the present study to test parts of the digestive system for the presence of *R. salmoninarum* DNA, since the fecal-oral route contributes to horizontal transmission (Balfry et al., 1996). Although positive PCR results were obtained from this testing, it could not be ascertained that all *R. salmoninarum* DNA detected was from bacteria in the tissues rather than attached to the surface of the organs. Nevertheless, about half of fish in which bacterial DNA was detected in oesophagus or mid-gut also presented with positive results in spleen and/or head-kidney. The interest in using gills for *R. salmoninarum* screening was based on their potential utility as non-lethal samples (Elliott et al. 2015). In the population studied results did not indicate the gills as an organ of choice. The question of the bacterium being "in or on" the tissue is also valid for gills but positive gill samples suggest recent exposure to *R. salmoninarum*. Tests of potential non-lethal samples for *R. salmoninarum* detection in experimentally infected Chinook salmon (*Oncorhynchus tshawytscha*) identified mucus scrapings as the best alternative candidate to lethal kidney sampling in comparison to gill snips and fin clips (Elliott et al., 2015).

In summary, although our results indicated that every fish tested had encountered *R. salmoninarum*, they did not reveal whether the fish harboured viable bacteria. These results also supported those of earlier research indicating a substantial increase in prevalence and levels of *R. salmoninarum* antigens in Lake Ellidavatn

brown trout between 1993 and 2008/2009. In our survey prevalence by culture was nil in contrast to 100% by pELISA and 92.9% by combined results from testing five organs by three PCR methods. However, if only a single organ or PCR test had been used, the observed prevalence would have been much lower. Whether we could expect this level of test discrepancy in all trout populations is uncertain, but clearly the choice of organs and detection methods can influence the outcome of surveys conducted in wild populations.

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