RESEARCH ARTICLE

Non-lethal sampling for the detection of *Renibacterium salmoninarum* by qPCR for diagnosis of bacterial kidney disease

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Abstract

Bacterial kidney disease (BKD), caused by *Renibacterium salmoninarum* (Rs), can be transmitted both horizontally and vertically and there is no available cure or prophylaxis. The control of BKD requires continuous surveillance, which is challenging in aquaculture as well as in programs for conservation and restoration of salmonid fish strains. BKD is a notifiable disease in Sweden and is monitored through the mandatory health control program using a polyclonal ELISA for detection of the Rs p57 protein in kidney. Fish must be killed for sampling, an obvious disadvantage especially regarding valuable broodfish. The present study shows that gill-/cloacal swabs collected in vivo for real-time PCR ($qPCR_{gc}$), allow a sensitive and specific detection of Rs. The sensitivity of $qPCR_{gc}$ was estimated to 97.8% (credible interval (ci) 93.8%–100%) compared to 98.3% (ci 92.7%–100%) and 48.8% (ci 38.8%–58.8%) of kidney samples for qPCR ($qPCR_k$) and ELISA ($ELISA_k$) respectively, by use of the Bayesian Latent Class Analysis (BLCA). Since the goal of the program is eradication of BKD the most sensitive test is preferrable. Using $qPCR_{gc}$ instead of $ELISA_k$ will result in a lower false negative rate and can be useful for surveillance in aquaculture and in breeding programs with valuable fish. However, a higher false positive rate warrants confirmatory lethal testing before a previously Rs negative farm is subject to restrictions.

KEYWORDS
Bayesian Latent Class Analysis, BKD, diagnostics, non-lethal sampling, *Renibacterium salmoninarum*

1 | INTRODUCTION

The chronic progressive bacterial kidney disease (BKD) can cause severe morbidity and mortalities in salmonid fish and acute disease outbreaks occasionally occur. Anorexia, abdominal distension, loss of balance, exophthalmia, pale gills and haemorrhaging skin lesions are examples of externally visible signs of BKD. Advanced cases show extensive internal lesions such as a swollen kidney with granulomas, which may also appear in the heart, liver and spleen, an accumulation of ascites, congestion, and splenomegaly. Chronic stages of BKD occur with minor external pathological signs and there are also asymptomatic carriers without any clinical signs of disease (Austin & Rayment, 1985). Despite a lack of pathological changes, asymptomatic carriers can transmit the infection (Fryer & Sanders, 1981).

Transmission occurs both horizontally, through direct contact and through the water (Mitchum & Sherman, 1981), and vertically from infected females to their offspring (Evelyn et al., 1986). There are no commercial vaccines or cures available. The possibility of control depends on an active surveillance of the causative agent, the Gram-positive *Renibacterium salmoninarum* (Rs), combined with transport restrictions and slaughter of fish on infected farms. Destruction of eggs from infected females is used to prevent vertical transmission in restocking broodstock farms. Immunological or molecular diagnostic techniques are commonly used for routine diagnostics and in screening programs (Chambers et al., 2008; Kristmundsson et al., 2016; Suzuki et al., 2017) as Rs is slow-growing and has special medium requirements that hampers in vitro cultivation.

BKD occurs in salmonid wild and farmed populations in America, Asia and in most countries of Europe, in fresh as well as in marine waters. Australia and New Zealand belongs to the few countries where BKD has not been encountered. Epizootics with high mortalities in wild Chinook salmon (*Oncorhynchus tshawytscha*) and brook trout (*Salvelinus fontinalis*) have been reported from wild stocks in America (Eissa et al., 2007; Holey et al., 1998) and clinical disease observed occasionally in wild salmonid stocks collected for artificial fertilization and breeding in Iceland (Guðmundsdóttir et al., 2017) Wild white-fish (*Coregonus* sp.) and grayling (*Thymallus thymallus*), sampled in a Swedish river where Rs infected fish farms are located, have recently tested positive for Rs (Persson et al., 2022). Elliott et al. (1995) demonstrated impaired health and higher mortality in progeny of Rs positive females, compared to progeny of females with low Rs levels, an effect observed up to 21 months after hatching. Rs infection was found to be associated with reduced growth of juvenile Chinook salmon and this was suggested to affect the regulation of these populations in the Northeast Pacific Ocean as growth during the first year at sea has been linked to survival (Sandell et al., 2015). Thus, ethical issues in the context of fish welfare in aquaculture and the success of breeding programs advocates that the disease must be monitored and combatted.

BKD was first diagnosed in Sweden in 1985, probably introduced by import of whole, frozen fish for processing before human consumption and/or of eggs for fish production. During 1985–2003, clinical BKD or Rs infection without clinical signs was diagnosed in 70 fish farms, several belonging to the same company. The mode of transmission was investigated, and sanitation plans prepared for each case. Transport of live fish between farms was found to be the main factor for spread of the disease. There was no evidence that the infection originated from feral fish (Wichardt, 2004). After an initially high incidence with up to 26 annual index cases, the number of cases has declined to none or up to a few annual index cases (Figure 1). In the last ten years, cases have mostly occurred as re-infections in farms recently diagnosed and sanitized and the goal of eradication has not been reached yet. One reason can be that infected cage farms have been allowed to keep the fish for one to a few years until slaughter weight is reached, and the infection has become manifested in wild fish in the area. It is also possible that low levels of Rs have been maintained in brood stock, resulting in occasional transmissions of the infection to the progeny.

To protect wild salmonids, screening of Rs was initiated in the early 1990´s. The screening is based on samples from 30 fish from each sampled farm, according to the Swedish regulation on mandatory health control of farmed fish (SJVFS 1994:94). This program was further supported by an eradication program approved through additional guarantees by the EU in 2004. The guarantees ended in 2021 but has been renewed as national measures under Article 226 in the Animal health law (EU) 2016/429 until 2027. The Swedish government has decided that surveillance of Rs infection shall continue to be mandatory when our guarantees/national measures end, and national regulations are currently under development.

Initially, Swedish Rs screening was based on necropsies and bacterial cultivation from kidney on selective kidney disease medium (SKDM) agar (Austin et al., 1983; Benedictsdóttir et al., 1991). Since 1994, kidney samples are tested by a polyclonal ELISA detecting the p57 protein from Rs (Jansson et al., 1996) and, since 2008, positive results are confirmed by qPCR, identifying the 16S rRNA gene of Rs (Jansson et al., 2008). In addition to the mandatory health control program, kidney samples for ELISA are collected after stripping of roe in all wild brood stock females used for artificial fertilization in the national restocking program for salmonids. In small local sea trout or brown trout strains, each female is extremely genetically valuable. Thus, to allow repeated spawning, ovarian fluid is collected for ELISA although the analytic sensitivity is lower for ovarian fluid than for kidney tissue (Arnason et al., 2013; Pascho et al., 1991). Eggs from ELISA positive females are destroyed. Brood stock of farmed fish is, with a
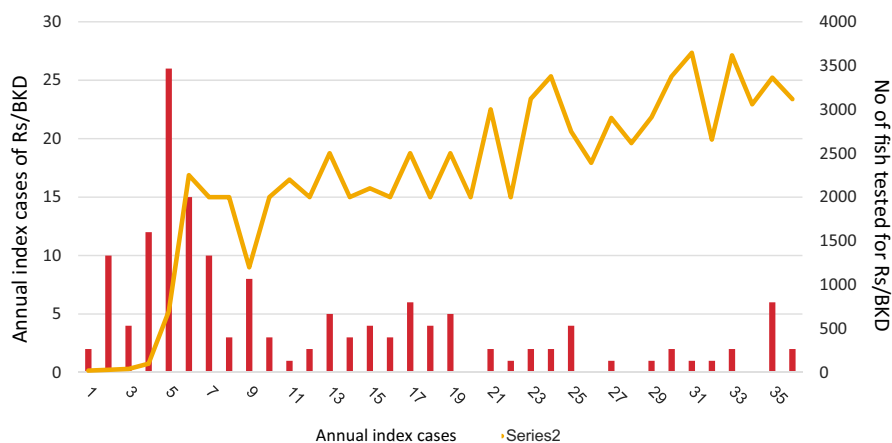

FIGURE 1 Annual index cases of Rs/BKD (left y-axis) and the number of fish tested for Rs (right y-axis) during 1985–2020. Diagnostics were performed by cultivation on SKDM-agar 1985–1993 and by polyclonal ELISA 1994–2020

few exceptions, not sampled at stripping. A sensitive non-lethal sampling strategy would reduce the number of fish that must be killed for sampling. In addition, it offers a motivation for sampling of farmed brood stock, provided that the method can be performed on anesthetized fish and that the sensitivity is at the same level as examination of the kidney, preferably better. For terrestrial animals, blood sampling for antibody detection is a well-established technique. Detection of Rs antibodies is possible, but due to individual variation in immunological response, it is not suitable for tracing individual Rs infected fish (Jansson & Ljungberg, 1998). Blood, mucus and urine-faecal samples have been used to identify Rs infected fish (Bruno et al., 2007; Elliott et al., 2015; Richards et al., 2017; Riepe et al., 2021).

The aim of the present study was to investigate the accuracy of detection of Rs in blood and gill/cloacal swabs in comparison to kidney tissue in salmonid fish collected from fish farms and from experimentally Rs-challenged fish. Since there is no gold standard recommended for Rs diagnosis and in the absence of perfect reference samples that cover all different stages of the infection, we have used the Bayesian Latent Class Analysis (BLCA) model for estimation of sensitivity and specificity of ELISA and qPCR of selected tissues.

2 | MATERIAL AND METHODS

2.1 | Field samplings

Fish were collected from six fish farms included in the mandatory health control program. Samples from Arctic char (*Salvelinus alpinus*; $n = 91$) and rainbow trout (*Oncorhynchus mykiss*; $n = 79$) were collected from four farms (Farm A-D), with ongoing BKD outbreaks, and from salmon (*Salmo salar*; $n = 27$) and rainbow trout ($n = 59$) from two farms (Farm E-F) diagnosed as Rs negative through annual screening of all brood stock females.

2.2 | Experimental challenges

Rainbow trout were obtained from a fish farm included in the mandatory health control program since the 1980's and with no history of BKD. After arrival, the fish were held in 250 L tanks, supplied with continuous flow through aerated tap water at a temperature of $11 \pm 1°C$ and with intervals of equal night (dark) and day (light) periods. There was an acclimatization period of at least two weeks before the start of experiments. Rs (Rs CIP 10,778; SVA4/86, initially isolated from a rainbow trout with clinical BKD) was propagated in Peptone broth (1% peptone, 1% yeast extract and 0.1% L-cysteine-HCl; Daly & Stevenson, 1993) and incubated with mild agitation at $18 \pm 1°C$ for 12 days. Bacteria were harvested by centrifugation for 20 min at 3000*g* and resuspended in water for the challenge. Fish were challenged by immersion in a tank with oxygenated water and thereafter distributed, using a net, into two 100 L tanks, containing water with a temperature of 15–17°C and a waterflow of 300 ml/min. The fish was inspected and fed daily with 25–50 g/day of commercial

feed (EFICO, Enviro 4,5 mm 11.3284 Biomar), except the day before sampling to avoid faecal contamination of the anaesthetic bath. Tanks were regularly siphoned to remove feed residues and faeces.

Experiment 1: Rainbow trout ($n = 63$; average weight 326 gram) were immersed for 20 min in water containing 1×10^7 CFU of Rs/ml water, determined by viable count. Fish in the control group were treated in the same way except that no bacteria were added to the water. All fish were killed week 6 or 7 post-immersion (p.i.) for sampling of blood, gills, cloaca and kidney.

Experiment 2: Rainbow trout ($n = 21$, average weight 840 g) were individually tagged by insertion of a Passive Integrated Transponder (PIT) tag (AVID Microchip I.D. Systems) in the peritoneal cavity three weeks prior to the experiment. In this experiment, the fish was immersed for 20 min in water containing 5×10^7 CFU of Rs/ml water. Non-lethal sampling of gills and cloaca was done on 10–13 fish every other week for five weeks p.i.

Four fish were killed during the experiment (3 and 4 weeks p.i.) and four fish died day 36 p.i. in connection with a technical aeration failure. These fish and remaining fish killed week 7 p.i. were all tested by sampling of gills, cloaca and kidney.

2.3 | Sampling procedures

The fish was anesthetized with buffered tricaine methane-sulphonate (MS222; 75 mg/L, Pharmaq) prior to sampling. When both non-lethal and lethal sampling was performed, fish was killed by a sharp blow to the head. In Experiment 2, where repeated non-lethal sampling was performed, the fish was allowed to recover in fresh running water under observation after anaesthesia and sampling. For the non-lethal sampling, a cotton swab was drawn over the gill lamellae and another swab brought into the cloaca (2–10 mm depending on the size of the fish) of each fish. Initially, swabs from gills and cloaca were individually analysed by qPCR. In fish from Farm D ($n = 65$) and from Experiment 2 ($n = 54$), gill- and cloacal swabs were collected both separately and pooled to investigate whether qPCR of gill/cloacal pools ($qPCR_{gc}$) would increase the ability to detect infected individuals. Swabs were transferred to Eppendorf tubes and kept at −20°C until DNA extraction and subsequent PCR analysis. Swabs were simultaneously collected for ELISA (at Farm D and E and in Experiment 2) to Eppendorf tubes containing PBS. Killed fish was examined externally and internally for disease signs compatible with BKD. Blood was collected in EDTA vacutainer tubes for qPCR ($qPCR_b$) analysis. Kidney tissue (about 1 cm³) was aseptically dissected and put in stomacher bags for ELISA ($ELISA_k$) analysis. Kidney tissue was also collected on cotton swabs for qPCR ($qPCR_k$) analysis. Sampling was performed with single use forceps and scalpels.

2.4 | Polyclonal ELISA

Kidney samples were homogenized in PBS (1:4) with limonene 145, containing butylated hydroxyanizol (0.2 g/L) (Fluka Chemika, Sigma-Aldrich) in a stomacher (Stomacher Lab Blender 80, Seward

TABLE 1 Detection of *Renibacterium salmoninarum* (Rs) by polyclonal ELISA and qPCR in samples collected from the kidney (ELISA$_k$, qPCR$_k$), gills (qPCR$_g$), cloaca (qPCR$_c$), gill-cloacal swabs (ELISA$_{gc}$, qPCR$_{gc}$) or blood (qPCR$_b$)

Population	Fish species	No of fish	Size Average: Weigh, g (*SD*) Length, cm (*SD*)	Kidney ELISA$_k$ positive (total tested)	qPCR$_k$ positive (total tested)
Farm A: Rs positive, clinical BKD	Arctic char	35	W: 514 (150) L: 35 (3)	13 (35)	27 (35)
Farm B: Rs positive, clinical BKD	Arctic char	50	W & L not reported 2 years of age	20 (48)	50 (50)
Farm C: Rs positive, clinical BKD	Rainbow trout	20	W & L not reported 1–2 years of age	10 (20)	12 (20)
Farm D: Rs positive, diffuse signs of BKD	Arctic char Rainbow trout	6 59	W: 680 (248) L: 39 (5) W: 2 500 (800) L: 53 (7)	0 (6) 0 (59)	0 (6) 2 (59)
Farm E: Rs negative	Salmon	27	W: 4 900 (1 500) L: 77 (7)	0 (27)	NT
Farm F: Rs negative	Rainbow trout	51	W: 6 269 (861) L: 72 (3)	0 (51)	NT

Note: Samples were collected from fish farms diagnosed as positive for Rs (Farms A–D) with varying expression of the resulting bacterial kidney disease (BKD) or from Rs negative farms (Farms E and F).

Laboratory) and autoclaved 30 min at $104 \pm 2°C$. The aqueous phase was collected after centrifugation and the presence of Rs antigens was determined by ELISA. The negative-positive threshold value was set to OD 0.1 based on the mean OD value +3SD of samples collected from negative fish and after correction against the negative control absorbance value (Jansson et al., 1996).

Gill and cloacal samples were supplemented with Limonene, before mixture by vortex, autoclaving and used in ELISA as above.

2.5 | qPCR

A fragment of the 16S rRNA gene of Rs was detected from tissue samples by real-time qPCR (Jansson et al., 2008.) In short, swabs from sampled tissue (gills, cloaca or kidney) were incubated in lysis buffer consisting of 540 μl G2 buffer and 60 μl; proteinase K; (QiaGene Hilden) for 30 min at 56°C with 600 rpm agitation using a Thermomixer Comfort (Eppendorf, Hamburg, Germany). The samples were allowed to cool to room temperature before addition of 23 μl lysozyme,100 mg/ml (Roche, Basel, Schweiz followed by 2 h incubation at 37°C with 300 rpm agitation. DNA was extracted from 200 μl lysate with the EZ1 Biorobot and EZ1 DNA Tissue kit (QiaGene) according to the manufacturer's instructions. DNA was also extracted from 10 μl EDTA blood using the same robot and extraction kit. The forward primer (5'TGGATACGACCTATCACCGCAT-3') and reverse primer (5'-TCGCCTTGGTTAGCTATTACC-3') produces an amplicon detected by a FAM labelled probe 5'TTTTTGCGGTTTTGGATGGACTCG-3'. The primers also amplify an internal control plasmid which is detected by a CY5 labelled probe 5'-CAACCAATGATGCCCGTTCCT-3' used to control for inhibition in the PCR reaction, as described by Jansson et al., (2008). Each 15-μl PCR reaction mixture contained PerfeCTa

qPCR Toughmix, with Low Rox (Quanta BioSciences Inc.), 500 nM of each primer, 100 nM of each probe,$75*10^{-8}$ ng of internal control plasmid DNA and 2 μl template DNA. Real-time PCR was performed in an Applied Biosystems 7500 Fast Real-Time PCR System (Thermo Fisher Scientific Inc.). The PCR program comprised of an initial denaturation step of 3 min at 95°C, followed by 45 cycles of 3 s at 95°C and 30 s at 60°C. A cycle threshold (Ct) value of 38 was applied for classification of samples as positive or negative.

2.6 | Statistical analysis

The degree of association between Ct values in qPCR$_{gc}$ and qPCR$_k$ or optical density (OD) values in ELISA$_k$ was estimated by calculation of the Pearson correlation coefficient in Excel (Version 2101).

We applied Bayesian Latent Class Analysis (BLCA) to estimate the diagnostic sensitivity and specificity of three testing strategies aimed at detecting the presence of Rs. The strategies under evaluation were ELISA on kidney tissue (ELISA$_k$, i.e. the current screening test strategy), qPCR on kidney tissue (qPCR$_k$, i.e. the current confirmatory test strategy) and qPCR on gill/cloacal swabs (qPCR$_{gc}$, i.e. an alternative non-lethal strategy). BLCA is an established method to estimate diagnostic test accuracy when the reference test is imperfect or unavailable, which has been recently endorsed by the World Organisation for Animal Health (OIE) in the Manual of Diagnostic Tests for Aquatic Animals (OIE, 2019). One of the assumptions of BLCA is that the tests under evaluation perform constantly across populations. Given that experimental and field conditions are very different, we restricted the analysis to the farm data only, to ensure that the populations are comparable. The final model included three testing strategies and six populations (i.e. farms A-F) assuming some degree of covariance

Gills	Cloaca	Gill-/cloacal swabs		Blood
qPCR$_g$ positive (total tested)	qPCR$_c$ positive (total tested)	ELISA$_{gc}$ positive (total tested)	qPCR$_{gc}$ positive (total tested)	qPCR$_b$ positive (total tested)
23 (35)	21 (35)	NT	NT	7 (13)
NT	NT	NT	50 (50)	12 (50)
NT	NT	NT	20 (20)	6 (20)
2 (6)	2 (6)	0 (6)	1 (6)	NT
19 (59)	12 (59)	1 (59)	28 (59)	0 (30)
0 (27)	0 (27)	0 (27)	0 (27)	NT
NT	NT	NT	0 (51)	NT

between qPCR$_k$ and qPCR$_{gc}$ (which shared the same method) and between qPCR$_k$ and ELISA$_k$ (which shared the same matrix). Weakly informative prior information was used for sensitivity and specificity of ELISA$_k$ as well as for prevalence in farms free from disease (Table S1), as they gave a better model fit (i.e. lower deviance information criterion). All the other parameters used uninformative priors. The posterior estimates were based on three Markov chains with different starting values, 20,000 iterations per chain after a burn-in of 5000 and a sampling lag of 50 to avoid autocorrelation. A further sensitivity analysis was performed by looking at the changes in the posterior estimates when excluding one population at the time.

3 | RESULTS

3.1 | Field sampling

Farms A-C, fattening Arctic char or rainbow trout, had ongoing clinical outbreaks of BKD at sampling. Forty percent of sampled Arctic char from Farm A and approximately 15% of sampled rainbow trout and Arctic char in Farms B and C had one or more of the following disease signs at necropsy: swollen kidney, granuloma formation in internal organs, ascites, splenomegaly, liver petechiae and pale gills. In fish from Farm D, fattening rainbow trout and Arctic char, necropsies showed no fish with advanced lesions of BKD. In 23% of the fish, one or more of the following disease signs were noted: fibrous adhesions on internal organs, thickening of the swim-bladder wall, slightly enlarged kidney or spleen, discoloration or liver petechiae No indications of BKD were recorded at necropsy in farms classified as free from the infection (Farm E-F).

The results per farm and analytical method are summarized in Table 1. In Farms with clinical BKD A-C 42% (43 out of 103) and 85% (89 out of 105) of the samples were positive for Rs by ELISA$_k$ and qPCR$_k$, respectively. In farm A, gills and cloacal samples were analyzed separately and 19 of 35 (54%) fish, sampled for qPCR$_g$ and qPCR$_c$ were positive by both analyses, whereas another six fish were positive by either qPCR$_g$ (4 fish) or qPCR$_c$ (2 fish). Non-lethal sampling for qPCR$_{gc}$ in Farm B and C identified 100% (50 + 20 samples) as positive for Rs but only 30% (12 + 6 samples) were positive for Rs by qPCR$_b$. In Farm D, 0% and 3% (0 and 2 samples, respectively) were Rs positive by ELISA$_k$ and qPCR$_k$, respectively, whereas 45% (29 out of 65 samples) were Rs positive by qPCR$_{gc}$. All qPCR$_b$ samples from Farm D were negative. All samples collected from fish farms without suspicion of BKD (Farm E-F), tested negative by qPCR$_{gc}$ and ELISA$_k$.

3.2 | Experimentally Rs exposed rainbow trout

In both experiments, there were few signs of disease at necropsy. A slightly swollen kidney, splenomegaly and a thickened swim bladder wall was observed in a few fish. In Experiment 1, 19 out of 63 animals showed such diffuse signs, and in Experiment 2 seven of 21 animals showed tendencies of a swollen kidney and tissue adherens between the kidney and the swim bladder. In Experiment 2, four fish died on day 36 p.i. in connection with a technical aeration problem. The stomachs and intestines were filled with slightly degraded feed residues, demonstrating a good appetite, and indicating that the technical failure was the main reason for the mortality. No other mortalities were recorded in either experiment.

TABLE 2 Results from Experiment 1: Individual sampling of rainbow trout (*n* = 63) experimentally challenged with *Renibacterium salmoninarum* (Rs) by immersion

	Kidney		Gills	Cloaca
Rainbow trout	**ELISA$_k$**	**qPCR$_k$ positive (total tested)**	**qPCR$_g$ positive (total tested)**	**qPCR$_c$ positive (total tested)**
Challenged with Rs (*n* = 63)	Positive (*n* = 36)	0 (36)	3 (36)	3 (36)
	Negative (*n* = 27)	2 (27)	0 (27)	2 (27)
Control group (*n* = 10)	Negative (*n* = 10)	0 (10)	0 (10)	0 (10)

Note: Results from polyclonal ELISA of kidney (ELISA$_k$) tissue compared to results from qPCR of the kidney (qPCR$_k$), gills (qPCR$_g$) or cloaca qPCR$_c$, collected by separate swabs at the end of the experiment, weeks 6 or 7 post immersion.

There was no indication that non-lethal sampling had any adverse acute effects on the fish as the fish recovered in a few minutes after anaesthesia and ate when fed a few hours after sampling (Experiment 2). The results of the experiments are summarized in Tables 2 and 3.

Experiment 1: Week 6 and 7 p.i., 36 of 63 kidney samples tested positive by ELISA$_k$, with OD values of 0.1–0.2, i.e. just above the negative-positive threshold. All ELISA$_k$ positive samples tested negative by qPCR$_k$, whereas 2 out of 27 ELISA$_k$ negative samples were qPCR$_k$ positive. Three samples for qPCR$_g$ respective qPCR$_c$, collected from different ELISA$_k$ positive fish, tested positive. Two ELISA$_k$ negative fish where positive by qPCR$_c$ (Table 2).

Experiment 2: Non-lethal sampling: All 21 fish sampled in week 1 or week 3 p.i. tested positive by qPCR$_g$. Samples for qPCR$_c$ and qPCR$_{gc}$ from the same individuals tested positive in 18 and 20 fish, respectively. At week 4 p.i., 10 fish were sampled. Two tested positive by qPCR$_g$ and three tested positive by qPCR$_{gc}$. At week 5 p. i., 5 qPCR$_g$, 1 qPCR$_c$ and 3 qPCR$_{gc}$ out of 13 non-lethal samples were positive. At week 7, 4 qPCR$_g$, 2 qPCR$_c$ and 3 qPCR$_{gc}$ tested positive out of 13 fish tested. Lethal sampling at week 3, 4, 5 and 7 identified 1 positive by qPCR$_k$ and 2 by ELISA$_k$ of the 21 Rs challenged fish (Table 3). All ELISA$_{gc}$ samples were negative.

3.3 | Comparison of diagnostic strategies

A summary of the results of detection of Rs in samples collected from kidney (ELISA$_k$ and qPCR$_k$) and from blood (qPCR$_b$), gills and/or cloaca (qPCR$_g$, qPCR$_c$ and qPCR$_{gc}$) in samples collected from fish farms previously diagnosed as positive for BKD (Farm A-D) and from experimentally exposed to Rs in Experiment 1 and 2, are shown in Figure 2. Samples from gills-cloaca were also examined for the presence of the p57 antigens by ELISA but just a few positive individuals were identified.

All fish from farms A-D and from Rs-challenged fish in Experiments 1 and 2, were allocated into one of three infection levels based on the ELISA OD values: High (OD>0.3), Low (OD 0.1–0.3) and Negative (OD<0.1). In fish classified as having a high Rs infection level, all analyses of qPCR$_k$ and qPCR$_{gc}$ were positive. In fish with a low infection level, 24% were positive by qPCR$_k$. Only seven fish in this category were tested by qPCR$_{gc}$ but six of these were positive.

Individual qPCR$_g$ and qPCR$_c$ were positive in 18 and 20% fish in this category respectively. In fish tested negative by ELISA$_k$, 29% tested positive by qPCR$_k$ and 60% tested positive by qPCR$_{gc}$ (Table 4).

Pooling of gill- and cloacal swabs before extraction for qPCR$_{gc}$ was done in Farm B-D and Experiment 2. In fish from these four groups, lethal sampling for Rs detection by ELISA$_k$ and qPCR$_k$ identified 21% and 42% of sampled fish as positive respectively, compared with 68% positives by qPCR$_{gc}$. Individual gill- and cloacal swabs were not analysed in Farm B and C. Whereas qPCR$_{gc}$ identified 51% (61 of 119) of the tested samples in Farm D and Experiment 2 as positive, individual analyses identified 45% (54 of 119) and 29% (35 of 119) of the same samples as positive by qPCR$_g$ and qPCR$_c$ respectively.

A positive association of the Ct values in qPCR$_{gc}$ and qPCR$_k$ was identified by the Pearson correlation coefficient (*r* = .61, *p* < .001), based on sampling in Farm B and C, indicating a moderate strength of agreement (Figure 3a). No agreement was observed between Ct values in qPCR$_{gc}$ and OD values in ELISA$_k$ (*r* = .2, *p* > .05, Figure 3b).

3.4 | Estimation of the sensitivity and specificity of diagnostic strategies

The posterior estimates of sensitivity and specificity of the three testing strategies alongside with the prevalence in the six populations are reported in Table 5. The ELISA$_k$ had a sensitivity of 48.8% (credible interval (ci) 38.8%–58.8%) and a specificity of 99.2% (ci 97.4%–100%). The qPCR$_k$ had a sensitivity of 98.3% (ci 92.7%–100%) and a specificity of 99.5% (ci 95.9%–100%). The qPCR$_{gc}$ had a sensitivity of 97.8% (ci 93.8%–100%) and a specificity of 75.8% (ci 68.7%–82.5%). The prevalence in the six farms met the expectations, with high values for farms where clinical signs were recorded (A, B and C), low values for the farm with diffuse signs (D) and zero in farms where there was no suspicion of BKD (E and F). It is interesting to notice that the post-estimates did not change when only uninformative priors were used, indicating that the data alone was sufficient to provide robust estimates. The final choice of weakly informative priors for some of the parameters was justified by a better fit of the model, assessed through the deviance information criterion. The sensitivity analysis showed that the post-estimates did not change significantly when removing one population at the time, suggesting that the estimates could be considered robust and reliable.

TABLE 3 Results from Experiment 2: Individual sampling of rainbow trout (*n* = 21), experimentally challenged with *Renibacterium salmoninarum* by immersion

		Non-lethal sampling																Lethal sampling			
Week p.i.	0	1			3			4			5			7							
Fish no.	$qPCR_g$	$qPCR_g$	$qPCR_c$	$qPCR_{gc}$	$qPCR_g$	$qPCR_c$	$qPCR_{gc}$	$qPCR_g$	$qPCR_c$	$qPCR_{gc}$	$qPCR_g$	$qPCR_c$	$qPCR_{gc}$	$qPCR_g$	$qPCR_c$	$qPCR_{gc}$	Week p.i	$qPCR_k$	$ELISA_k$	Fish no.
1	NEG	POS	POS	POS	POS	POS	NEG										3	NEG	NEG	1
2	NEG	ND	ND	ND	POS	POS	POS										3	POS	NEG	2
3	NEG	ND	ND	ND	POS	NEG	POS	NEG	NEG	NEG							4	NEG	NEG	3
4	NEG	POS	POS	POS	ND	ND	ND	NEG	NEG	NEG							4	NEG	NEG	4
5†	NEG	POS	POS	POS	ND	ND	ND	POS	NEG	POS	NEG	NEG	ND				5	NEG	NEG	5†
6†	NEG	POS	POS	POS	ND	ND	ND	POS	NEG	NEG	NEG	NEG	ND				5	NEG	POS	6†
7†	NEG	POS	POS	POS	ND	ND	ND	NEG	NEG	POS	NEG	NEG	ND				5	NEG	NEG	7†
8†	NEG	POS	POS	POS	ND	ND	ND	NEG	NEG	NEG	NEG	NEG	ND				5	NEG	NEG	8†
9	NEG	ND	ND	ND	POS	POS	POS	ND	ND	ND	NEG	NEG	POS	NEG	NEG	NEG	7	NEG	NEG	9
10	NEG	ND	ND	ND	POS	POS	POS	ND	ND	ND	NEG	NEG	NEG	POS	NEG	NEG	7	NEG	NEG	10
11	NEG	ND	ND	ND	POS	NEG	POS	ND	ND	ND	POS	NEG	NEG	NEG	NEG	NEG	7	NEG	NEG	11
12	NEG	ND	ND	ND	POS	NEG	POS	ND	ND	ND	NEG	NEG	NEG	NEG	NEG	NEG	7	NEG	NEG	12
13	NEG	ND	ND	ND	POS	POS	POS	ND	ND	ND	POS	POS	NEG	NEG	NEG	NEG	7	NEG	POS	13
14	NEG	ND	ND	ND	POS	POS	POS	ND	ND	ND	POS	NEG	POS	POS	NEG	NEG	7	NEG	NEG	14
15	NEG	ND	ND	ND	POS	POS	POS	ND	ND	ND	POS	NEG	NEG	NEG	NEG	POS	7	NEG	NEG	15
16	NEG	ND	ND	ND	POS	POS	POS	ND	ND	ND	NEG	NEG	NEG	POS	NEG	NEG	7	NEG	NEG	16
17	NEG	ND	ND	ND	POS	NEG	POS	ND	ND	ND	POS	NEG	POS	NEG	POS	NEG	7	NEG	NEG	17
18	NEG	POS	POS	POS	ND	ND	ND	NEG	NEG	POS	ND	ND	ND	NEG	NEG	POS	7	NEG	NEG	18
19	NEG	POS	POS	POS	ND	ND	ND	NEG	NEG	NEG	ND	ND	ND	NEG	NEG	NEG	7	NEG	NEG	19
20	NEG	POS	POS	POS	ND	ND	ND	NEG	NEG	NEG	ND	ND	ND	POS	NEG	NEG	7	NEG	NEG	20
21	NEG	POS	POS	POS	ND	ND	ND	NEG	NEG	NEG	ND	ND	ND	NEG	POS	POS	7	NEG	NEG	21

Fish were non-lethally sampled of gills ($qPCR_g$,) cloaca ($qPCR_c$) and gill-cloacal swabs ($qPCR_{gc}$) repeatedly up to seven weeks post-challenge/immersion (weeks p.i.). Four fish were euthanized during the experiment (weeks 3 and 4 p.i.), four fish died due to a technical failure (week 5 p.i) and the remaining fish were euthanized at the end of the experiment (week 7 p.i.). Dead fish allowed analysis of kidneys by ELISA ($ELISA_k$) and qPCR ($qPCR_k$).

[a]Fish died due to a technical failure at day 36 post immersion. ND, sampling not done. Striped cells indicates euthanized or dead fish, Grey cells indicates non-sampled fish, Red cells indicates Rs positiv fish, White cells with the text "NEG" indicates Rs negative fish.

FIGURE 2 Rs diagnostics by polyclonal ELISA and by qPCR in samples of kidney ($ELISA_k$, $qPCR_k$) blood ($qPCR_b$), gill ($qPCR_g$), cloaca ($qPCR_c$) and gill-/cloacal swabs ($qPCR_{gc}$). Samples were collected from farms with clinical BKD (Farm A-C, *n* = 20 rainbow trout and *n* = 85 Arctic char) and from fish with diffuse signs of BKD (Farm D *n* = 59 rainbow trout *n* = 59 and *n* = 6 Arctic char; Experiment 1 and 2 Rs *n* = 84 rainbow trout)
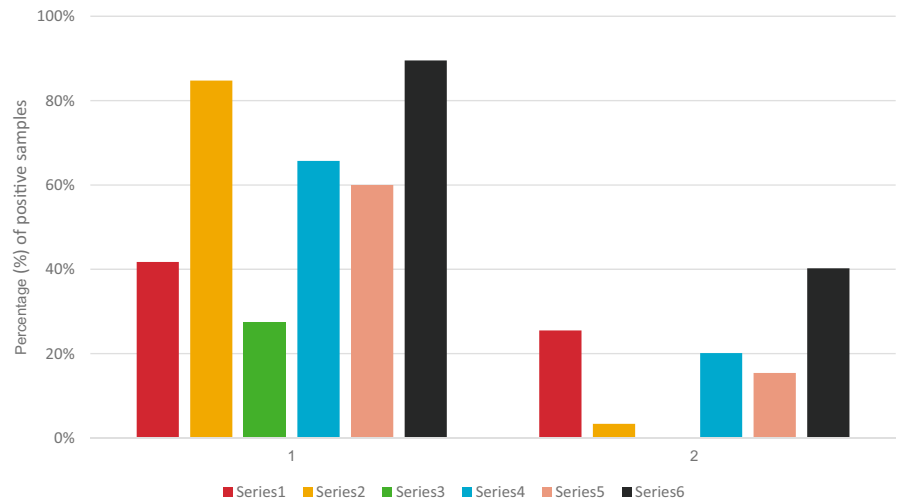
4 | DISCUSSION

BKD impairs the success of programs for restocking of salmonids in natural waters and cannot be accepted for a satisfactory fish welfare in aquaculture. Although an eradication program has been in practise for more than 30 years in Sweden, the infection is still diagnosed annually. To trace Rs positive individuals in populations with a low prevalence and non-clinical infection, a high sensitivity of the diagnostic test is crucial. No gold standard assay for detection of Rs exists and applying a combination of tests would provide the optimal information about the true Rs status (Bruno et al.. 2007; Elliott et al., 2015). Screening all sampled fish by double methodology is however time consuming and not acceptable for economic reasons. The BLCA method allow estimations of sensitivity and specificity of testing strategies without assuming a gold standard and is recommended by the OIE (Gardner et al., 2021). Collection of samples for

TABLE 4 Results of polyclonal ELISA of kidney tissue compared to results from qPCR of gills (qPCR$_g$), cloaca (qPCRc), gill-/cloacal swabs (qPCR$_{gc}$) and kidney

ELISA infection level	Number of fish	qPCR$_g$ Positive (total tested[a]); %	qPCR$_c$ Positive (total tested[a]); %	qPCR$_{gc}$ Positive (total tested[a]); %	qPCR$_k$, Positive (total tested); %
Negative, OD<0.1	171	40 (133); 30%	32 (133); 24%	71 (119); 60%	50 (171); 29%
Low, OD 0.1–0.3	51	8 (45); 18%	9 (45); 20%	6 (7); 86%	12 (51); 24%
High, OD>0.3	30	5 (6); 83%	3 (6);50%	24 (24); 100%	30 (30); 100%

Note: The infection level, based on the optical density (OD) values in ELISA, were divided in three categories: negative (OD <0.1), low infection level (OD 0.1–0.3) and high infection level (OD>0.3). Samples were from BKD infected Farms A–D and from challenged rainbow trout in Experiments 1 and 2.

[a]In the first sampling occasions (at Farm A and in Experiment 1) gills (qPCR$_g$) and cloaca samples(qPCRc) were tested separately. During the study, we found that analysing gill-/cloacal swabs by qPCR (qPCR$_{gc}$) increased the number of positive individuals.

diagnostics without killing the fish facilitates the sampling procedure and makes it possible to keep fish that test negative. Sampling of individual broodfish in connection with spawning can identify females at risk of transmitting the infection to their progeny. In the present study, we showed by use of BLCA, that qPCR$_{gc}$ is a reliable non-lethal alternative for diagnosis of BKD compared to ELISA$_k$ and qPCR$_k$. The sensitivity of qPCR$_{gc}$ was not different from the sensitivity of qPCR$_k$ but significantly higher than the sensitivity of ELISA$_k$ (Table 5). The specificity of qPCR$_{gc}$ was however significantly lower than the specificity for the other two testing strategies. Consequently, a confirmatory test with ELISA and qPCR on kidney tissue is recommended if positive findings on a farm earlier declared free from Rs, as a positive diagnosis will have significant impact on the farm's activities. Elliott et al. (2013) estimated the diagnostic sensitivity of qPCR$_k$ and ELISA$_k$ to 25% and 70% respectively based on samples collected from Rs challenged Chinook salmon, considering all Rs injected fish as true positives. Our calculations were based on results from field samplings in six fish farms. The calculated population prevalence in these farms agreed with the expectation about their status, based on earlier results and clinical indications at sampling: high prevalence (farm A-B-C), low prevalence (farm D) and negative (Farm E, F).

A significant correlation between Ct values of qPCR$_{gc}$ and qPCR$_k$ was demonstrated in farms B and C with a high Rs prevalence (Figure 3a), but there was no correlation between qPCR$_{gc}$ and ELISA$_k$ OD values (Figure 3b). All individuals classified with a high Rs level by ELISA$_k$ were positive by both qPCR$_{gc}$ and qPCR$_k$ whereas among the ELISA$_k$ negative samples, 31% tested positive by qPCR$_k$ and 59% by qPCR$_{gc}$. Similarly, Powell et al., (2005) demonstrated a high correlation between ELISA$_k$ samples with high OD values and the estimated copy numbers of qPCR$_k$, but a low correlation in the low-level ELISA$_k$ samples. Disagreement of results between assays based on different principles has been suggested to reflect different phases of Rs infection at the time for sampling (Faisal & Eissa, 2009; Nance et al., 2010). In farms B and C, there was a disagreement between ELISA$_k$ and qPCR$_k$ results in that 44% and 89% were positive, respectively. Sampling was performed in the beginning of the summer period during increasing water temperatures. A sudden rise of the water temperature is stressful for fish and the obvious higher
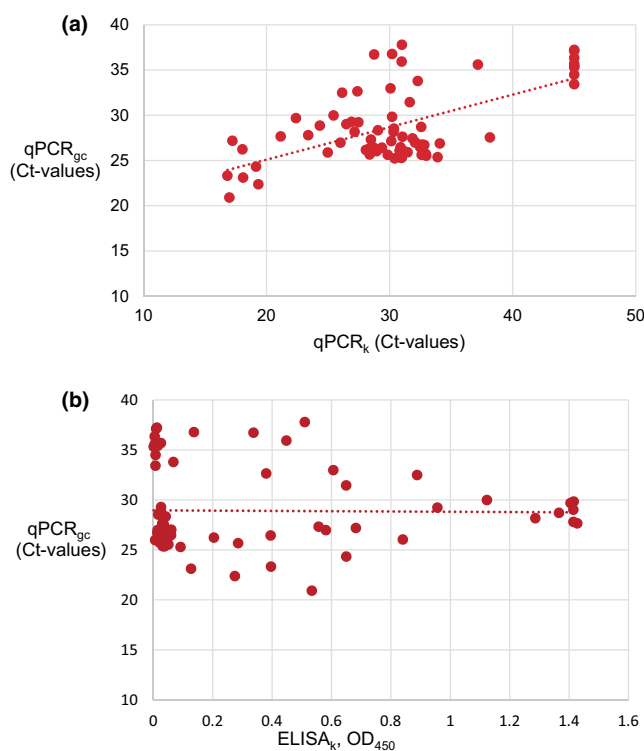

FIGURE 3 (a) Association between qPCR Ct values of gill/cloacal swabs (qPCR$_{gc}$) and kidney (qPCR$_k$) from Arctic char and rainbow trout with clinical BKD (Farm B and C; $n = 70$) for detection of the 16S rRNA gene of Rs. A positive correlation was found (Pearson´s correlation coefficient 0.61, $p < .001$). (b) The association between qPCR Ct values in for detection of the 16S rRNA gene of Rs in gill/cloacal pools (qPCR$_{gc}$) and the optical density (OD) values obtained from ELISA (ELISA$_k$), reflecting the amount of the p57 antigen(s) in kidney tissue from Arctic char and rainbow trout with clinical BKD (Farm B and C; $n = 70$). No correlation was observed (Pearson´s correlation coefficient 0.02, $p > .05$)

percentages of positives identified by qPCRk compared with ELISAk, might reflect a rapid multiplication of Rs in the kidney, whereas measurable p57 levels are produced more slowly.

The significantly higher numbers of positives by ELISA$_k$ compared to qPCR$_k$ in Experiment 1, might be due a recovery from infection,

TABLE 5 Posterior Bayesian estimates of the parameters

Parameter	Posterior estimate	95% credible interval	Effective sample size	psrf
Sensitivity ELISA$_k$	0.488	[0.388–0.588]	60,810	1.00000
Sensitivity qPCR$_{gc}$	0.978	[0.938–1.000]	56,359	1.00010
Sensitivity qPCR$_k$	0.983	[0.927–1.000]	51,930	1.00000
Specificity ELISA$_k$	0.992	[0.974–1.000]	55,725	0.99999
Specificity qPCR$_{gc}$	0.758	[0.687–0.825]	60,269	1.00010
Specificity qPCR$_k$	0.995	[0.959–1.000]	34,131	0.99999
Prevalence Farm A	0.788	[0.641–0.917]	59,464	1.00000
Prevalence Farm B	0.983	[0.938–1.000]	61,171	1.00000
Prevalence Farm C	0.611	[0.392–0.833]	60,201	1.00000
Prevalence Farm D	0.039	[0.001–0.093]	58,682	0.99998
Prevalence Farm E	0.004	[0.000–0.037]	59,068	1.00010
Prevalence Farm F	0.003	[0.000–0.026]	54,216	1.00010

Note: The sensitivity and specificity of three testing strategies, ELISA on kidney tissue (ELISA$_k$), qPCR on kidney tissue (qPCR$_k$) and on gill/cloacal swabs (qPCR$_{gc}$), used for detection of Rs were estimated by the Bayesian Latent Class Analysis (BLCA). The prevalence of BKD was calculated in sampled farms (A–F) by the same method.

whereby Rs DNA had been degraded and only the p57 protein was possible to detect. Rs antigen was detectable by ELISA in salmonid kidney for up to 43 weeks after injection challenge, when both cultivation and PCR for Rs were negative (Gudmundsdottir et al., 2020). There is also a possibility that Rs and its antigens have an uneven distribution within and between tissues as circulating p57 might be starting to accumulate in the kidney from Rs bacteria growing in other organs. Initially, gill- and cloacal swabs were analysed separately, and the results were not always consistent. A higher proportion of positives were identified when the swabs were pooled before analysis. Pooling can increase the amount of target DNA from each individual and thereby increase the possibility to find Rs in the sample. However, pooling of tissue also imposes a risk for dilution of the target DNA because it also increases the amount of non-target tissue material originating from the host. All DNA samples in this study were also tested with an internal amplification control (IAC) to identify inhibition of tissue or DNA excess in the extraction. The IAC mimic molecule gave a Ct value of 30–35 in all samples, demonstrating that the qPCR reaction was not affected. In the second experiment, pooling for qPCR$_{gc}$ was used to follow the fish's BKD status in vivo during the first seven weeks after Rs exposure. All 21 fish were positive in gills and/or cloaca at the first sampling occasions at weeks 1 or 3, whereas the proportion of Rs positive fish declined the following four weeks indicating either a recovery from infection, as in the first experiment, or lack of an established systemic infection in fish (18 of 21 individuals) that where not positive by ELISA$_k$ or qPCR$_k$. This experiment demonstrates that useful information about the fish's Rs status can be obtained without killing the fish. There were no indications that sampling of gills and cloaca had any immediate adverse effects on the fish, as they recovered quickly. Farmed fish are sedated for vaccination, fin clipping or size sorting without significant adverse effects, and in vivo Rs sampling should not pose a larger threat to fish health than those procedures.

Detection in gills and cloaca do not necessarily mean that the fish suffer from generally spread BKD in internal organs. Gill epithelium is the primary route of entry for several fish pathogens, and the capacity of Rs for intracellular survival indicates that this is also the case for Rs (Evelyn, 1996). Both gills and cloaca represent tissues for excretion of wastes. Mucus is a complex viscous adherent secretion that represents an interface between the environment and the interior milieu of the fish, that encounter pathogens (Benhamed et al.. 2014). Mucous is predominantly produced on the surface of the skin, gills and the gut lining. In the present study, gill and cloaca samples were collected with cotton swabs on the tissue surface. Consequently, the samples most certainly contain epithelial cells and mucus. Elliott et al. (2015) found scrapings of skin surface mucus for qPCR as the most promising candidate for non-lethal sampling, followed by gill filament and pelvic fin clips in experimentally Rs challenged Chinook salmon. The sensitivity of qPCR on mucus was 92% compared with 72% on gill tissue. Skin-mucus and kidney Ct levels in qPCR were correlated. This is in agreement with the results from Riepe et al., (2001), who found that, when comparing PCR on mucus, anal and buccal swabs to a kidney antibody test, mucus testing was to be preferred. Arnason et al., (2013) got poor results of a seminested PCR on gill tissue during an escalating outbreak of BKD in Atlantic salmon broodfish. In comparison, external swabs of mucus samples were efficient for detection of koi herpes virus in carp, while biopsies from gills and kidney were negative, using the same PCR assays (Monaghan et al., 2015). Urine-faecal samples for nested PCR were suggested to have the greatest potential for non-lethal Rs sampling by Richards et al., (2017), based on samplings of experimentally challenged Chinook salmon. That study demonstrated that the exposure route (intraperitoneal or immersion) and the severity of disease reflected the possibility to identify Rs infected fish by both lethal and non-lethal sampling. All urine-faecal samples tested positive up to three weeks after injection, then the number of positive

samples decreased to 33% in the last sampling, four weeks after the challenge. In contrast, skin-mucus samples were negative in the first samplings but produced 100% positives at the last sampling occasion. In the immersion challenge however, mucus was found to be the least effective matrix and only urine-faecal sampling identified positive fish in the non-lethal samplings. It was suggested that positive Rs findings in skin-mucus originated from the interior, transmitted through the blood circulation. As sampling of the kidney for ELISA identified a higher frequency of positives than PCR of urine-faecal samples, it was suggested that a combination of lethal and urine-faecal samplings should be used as this reduces the number of fish that have to be killed. This was also suggested by Riepe et al., (2021) where adult reared brook trout were tested and where surface mucus came second to kidney samples in sensitivity, while anal and buccal samples gave poorer results. The urine-faecal samples collected by Richards et al., (2017) was made after ethanol treatment of the vent area to remove mucus. Possibly, excluding the step with ethanol would increase the sensitivity of their assay. The reason for the high sensitivity obtained by $qPCR_{gc}$ in our study, might be that both uptake (gills) and excretion (gills, cloaca) of Rs was traced. Because gill swabs represent external sampling, this could reflect the presence of Rs in the environment as well as excretion of bacteria from the individual fish. However, the small amounts of water present in the gill/cloacal swabs probably have a limited influence on the results. The occurrence of Rs in water after a challenge was demonstrated to be less than ten Rs bacteria/ml water by Elliott et al., (2015).

Our study demonstrates a limited usefulness of blood for sensitive detection of Rs. Thirty percent of blood samples, collected from Farms B and C with high prevalence of Rs, tested qPCR positive, compared with 85% of the samples for $qPCR_k$ and 100% of the samples for $qPCR_{gc}$. All blood samples collected from Farm D, with a low Rs prevalence, were negative. This is in line with the results of Elliott et al., (2015) and Richards et al., (2017), where blood was not recommended for Rs sampling due to the low sensitivity.

Richards et al., (2017) found that nearly 10% of challenged Chinook salmon, positive by non-lethal sampling, were negative by lethal sampling of the kidneys. This supports our findings with $qPCR_{gc}$ positives when $qPCR_k$ and $ELISA_k$ were negative, and that the different methods are useful at different timepoints of infection. A sensitive diagnostic tool is imperative to defeat the disease in an eradication program.

5 | CONCLUSIONS

Non-lethal sampling using $qPCR_{gc}$ allow a sensitive detection of Rs. This sampling strategy makes it possible to trace individual Rs carriers in control programs as well as to remove positive farmed brood stock females to avoid vertical disease transfer. In addition, the non-lethal sampling can be used to spare brood stock of vulnerable wild salmonid strains in re-stocking programs, as fish can be released after stripping. Using the $qPCR_{gc}$ strategy instead of $ELISA_k$

will reduce the risk of false negative Rs individuals d to the price of an increased probability of false positive diagnosis. Since the goal of the BKD screening is eradication, the higher sensitivity of $qPCR_{gc}$ compared with $ELISA_k$ is preferrable. A lower specificity of $qPCR_{gc}$ compared with $ELISA_k$, suggests that positive $qPCR_{gc}$ diagnoses on previously Rs negative farms still must be verified by kidney sampling in line with the present program. This is to ensure that the farmer is not subjected to restrictions due to a false positive diagnosis.

CONFLICT OF INTEREST

The authors declare that they do not have any conflict of interest.

PATIENT CONSENT STATEMENT

Not applicable.

PERMISSION TO REPRODUCE MATERIAL FROM OTHER SOURCES

Not applicable.

CLINICAL TRIAL REGISTRATION

Ethical permit C72/13, Swedish Board of Agriculture.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

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