RESEARCH ARTICLE

qPCR screening for *Yersinia ruckeri* clonal complex 1 against a background of putatively avirulent strains in Norwegian aquaculture

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Abstract

Although a number of genetically diverse *Yersinia ruckeri* strains are present in Norwegian aquaculture environments, most if not all outbreaks of yersiniosis in Atlantic salmon in Norway are associated with a single specific genetic lineage of serotype O1, termed clonal complex 1. To investigate the presence and spread of virulent and putatively avirulent strains in Norwegian salmon farms, PCR assays specific for *Y. ruckeri* (species level) and *Y. ruckeri* clonal complex 1 were developed. Following extensive screening of water and biofilm, the widespread prevalence of putatively avirulent *Y. ruckeri* strains was confirmed in freshwater salmon hatcheries, while *Y. ruckeri* clonal complex 1 was found in fewer farms. The formalin-killed bacterin yersiniosis vaccine was detected in environmental samples by both PCR assays for several weeks post-vaccination. It is thus important to interpret results from recently vaccinated fish with great care. Moreover, field studies and laboratory trials confirmed that stressful management procedures may result in increased shedding of *Y. ruckeri* by sub-clinically infected fish. Analysis of sea water sampled throughout thermal delousing procedures proved effective for detection of *Y. ruckeri* in sub-clinically infected populations.

KEYWORDS
aquaculture, Atlantic salmon, delousing, qPCR, *Yersinia ruckeri*

1 | INTRODUCTION

The gram-negative bacterium *Yersinia ruckeri* is the causative agent of yersiniosis, also known as enteric redmouth disease, a haemorrhagic septicaemia prevalent in farmed salmonids throughout the world (Bastardo et al., 2015; Davies & Frerichs, 1989; Ross et al., 1966). While yersiniosis is most commonly associated with farmed rainbow trout internationally, in Norway, the disease is restricted to farmed Atlantic salmon, *Salmo salar*, with occasional outbreaks in farmed Arctic char, *Salvelinus alpinus*. Although currently under relatively good control due to recent widespread adoption of intraperitoneal (ip) vaccination, the incidence of yersiniosis in Norwegian freshwater farms increased in the period 2006–2010, and in the sea-phase of culture c. 2013–2017 (Gulla & Olsen, 2020).

A number of different *Y. ruckeri* serotypes have been recognized (Davies, 1990; Romalde et al., 1993), but most disease-associated

variants belong to serotype O1 (McCarthy & Johnson, 1982; Davies, 1990; Barnes et al., 2016; Gulla, Barnes, et al., 2018). Recent studies have further revealed that this serotype is shared across a number of discrete genetic lineages, with some displaying a strong affinity towards specific host species (Barnes et al., 2016; Gulla, Barnes, et al., 2018). In Norwegian salmon farming, one such lineage, termed clonal complex 1 (CC1), was found responsible for all major yersiniosis outbreaks diagnosed since the late 1980s, while the serotype O2 lineage CC3 had caused sporadic and often less severe disease (Gulla, Barnes, et al., 2018). This study also identified a number of genetically diverse, putatively avirulent genotypes from freshwater environments and ovarian fluid of clinically healthy brood-fish. This verifies the presence in Norwegian salmon-farming environments of both established virulent and putatively avirulent *Y. ruckeri* strains, although their relative prevalence, within and across farms, remains uncharted.

Yersinia ruckeri is capable of establishing latent sub-clinical infections in salmonid fish (Ross et al., 1966). The infection may then be maintained within the population by intermittent shedding of the bacterium by asymptomatic carriers (Busch & Lingg, 1975; Hunter, Knittel & Fryer 1980). While the cause/s of the recurring outbreaks experienced during the freshwater phase of culture and the recent increase in the incidence of yersiniosis in large sea-farmed salmon in Norway remain unclear, these outbreaks could conceivably be associated with activation of such latent infections. Outbreaks in salmon at sea have been associated with transfer from freshwater or other stressful handling operations (Carson & Wilson, 2009; Gulla et al., 2019; Sparboe et al., 1986). In recent years, the introduction of non-medicinal delousing, where large numbers of fish are treated in a relatively small volume of water in closed systems (Roth, 2016), represents an additional stressful handling event for large fish at sea (Overton et al., 2019). Stress-induced outbreaks of disease, including yersiniosis, may follow such treatment (Gismervik et al., 2019; Gulla et al., 2019).

While subclinical infections are generally difficult to detect at low prevalence, screening for aquatic infectious agents utilizing a non-invasive, environmental DNA (eDNA)-based polymerase chain reaction (PCR) approach offers the possibility of screening the population as a whole (Bernhardt et al., 2021; Rusch et al., 2018; Shea et al., 2020; Strand et al., 2019). This methodology should be suitable to monitor levels of *Y. ruckeri* shedding from carrier fish. Several PCR assays have been previously developed for the detection of *Y. ruckeri* in both fish and environmental samples (Gibello et al., 1999; LeJeune & Rurangirwa, 2000; Temprano et al., 2001; Del Cerro et al., 2002; Bastardo et al., 2012; Keeling et al., 2012; Ghosh et al., 2018, Lewin et al., 2020). In recent years, PCR-based screening for *Y. ruckeri* has been commonly used as a biosecurity tool in Norwegian freshwater salmon farms, but reports from the industry of false-positive PCR test results have complicated their interpretation. Additionally, as both virulent and avirulent strains may be detected by *Y. ruckeri* PCRs specific at the species level, there is a need for assays, targeting relevant pathogenic strains, which in the current Norwegian context is *Y. ruckeri* CC1. We therefore sought to develop novel PCRs, specific

to the *Y. ruckeri* species and *Y. ruckeri* CC1, respectively. These assays were utilized in tandem to assess eDNA-based PCR-screening for the general presence of *Y. ruckeri* and the virulent *Y. ruckeri* CC1 in Norwegian salmon aquaculture and to monitor shedding from subclinical and active infections.

2 | MATERIALS AND METHODS

2.1 | Strains and culture

Bacterial strains used for specificity testing (Table 2; Table 3) were cultured on suitable agar media and incubated at appropriate temperatures and durations. Species verification was performed with MALDI-TOF (Biotyper Microflex LT; Bruker Daltonics). Isolates of uncertain taxonomic status were classified and confirmed as non-*Y. ruckeri* by whole-genome-based analyses (Figure S1) as described previously (Riborg et al., 2022).For spiking experiments, *Y. ruckeri* CC1 strain NVI-10705 was cultured in Tryptic Soy Broth at 22°C with shaking until mid-log phase, from which a dilution series was prepared with sterile phosphate-buffered saline (PBS) chilled on ice and enumerated by plating on 5% bovine blood agar (BA) in triplicate. For the challenge trials, *Y. ruckeri* NVI-10705 was grown in Brain Heart Infusion Broth at 15°C with shaking for 20hr, harvested by centrifugation and re-suspension in PBS, followed by enumeration on a cell counter (Casy Inovatis; Roche Diagnostics) and by plating of a 10-fold dilution series on BA. All BA plates were incubated at 22°C for 2days prior to counting.

2.2 | DNA extraction

DNA templates for specificity testing were prepared from cultured colonies using the QIAamp DNA kit (Qiagen) according to the manufacturer's recommendations for Gram-positive or Gram-negative bacteria as appropriate, and assessed for purity and quantity with a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific).

DNA for standard curves and determination of limits of detection were extracted from NVI-10705 with Gentra puregene (Qiagen) as recommended by the manufacturer for Gram-negative bacteria. Fluorometric quantification of DNA was done with a Qubit dsDNA HS Assay Kit on a Qubit 4 Fluorometer (Thermo Fisher Scientific).

Samples consisting of salmon tissue, biofilm swabs (eSwab with 1 ml Liquid Amies transport medium; Copan Diagnostics) and water filters (47 mm nitrocellulose 0.45 μm pore-size filters; Whatman; Cytiva) were processed by individual protocols prior to lysis and purification (using a common protocol) with the DNeasy Blood & Tissue kit (Qiagen). As such, environmental swabs were processed by vortexing for 5 s followed by transfer of 1 ml transport medium to a 1.5 ml Eppendorf tube. The sample material was then pelleted by centrifugation at 8000*g* for 10 min, and the supernatant was discarded. Tissue samples were transferred to 1.5 ml Eppendorf tubes and macerated with a sterile scalpel. Filters were gently folded and

transferred to 1.5 ml Eppendorf tubes. Macerated tissue and swab pellets were subsequently suspended in a lysis buffer consisting of 180 μl buffer ATL and 20 μl proteinase-K. Twice the volume of lysis buffer (400 μl) was added to tubes with filters to keep them submerged during lysis. Tissue, swab and filter samples were then lysed overnight at 56°C with agitation on a Thermomixer (Eppendorf AG). Post-lysis, filters were carefully removed with sterile tweezers while compressing the filter with a pipette to recover all of the lysate, prior to the addition of twice the volume of buffer AL and ethanol as described in the DNeasy manual. The resulting 1200 μl mixture was loaded onto a single spin column in two separate aliquots of 600 μl. All lysates were otherwise further processed according to the manufacturers description for purification of total DNA from animal tissues with the DNeasy Blood & Tissue kit. Extracted DNA was eluated in 100 μl buffer AE.

2.3 | PCR development

The CC1-specific locus was identified by alignment of genomes in Mauve (development version 20,150,226) (Darling et al., 2010), comparing *Y. ruckeri* CC1 and non-CC1 MIVA genotypes identified previously (Riborg et al., 2022), followed by BLAST searches (Altschul et al., 1997) on local and public databases with candidate sequences to identify CC1-specific targets suitable for PCR analysis. An intergenic region between the class C beta-lactamase (acc. no. WP_004721718) and a predicted AAA family ATPase (acc. no. WP_096823432) was identified as specific for, and ubiquitous within, *Y. ruckeri* CC1, and formed the basis for the CC1-specific PCR assay. By the same approach, a LuxR family transcriptional regulator (acc. no. WP_038241605) was found to be conserved across all of the investigated *Y. ruckeri* genomes and used as target for the *Y. ruckeri* species-specific assay. Primer and probe sequences (Table 1) were determined with Primer Express Software v3.0.1 (Applied Biosystems) and purchased from Thermo Fisher Scientific.

PCR reactions comprised 10 μl TaqMan Fast Advanced Master Mix (Applied Biosystems), primers and probes at 600 nM and 200 nM, respectively, and 5 μl of template DNA in 20 μl total reaction volume. The reactions were cycled in a CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories) with the following PCR program: UNG incubation at 50°C for 2 min, polymerase activation at 95°C for 20 s, followed by 50 cycles of 95°C for 3 s and 62°C for 20 s. Samples yielding exponential amplification curves with cycle threshold values of 42 or less were considered positive.

2.4 | PCR validation

A dilution series of *Y. ruckeri* CC1 DNA was analysed in triplicate to investigate the amplification efficiency and linearity of both assays, and serve as standard curve for quantification. Limits of detection (LOD) were established by analysing 20 parallels of DNA samples in relevant dilutions. LOD was defined as the lowest amount producing at least 95% (≥19/20) positive results. The specificity of both PCR assays was tested using 1 ng purified DNA from a diverse panel of bacterial reference strains, clinical isolates primarily from diseased fish and isolates from aquaculture environments (Table 2). Specificity was further investigated by assaying a broad panel of *Y. ruckeri* isolated from various geographical and biological origins, serotypes and biotypes, including representatives from all known MLVA clonal complexes and some singletons as described by Gulla, Barnes, et al. (2018) (Table 3). The assays were also challenged with 100 ng DNA extracted from heart tissue of healthy Atlantic salmon and from *Y. ruckeri*-free biofilm (collected from an in-house research aquarium). Possible interference from a background matrix of non-target DNA was investigated by adding purified *Y. ruckeri* CC1 DNA equivalent to LOD directly to PCR reactions together with the salmon heart tissue or biofilm DNA templates. The ability to detect a low number of *Y. ruckeri* cells in the various sample types was investigated by spiking triplicates of 200 ml sterile PBS, 25 mg macerated salmon heart tissue and 25 mg (wet-weight) biofilm, with 100 μl of a *Y. ruckeri* CC1 2-fold dilution series (starting at 9.6×10^3 CFU ml^{-1}).

2.5 | Screening of freshwater hatcheries

Screening of freshwater hatcheries consisted of two distinct sample sets. Biofilm samples for both sample sets were collected by swabbing effluent pipes and the inner walls of tanks at the air/water interface. Samples were transported chilled, overnight to the laboratory, and stored at 4°C for up to 48 hr pending further processing.

2.5.1 | Sample-set one

Twenty-four Norwegian salmon hatcheries that had not experienced yersiniosis problems in recent years were sampled in 2017 as part of a previous study (Gulla, Wiik-Nielsen, & Colquhoun, 2018). Samples from hatcheries which had been identified in 2017 as PCR positive for *Y. ruckeri* (data not shown) were then re-analysed in the current work with the two novel PCR assays. This sample set also included salmon tissues (kidney and intestine), sampled on-site, suspended in

TABLE 1 Primer and probe sequences used in qPCR assays

Assay specificity	Name	Sequence (5′–3′)
Y. ruckeri	YrF	CTAATGTGCAGAGCGCAGATG
	YrR	GCGGACTGAATAACGATGATTG
	YrP	FAM-CCTGTACCGTCGTCAGG-MGB
Y. ruckeri CC1	YrCC1F	GAATTAGGCGCAACTCAATTTGAC
	YrCC1R	GCTGGTAAGGGATGTTATGTTTCA
	YrCC1P	VIC-TATGACGACTGAGTGTTTAC-MGB

Note: Minor groove binding (MGB) probes were labelled with FAM (6-carboxyfluorescein) or VIC (proprietary, Life Technologies).
Abbreviations: F, forward; P, Probe; R, Reverse.

TABLE 2 Bacterial strains of various taxa used for specificity tests with the *Y. ruckeri* (Yr) and *Y. ruckeri* CC1 (YrCC1) qPCR assays

Bacterial species	Strain designation	Biological source	PCR Yr	PCR YrCC1
Aeromonas hydrophila	ATCC 14715	Silver salmon	–	–
Aeromonas salmonicida ssp. *salmonicida*	ATCC 14174	Brook trout	–	–
Arthrobacter globiformis	NCIMB 8907^T	Soil	–	–
Bacillus cereus	NVI-3588	Unknown	–	–
Brochothrix thermosphacta	NCFB 1676^T	Pork sausage	–	–
Carnobacterium piscicola	NCFB 2762^T	Cutthroat trout	–	–
Edwardsiella piscicida	NCIMB 14824^T	European eel	–	–
Escherichia coli	ATCC 25922	Clinical isolate	–	–
Francisella noatunensis ssp. *noatunensis*	NCIMB 14265^T	Atlantic cod	–	–
Moritella viscosa	NCIMB 13584^T	Atlantic salmon	–	–
Nocardia asterioides	NVI-6532	Unknown	–	–
Pasteurella sp.	NVI-9100	Lumpsucker	–	–
Pasturella skyensis	NCIMB 13593^T	Atlantic salmon	–	–
Photobacterium phosphoreum	NCIMB 1282^T	Unknown	–	–
Piscirickettsia salmonis	NVI-5692	Atlantic salmon	–	–
Proteus mirabilis	NCIMB 10823	Human, urine	–	–
Pseudomonas fluorescens	NCIMB 10067	Unknown	–	–
Renibacterium salmoninarum	ATCC 33209	Chinook salmon	–	–
Rhanella inusitata	NVI-11515	Biofilm, aquaculture	–	–
Rhanella sp.	NVI-11513	Biofilm, aquaculture	–	–
Undescribed *Yersiniaceae*	NVI-11511	Biofilm, aquaculture	–	–
Undescribed *Yersiniaceae*	NVI-11512	Biofilm, aquaculture	–	–
Undescribed *Yersiniaceae*	NVI-11514	Biofilm, aquaculture	–	–
Rhodococcus equi	NVI-6122	Unknown	–	–
Rhodococcus erythropolis	NCIMB 11148^T	Soil	–	–
Serratia marcescens	NCIMB 10351	Sheep	–	–
Serratia sp.	NVI-11516	Biofilm, aquaculture	–	–
Staphylococcus aureus	NCIMB 11787	Human, septic arthritis	–	–
Tenacibaculum finnmarkense	NVI-5134	Atlantic salmon	–	–
Vibrio salmonicida	NCMB 2262^T	Atlantic salmon	–	–
Vibrio splendidus	NVI-7628	Ballan wrasse	–	–
Vibrio tapetis	NVI-7627	Ballan wrasse	–	–
Yersinia entomophaga	NVI-2267	Aquaculture	–	–
Yersinia fredriksenii	NVI-1098	Unknown	–	–
Yersinia intermedia	NVI-11508	Biofilm, aquaculture	–	–
Yersinia intermedia	CCUG 26592	Domestic pig	–	–
Yersinia kristensenii	CCUG 26588	Domestic pig	–	–
Yersinia kristensenii	NVI-11509	Biofilm, aquaculture	–	–
Yersinia kristensenii	NVI-11510	Biofilm, aquaculture	–	–
Yersinia sp.	NVI-11061	Biofilm, aquaculture	–	–
Yersinia ruckeri (CC2)	ATCC 29473^T	Rainbow trout	+	–
Yersinia ruckeri (CC1)	NVI-10705	Atlantic salmon	+	+

TABLE 3 *Yersinia ruckeri* strains of various MLVA genotypes used for specificity tests with the *Y. ruckeri* (Yr) and *Y. ruckeri* CC1 (YrCC1) qPCR assays

Strain designation	Biological source	Country	Year	Serotype	Biotype	MLVA-CC[a]	PCR Yr	PCR YrCC1
NVI-1292	*Salmo salar*	Norway	1987	O1	1	1	+	+
NVI-8074	*Salmo salar*	Norway	2011	O1	1	1	+	+
NVI-9698	*Salmo salar*	Norway	2014	O1	1	1	+	+
NVI-9812	*Salvelinus alpinus*	Norway	2014	O1	1	1	+	+
NVI-10428	*Salmo salar*	Norway	2016	O1	1	1	+	+
NVI-10542	*Salmo salar*	Norway	2016	O1	1	1	+	+
NVI-10989	*Salmo salar*	Norway	2017	O1	1	1	+	+
NVI-10990	*Salmo salar*	Norway	2017	O1	2	1	+	+
DVJ-93010	*Oncorhynchus mykiss*	UK	1993	O1	1	2a	+	−
DVJ-93046	*Oncorhynchus mykiss*	UK	1993	O1	2	2a	+	−
CSF007-82	*Oncorhynchus mykiss*	USA	1982	O1	1	2b	+	−
TW-11.68	*Carassius auratus*	USA	1983	O1	1	2b	+	−
TW-F190	*Oncorhynchus mykiss*	USA	1995	O1	2	2b	+	−
NVI-1382	*Oncorhynchus mykiss*	Italy	1984	O1	1	2c	+	−
TW-F183	*Oncorhynchus mykiss*	USA	1995	O1	1	2c	+	−
NVI-9925	*Oncorhynchus mykiss*	Finland	2010	O1	2	2c	+	−
NVI-1347	*Salmo salar*	Norway	1988	O2	1	3	+	−
NVI-9681	*Salmo salar*	Norway	2014	O2	1	3	+	−
RD502	*Salmo salar*	UK	2010	O8/O1[b]	1	4	+	−
TW-11.43	*Oncorhynchus mykiss*	Australia	1959	O1	1	5	+	−
RD336	*Salmo salar*	UK	2001	O2	1	6	+	−
NVI-11077	Biofilm, aquaculture	Norway	2017	O1	1	7	+	−
NVI-11055	*Salmo salar*, egg-fluid	Norway	2017	O1	1	8	+	−
NVI-11054	*Salmo salar*, egg-fluid	Norway	2017	O1	1	9	+	−
RD154	*Salmo salar*	Norway	1985	O1	2	10	+	−
NVI-492	*Salmo salar*	Norway	1987	O1	1	10	+	−
NVI-11065	*Salmo salar*, egg-fluid	Norway	2017	O1	1	s	+	−
NVI-11073	Biofilm, aquaculture	Norway	2017	O1	1	s	+	−
TW-11.30	*Morone americana*	USA	1977	O1	N.D.	s	+	−
TW-11.57	*Salmo trutta*	USA	1980	O2	N.D.	s	+	−
NVI-1398	*Oncorhynchus mykiss*	Sweden	1986	O7	N.D.	s	+	−
YR122A	*Salmo salar*	Finland	1988	O6	N.D.	s	+	−
NVI-1389	*Salmo salar*	Norway	1989	O1	1	s	+	−
RD356	*Salmo salar*	UK	2005	O5/O1[b]	1	s	+	−
NVI-5635	*Salmo salar*	Norway	2006	N.R.	1	s	+	−
NVI-11065	*Salmo salar*, egg-fluid	Norway	2017	O1	1	s	+	−
NVI-11073	Biofilm, aquaculture	Norway	2017	O1	1	s	+	−
NCTC 12268	*Oncorhynchus mykiss*	Canada	1985	O5	1	s	+	−
NCTC 12269	*Oncorhynchus mykiss*	Canada	<1990	O6	1	s	+	−
NCTC 12270	*Anguilla anguilla*	Denmark	<1990	O7	1	s	+	−

Abbreviations: N.D., Not done; N.R., No reaction with any available *Y. ruckeri* antisera.

[a]MLVA clonal complex according to Gulla et al. (2018), with 's' indicating singleton or undefined clonal complex.

[b]Serotyping ambiguity dependent on strain-origin of antisera.

RNAlater (Qiagen) and kept chilled (during transport) for 24 h before being frozen at −20°C pending further processing.

2.5.2 | Sample-set two

The second sample set encompassed environmental samples alone, sampled as previously described from 16 Atlantic salmon hatcheries in Norway, collected between October 2019 and February 2020. These were analysed using the two novel quantitative PCR (qPCR) assays described in the present study. A single site was screened prior to vaccination, and then weekly for three consecutive weeks to investigate the possibility for detecting vaccine residues in the environment.

2.6 | Screening during active marine outbreak

To assess environmental *Y. ruckeri* levels during an active outbreak at sea, water samples were collected at a marine ongrowing site in Norway during a yersiniosis outbreak in large (4–5 kg), unvaccinated Atlantic salmon. Surface water samples (4 L) were collected in disposable plastic containers and filtered on-site through 0.45 μm pore-size nitrocellulose filters (Whatman) using a portable peristaltic pump (Masterflex portable sampler; Cole-Parmer) with a 47 mm inline filter-holder (Millipore). All equipment was thoroughly rinsed with ambient water between samplings. Filters were transported chilled to the laboratory for storage at −20°C pending further processing. Tissue samples (head kidney) from fish in the affected and neighbouring cages were collected with sterile scalpel and tweezers, suspended in RNAlater and transported chilled to the laboratory.

2.7 | Screening during thermal delousing

To investigate the presence of *Y. ruckeri* during delousing operations, water samples were collected from three different marine salmon farms in Norway during thermal delousing. The studied fish at site A were unvaccinated, clinically healthy but were exposed to *Y. ruckeri* at sea via an infected neighbouring cage. Fish treated at site B were vaccinated against yersiniosis (unknown method) but had experienced an outbreak of yersiniosis during the freshwater phase. Yersiniosis history and vaccination status for fish treated at site C are unknown. Clinical yersiniosis was not apparent in any of the populations at the time of sampling. Thermal delousing involves crowding of the fish which are then pumped into a treatment barge where they are exposed to heated sea water (28–34°C dependent on ambient sea water temperatures) for approximately 30 s, prior to being pumped back into the sea-cage (Roth, 2016).

Approximately 60,000–120,000 fish were treated in each of the treatments sampled. Water samples (0.5 L) were collected in disposable plastic containers from the sea cage prior to treatment, and from the treatment chamber prior to, during (twice) and post-treatment.

Water samples were shipped to the lab chilled overnight and immediately filtered through an analytical test filter funnel (Nalgene Analytical Test Filter Funnel; Thermo Fisher Scientific) with 0.45 μm pore-size nitrocellulose filters (Whatman) using a peristaltic pump (Masterflex; Cole-Parmer). High water turbidity necessitated the use of multiple filters for some samples, in which case DNA was extracted from individual filters and independently analysed by qPCR with average values presented as results.

2.8 | Simulated thermal delousing in latently infected salmon

A trial was designed to emulate a field situation involving fish subclinically infected with *Y. ruckeri* from the freshwater phase being subjected to a stressful handling event in sea water, while monitoring shedding of *Y. ruckeri* by weekly sampling of eDNA. The trial made use of a cohabitant infection model where ip injected shedder fish were used to infect naïve cohabitants, as described below.

The challenge trials were conducted at the Industrial and Aquatic Laboratory (ILAB) with ~35 g Atlantic salmon of mixed sex. The fish were fed daily with a commercial diet (Nutra Olympic; Skretting AS). A water temperature of 14°C was maintained throughout the trial with water flow adjusted to maintain adequate dissolved oxygen levels. When necessary, fish were killed by tricaine methanesulfonate (Finquel MS-222) overdose. Shedders ($n = 60$) anaesthetized with MS-222 were marked subcutaneously using Visual Implant Elastomers (Northwest Marine Technology) and ip injected with 100 μl PBS suspension containing 3×10^6 CFU *Y. ruckeri* CC1 using a 0.5 mm gauge needle. Negative control shedders ($n = 60$) were injected with 100 μl sterile PBS. Naïve cohabitants ($n = 480$) were randomly distributed between eight 150 L tanks with freshwater, each tank receiving 60 fish. Fifteen infected shedders were then added to four of the tanks, while 15 negative control shedders were added to the four remaining tanks.

Following an initial period of mortality in shedders and cohabitants in the tanks holding infected shedders, mortality ceased and the fish were maintained as previously. After 7 weeks, the photoperiod was changed from 12 to 24 h light to induce smoltification, followed by a change from freshwater to natural sea water (32 ± 2 ppt) at week 10. During week 13, the number of fish in all tanks was adjusted to 42, with excess fish being killed and sampled (head kidney) for bacteriology (streak on BA) and qPCR to assess subclinical *Y. ruckeri* carrier status. One week later, all the fish were fasted for 24 h before two tanks containing presumptive sub-clinically infected fish and two control tanks were subjected to simulated thermal delousing. For each of these tanks, groups of three fish were sequentially held in a fine-meshed net and submerged for 30 s in a 100 L tank containing heated sea water (33.8°C). Additional oxygenation held O_2 values at or above saturation level during treatment. Treated fish were then immediately transferred to a recovery tank (50 L) containing 14°C oxygenated sea water. Following treatment of each tank, fish were returned to the 150 L holding tanks and water samples (1 L) were collected from the treatment and

recovery tanks for qPCR analysis. Fish from the remaining four tanks (two infected and two control tanks) not subjected to hot water were instead subjected to stress by handling and confinement by first reducing the water volume to 50 L and then further confining the fish by netting all of the fish for 1 min, repeated three times. After experimental stress (thermal or confinement), all fish were monitored until termination of the trial and were killed after a total of 20 weeks before being sampled for bacteriology and qPCR.

3 | RESULTS

3.1 | qPCR performance

Both PCR assays demonstrated linear performance over a 6 log range (Figure 1) with PCR efficiency of 99% and LODs equal to five *Y. ruckeri* genome equivalents. No amplification signals were observed on analysis of pure cultures of non-target bacterial species with either assay (Table 2). The *Y. ruckeri* species-specific assay was positive for all *Y. ruckeri* isolates tested, while amplification with the CC1 assay was observed exclusively for isolates confirmed as CC1 by MLVA genotyping (Table 3). No amplification was observed with DNA extracted from healthy salmon or presumed *Yersinia*-free biofilm, and no inhibition was observed when these templates were combined with *Y. ruckeri* CC1 genomic DNA. Spiking experiments verified the ability of both assays to detect low numbers of *Y. ruckeri* cells with 240 CFU per spiked sample detected in all triplicates of all sample types (water, salmon tissue and biofilm).

3.2 | Screening of freshwater hatcheries

Sample-set one consisted of DNA extracts from 11 freshwater hatcheries positive for *Y. ruckeri* in a screening study concluded in
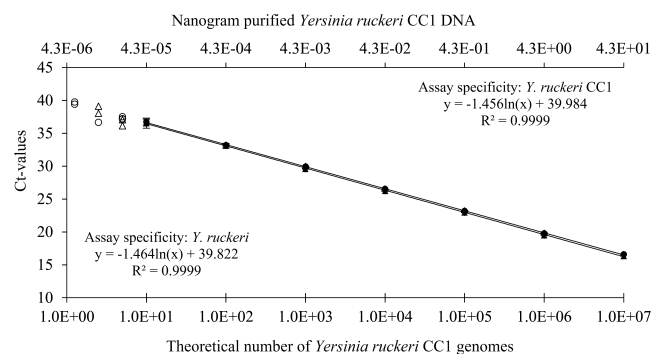

Nanogram purified *Yersinia ruckeri* CC1 DNA

Assay specificity: *Y. ruckeri* CC1
y = -1.456ln(x) + 39.984
R^2 = 0.9999

Assay specificity: *Y. ruckeri*
y = -1.464ln(x) + 39.822
R^2 = 0.9999

Theoretical number of *Yersinia ruckeri* CC1 genomes

FIGURE 1 Standard curves based on qPCR analysis of serial dilutions of *Yersinia ruckeri* CC1 strain NVI-10705 purified DNA in triplicate. Upper x-axis show ng DNA, lower x-axis show estimated number of *Y. ruckeri* CC1 genomes. The assay specific to *Y. ruckeri* is indicated by triangles, the assay specific *Y. ruckeri* CC1 by circles. Triplicates with negative samples, represented by empty shapes without means, were excluded from standard curve calculations. qPCR, quantitative polymerase chain reaction

2017 (Gulla, Wiik-Nielsen, & Colquhoun, 2018). Of 48 individual *Y. ruckeri* positive samples from these sites, CC1 was detected in only a single environmental sample from a single site, and in two kidney samples from dead fish at another site (Figure 2). Remaining kidney tissue samples from live fish were found negative by both assays. Positive environmental samples were thus heavily dominated by non-CC1 *Y. ruckeri*.

Sample-set two consisted of environmental swab samples collected from 16 Atlantic salmon hatcheries between October 2019 and February 2020. Due to a growing suspicion of detection of *Y. ruckeri* CC1 DNA from vaccine remnants in this sample set, the results were considered in relation to time since vaccination (Figure 3). While CC1 was detected at some sites that were not recently vaccinated (sites 5, 6 and 7), all produced high Ct-values (39–40) approaching the LOD. For recently vaccinated sites, the results for both assays correlated well, close to LOD. An exception was observed for site 15, which had a number of samples positive for *Y. ruckeri* with low Ct-values while being negative for CC1, indicating the presence of non-CC1 strains at this site. At sites 11 and 12, both of which were only partially vaccinated, *Y. ruckeri* CC1 was detected exclusively in samples from the recently vaccinated production units (Figure 4). Site 1 was screened prior to vaccination, and then weekly for three consecutive weeks using environmental swab samples. All samples were negative prior to vaccination, while all samples were positive 1 week post-vaccination. Most samples were still positive 2–3 weeks post-vaccination, albeit with increasingly high Ct-values (Figure 5).

3.3 | Screening during active marine outbreak

Yersinia ruckeri CC1 was detected in sea water (4 L) sampled adjacent to the affected sea cage and up to 100 m downstream from an active yersiniosis outbreak at a marine ongrowing site (Figure 6). Head kidney samples from salmon in the affected cage were all positive for *Y. ruckeri* CC1, while samples from neighbouring cages with ip vaccinated fish were negative with both assays (not shown).

3.4 | Screening during thermal delousing

Yersinia ruckeri and *Y. ruckeri* CC1 were detected in samples collected during thermal delousing treatment at two marine Atlantic salmon ongrowing sites (A and B), while at the third site (C) *Y. ruckeri* and *Y. ruckeri* CC1 were detected in the treatment chamber prior to, but not during treatment (Figure 7).

3.5 | Simulated thermal delousing

In the challenge trial designed to emulate a field situation involving stressful handling, mortality in ip infected shedder fish reached 100% in 10 days, while most of the cohabitant mortality occurred in weeks 2–4, eventually plateauing at 20–28% cumulative mortality
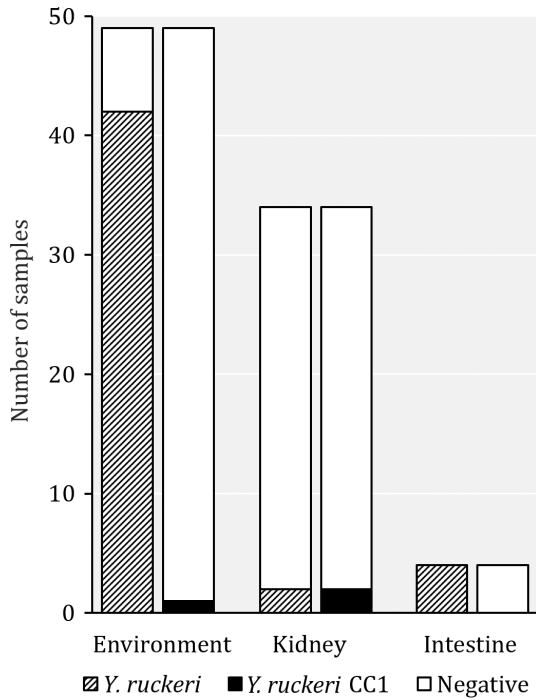
FIGURE 2 Stacked bar chart showing qualitative qPCR results from using the two novel assays on sample-set one. The sample material consisted of three different sample types, that is, environmental swabs, kidney and intestinal tissue, collected from 11 Atlantic salmon hatcheries. Samples were collected in 2017, prior to widespread use of ip vaccination against yersiniosis in Norway, from sites that had not experienced problems with the disease in recent years. qPCR, quantitative polymerase chain reaction

(Figure 8). *Y. ruckeri* CC1 was detected by qPCR in effluent water from all infected tanks during weeks 2–4, while mortality in cohabitants was ongoing. During the following weeks, *Y. ruckeri* CC1 was detected only sporadically from tank water, with Ct-values close to LOD and thus not readily visible in Figure 8. Change in photoperiod regimen or salinity did not seem to induce significant shedding. Thermal stress, however, resulted in significant shedding in both treatment and recovery tanks, with concentrations of *Y. ruckeri* CC1, as estimated by qPCR, similar to those observed during the active outbreak phase in the early weeks of the experiment (Figure 8). Unfortunately, water was not sampled for qPCR during the crowding treatment. *Y. ruckeri* was not detected in effluent water samples collected the week following stress exposures. Neither of the experimental stress events resulted in mortality during treatment, nor over the following weeks. From a total of 24 head kidney samples assayed for *Y. ruckeri* CC1 by qPCR in week 13, three were positive (12.5%), and at termination of the trial after 20 weeks, three out of 40 kidney samples were positive (7.5%). *Y. ruckeri* could not be isolated from kidney smears on BA at weeks 13 and 20. There were no mortalities nor *Y. ruckeri* detections in any of the negative control tanks throughout the experiment.

4 | DISCUSSION

The recent discovery in Norwegian salmon farming of a single virulent *Y. ruckeri* lineage (CC1), apparently co-existing alongside a
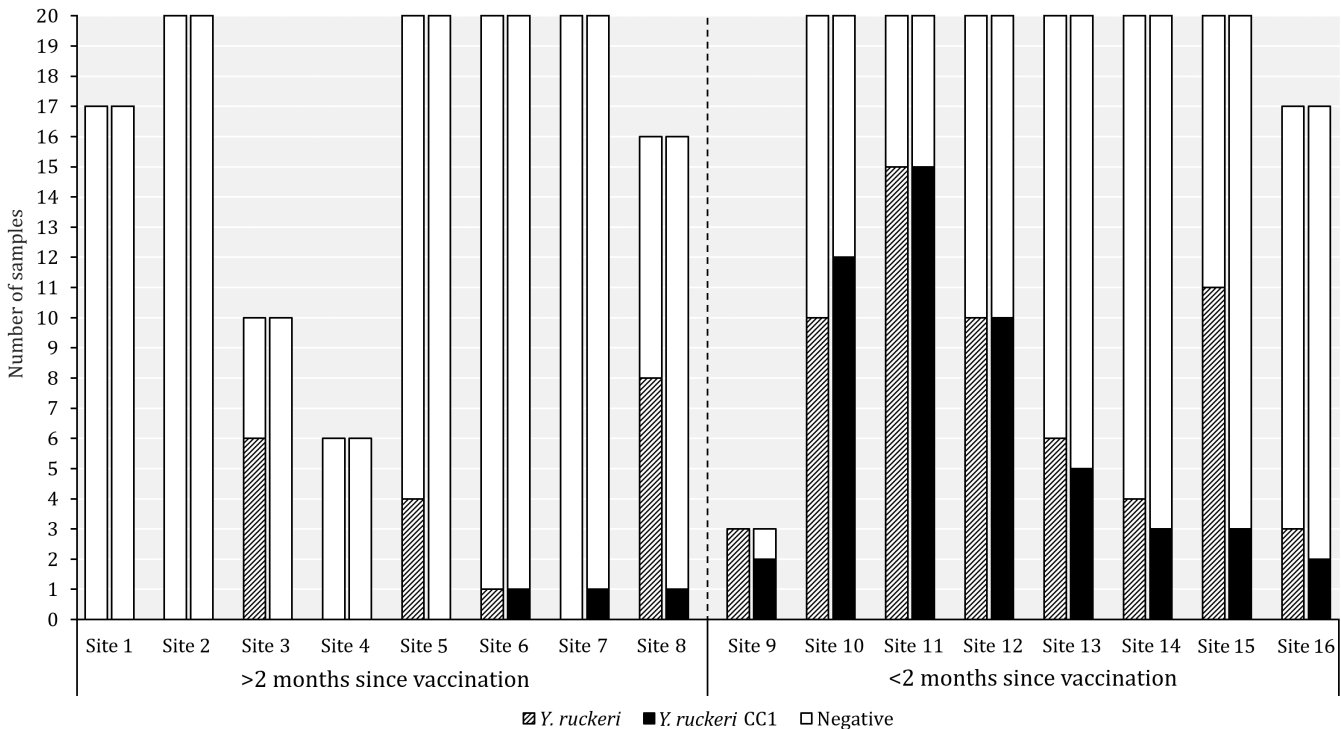

FIGURE 3 Stacked bar chart showing qualitative qPCR results from using the two novel assays on sample-set two. The sample material consisted of environmental swab samples from 16 Atlantic salmon hatcheries, collected in 2019 and 2020. Sites 9 through 16 had employed *Yersinia ruckeri* vaccines less than 2 months prior to sampling, while sites 1 through 8 did not. qPCR, quantitative polymerase chain reaction
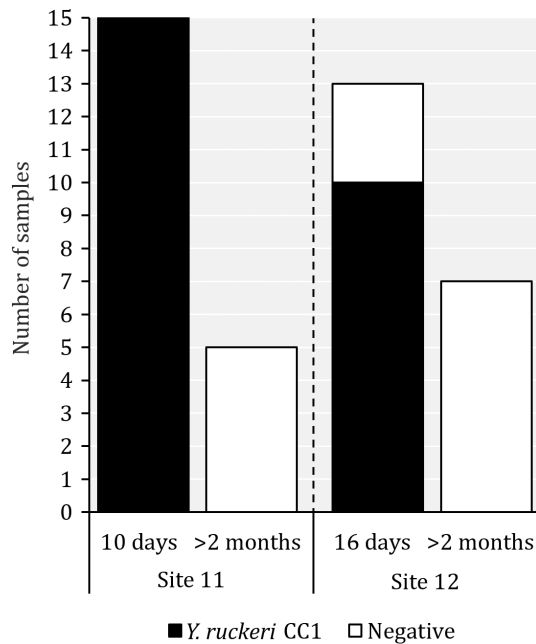
FIGURE 4 Stacked bar chart showing qualitative qPCR results for *Yersinia ruckeri* CC1 from environmental swab samples collected at two sites in sample-set two that were partially vaccinated, respectively 10 and 16 days prior to sampling (see also Figure 3). Results from production units that were not recently vaccinated ('>2 months') are also shown. The lowest Ct-values observed at each site were 28 (site 11) and 30 (site 12). qPCR, quantitative polymerase chain reaction
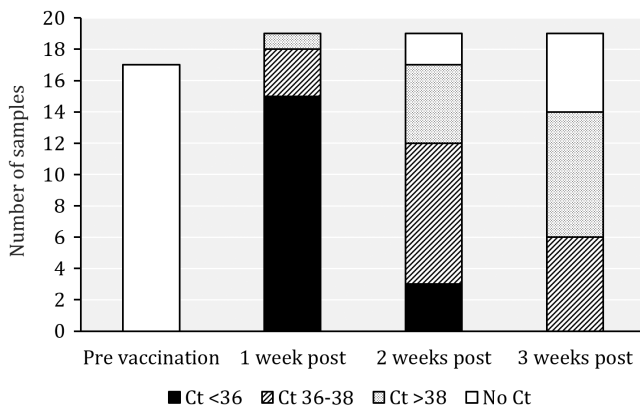

FIGURE 5 Stacked bar chart showing qPCR results for *Yersinia ruckeri* CC1 from environmental swab samples collected at site 1 (sample-set two, prevaccination data for this site are also depicted in Figure 3), a presumed *Y. ruckeri*-free freshwater aquaculture site, prior to vaccination against yersiniosis and weekly after vaccination. Samples were categorized by Ct-values to indicate relative amounts of *Y. ruckeri* DNA targets in them. The lowest Ct-values 1 week post-vaccination were 31 (one sample) and 32 (four samples). The lowest Ct-value 2 weeks post-vaccination was 35 (three samples). qPCR, quantitative polymerase chain reaction

FIGURE 6 Quantification of *Yersinia ruckeri* CC1 (estimated DNA targets per litre) in filtered sea water samples (4 L) collected upstream and downstream of an Atlantic salmon sea cage during an active yersiniosis outbreak

bacteria in salmon production. Via development and use of two highly sensitive PCR assays, respectively, specific at the species- (*Y. ruckeri*) and genotype- (*Y. ruckeri* CC1) level, we were able to verify non-CC1 *Y. ruckeri* as prevalent and dominating across freshwater salmon farm environments in Norway. Moreover, during PCR surveys of sea water sampled during both field and experimental thermal delousing, we observed rapid shedding of *Y. ruckeri* CC1 from sub-clinically infected fish, emphasizing the potential biosecurity risks involved during such stressful procedures.

PCR is widely used as a biosecurity screening tool for fish-pathogenic agents in Norway, and such assays used for detection of *Y. ruckeri* have generally targeted the 16S rRNA gene. Specific 16S-based qPCR detection at the species level and beyond is, however, challenging due to the often highly conserved nature of this gene between closely related species (Cloud et al., 2000; Linton et al., 1997; Nishio et al., 1997; Ryu et al., 2013). In our experience, such assays directed at *Y. ruckeri* may generate false-positive signals when used on environmental samples, likely due to undescribed members of the *Yersiniaceae* carrying 16S rDNA sequence motifs near identical to *Y. ruckeri* (e.g., NVI-11511, -11,512 and -11,514, Table 2 and Figure S1). In light of these experiences, we chose to forgo the potential benefits of increased sensitivity offered by the multi-copy 16S rRNA gene, and instead focus on genetic loci present in *Y. ruckeri* but absent in other *Yersiniaceae* native to aquaculture environments. Development of PCRs for specific detection of pathogenic strains against a background of less-virulent or avirulent members of the same bacterial species offers an additional challenge. The availability of continuously growing public genome databases has, however, eased identification of genetic loci specific to particular taxa. Through scrutiny of publicly available *Y. ruckeri* genomes and genomes sequenced in our laboratory, we could thus establish a PCR specific for the single *Y. ruckeri* lineage (CC1) currently associated with serious disease in Norwegian salmon farming.

It is generally accepted that *Y. ruckeri* infections may persist sub-clinically at a low prevalence in affected fish stocks (Bruno &

diverse array of putatively avirulent strains unrelated to clinical disease (Gulla, Barnes, et al., 2018), has highlighted the need for more information relating to the distribution and prevalence of these
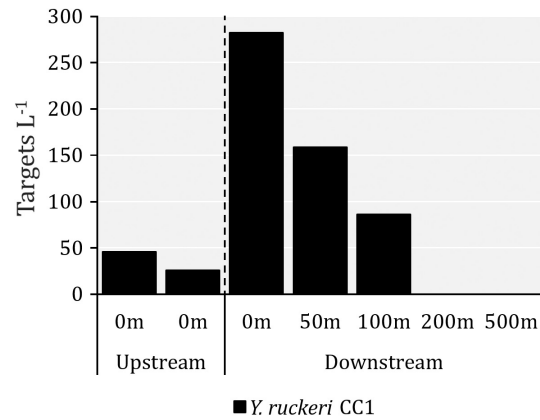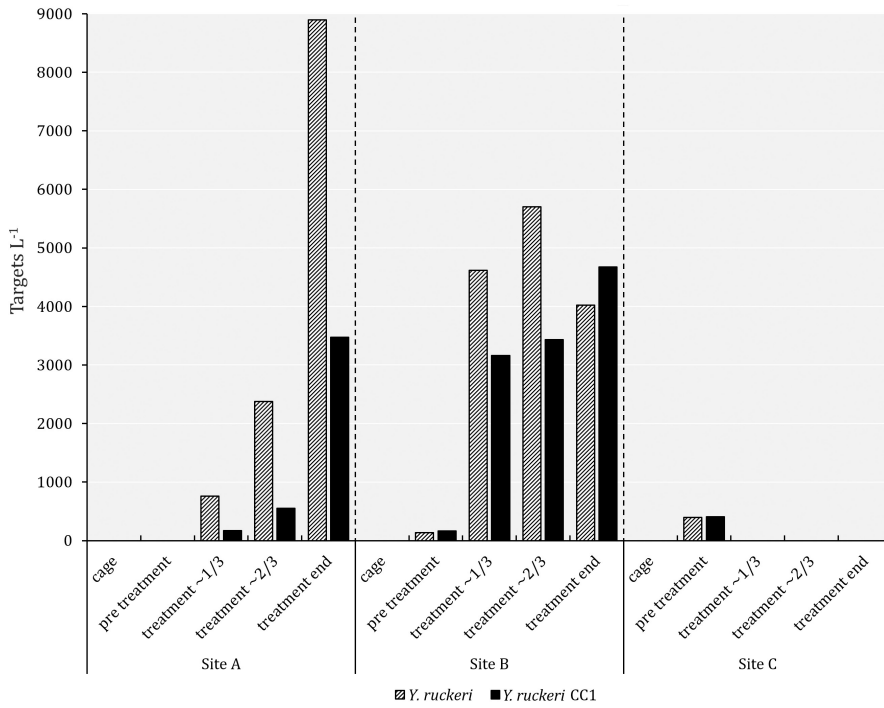
FIGURE 7 Quantification of *Yersinia ruckeri* and *Y. ruckeri* CC1 (estimated DNA targets per litre) in filtered sea water samples collected from sea cages ('cage') and treatment chambers ('pre treatment') prior to and during (at three intervals) thermal delousing. Two additional treatments were sampled at site C the following day, with the same equipment, where *Y. ruckeri* (not *Y. ruckeri* CC1) was only detected in the treatment chamber prior to one of the treatments (not shown)
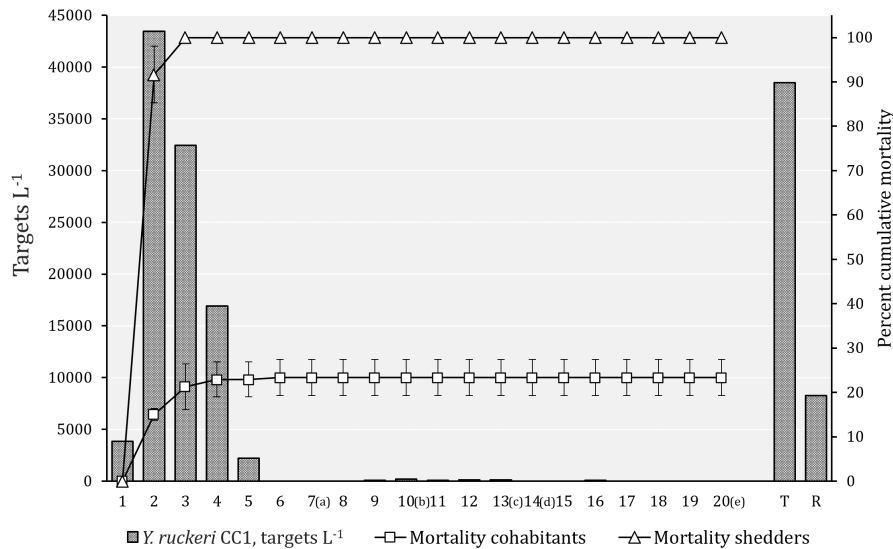

FIGURE 8 Cumulative mortalities (right vertical axis; percent) and quantification of *Yersinia ruckeri* CC1 (left vertical axis; estimated DNA targets per litre) in filtered water collected throughout the challenge trial. Sampling points as indicated on the horizontal axis are weekly samples (1–20), and sampling of tanks used for treatment (T) and recovery (R) of fish subjected to thermal stress (on far right). Sequential events indicated on the horizontal axis represent change in lightning regimen (a), change in salinity (b), fish number adjustments with kidney sampling (c), experimental stress (d) and trial termination with kidney sampling (e). *Y. ruckeri* CC1 quantification in weekly samplings are averages across four tanks, while quantification during thermal stress (T and R) are averages from two tanks. Cumulative mortality percentages are plotted as averages with bars indicating the observed range across four tanks

Munro, 1989; Willumsen, 1989) and that acute outbreaks of yersiniosis in Atlantic salmon may follow stressful management procedures (Gismervik et al., 2019). However, screening of fish-tissues for detection of such low-prevalence infections requires killing of many individuals and negative results will always be associated with a certain degree of uncertainty. On the other hand, screening

of environmental DNA (eDNA) offers the possibility of surveying the host population as a whole, but is dependent on the particular pathogen of interest being shed from the infected fish. Although we found both biofilm and filtered water equally reliable for *Y. ruckeri* screening in salmon-farming environments, biofilm sampling was chosen, as this was quicker and less technically demanding, while

also producing easily transportable samples. As mature biofilms are not normally found within experimental aquarium facilities, however, we based our eDNA sampling during the laboratory challenge trial on filtered water samples.

Our screening studies suggest that Norwegian salmon farms are commonly colonized by *Y. ruckeri* genotypes other than CC1 (Figures 2 and 3). Recently vaccinated sites excluded, CC1 constituted a low proportion of *Y. ruckeri* detections in Atlantic salmon hatcheries. While this may suggest CC1 to be less prevalent than non-CC1 *Y. ruckeri* in general, it does not exclude the possibility that sub-clinically CC1 infected fish remain undetected. In our laboratory trials, consistent detection of water-borne *Y. ruckeri* CC1 was only possible during the active outbreak phase and during experimental stressing of sub-clinical carriers (Figure 8). Detection of water-borne *Y. ruckeri* was otherwise sporadic with Ct-values close to the LOD, indicating infrequent shedding from carrier fish.

While culture of *Y. ruckeri* from environmental sample material is generally challenging, MLVA genotyping directly on eDNA templates from two of the freshwater sites that were positive for *Y. ruckeri* while negative for *Y. ruckeri* CC1 corroborated the qPCR results by producing MLVA-profiles incompatible with *Y. ruckeri* CC1 (not shown). Low-virulent *Y. ruckeri* strains associated with salmonid aquaculture have been reported also from Australia (Barnes et al., 2016) and the UK (Verner-Jeffreys et al., 2011), possibly indicative of a natural, non-pathogenic presence in such freshwater environments. Previous studies have demonstrated the capability of *Y. ruckeri* to survive for extended periods of time in sterile freshwater (Thorsen et al., 1992), sediments and sand (Bomo et al., 2004; Romalde et al., 1994), and to form hardy biofilms on various materials (Coquet, Cosette, Junter, et al., 2002; Coquet, Cosette, Quillet, et al., 2002; Wrobel et al., 2020). It remains unclear, however, whether the putatively avirulent strains documented here depend upon proximity to, and/or interaction with, a salmonid hosts in order to thrive in these environments.

While suitable for environmental *Y. ruckeri* screening in general, the high sensitivity of both PCR assays developed here also rendered them capable of detecting eDNA presumably originating from *Y. ruckeri* vaccines for at least 3 weeks after administration by ip injection (Figures 3, 4 and 5). These vaccines consist of killed *Y. ruckeri* CC1 cells and thus also contain genomic DNA from the *Y. ruckeri* CC1 vaccine strain. Still, this apparent persistence of intact PCR targets from the vaccine was surprising as relatively rapid degradation of inactivated bacteria was expected in such systems, where microbial activity is high. Likely explanations include vaccine residues gradually leaking from the injection site and/or being deposited in biofilms in the production environment. The specific vaccine technology used or the common practice of co-injection of this water-based vaccine together with an oil-based multi-component vaccine yielding a depot effect, may influence both the degree of leakage and persistence of inactivated *Y. ruckeri* in these environments. Nevertheless, as some freshwater farms employ up to two rounds of yersiniosis vaccination by immersion, followed by subsequent ip administration, interpretation of *Y. ruckeri* PCR detections at such sites will inevitably carry some degree of uncertainty. Although PCR technologies have been described that do not amplify DNA from dead cells, for example, by use of viable/dead staining with Ethidium monoazide bromide or derivatives thereof (Soejima et al., 2007), these methods require significant optimization and are critically reliant on low turbidity (Fu et al., 2020; Santander et al., 2019), making them generally unsuitable for sample materials such as environmental swabs and filtered water from fish farms.

Historically, yersiniosis in sea-farmed Atlantic salmon in Norway has been considered a minor problem, primarily occurring in subclinically infected stocks shortly after sea transfer. In recent years, however, the disease has also become more common in larger sea-farmed fish, often manifesting within a couple of weeks following non-medicinal delousing. Such procedures, introduced in the face of increasing development of salmon-louse resistance to chemotherapeutants, may often cause acute mortality, skin damage and poor fish welfare (Folkedal et al., 2021; Nilsson et al., 2019; Overton et al., 2019; Sviland Walde et al., 2021), and are undoubtedly extremely stressful to the subjected fish. In this study, we found eDNA from sea water equally suitable for *Y. ruckeri* detection by PCR as compared to freshwater, with unambiguous detection of the bacterium by both developed assays as far as 100 m downstream of a salmon sea-cage experiencing an active *Y. ruckeri* CC1 infection (Figure 6). However, considering the dilution effect and the relatively high Ct-values observed, eDNA analyses are likely not sensitive enough for reliable detection of sub-clinical carrier status in sea-cage held populations of salmon.

In light of these findings, our investigation of *Y. ruckeri* in salmon at sea sites was instead focused on eDNA sampling during thermal delousing, a form of non-medicinal delousing where large numbers of fish are treated within a limited volume of heated water. Here, high amounts of *Y. ruckeri* CC1 were detected in treatment water on two farms during delousing of suspected sub-clinically infected salmon stocks (sites A and B in Figure 7). Interestingly, we did not detect the bacterium in water sampled from these sea-cages prior to treatment, strongly suggesting that *Y. ruckeri* shedding was provoked by handling and treatment. It should be noted that detections in the treatment chamber prior to delousing at sites B and C may represent inactivated *Y. ruckeri* post-disinfection procedures. Simulated thermal delousing on sub-clinically infected fish in the laboratory subsequently confirmed that the combination of handling and thermal stress did in fact result in significantly increased *Y. ruckeri* shedding (Figure 8). Despite the apparently low number of sub-clinically infected fish present at the time of experimental thermal treatment, as assessed by sampling from fish 1 week prior (12.5% positive), the amount of *Y. ruckeri* released into the treatment water corresponded to levels observed during the acute phase of yersiniosis earlier in the same experiment. However, no further clinical disease was recorded subsequently to stress exposure, possibly indicating a partly immunized population following the initial outbreak when sub-clinical infection was established, and/or that the stress involved was insufficient to induce another outbreak.

In conclusion, we developed two *Y. ruckeri* qPCR assays, specific at the species- and genotype- (CC1) level, respectively. While the CC1-specific assay is most relevant under the current Norwegian situation, the species-specific assay is suitable for international application. Using these two assays in combination we could readily detect *Y. ruckeri* in freshwater salmon-farm environments in Norway, where putatively avirulent strains of this bacterium were found to dominate. While this approach proved highly sensitive for *Y. ruckeri* screening in such environments, it is important to be aware that yersiniosis vaccination several weeks in advance of sampling may give rise to false-positive results. Screening of treatment water in marine salmon-farms undergoing thermal delousing further corroborated that yersiniosis outbreaks may follow due to stress-related shedding of *Y. ruckeri* from sub-clinical carrier fish, possibly exacerbated by physical damage experienced by the fish during treatment. Lab trials further verified that the thermal delouser treatment chamber, in which large numbers of fish are treated within a relatively small volume of water, represents an ideal eDNA sampling site for evaluating the *Y. ruckeri* carrier status of salmon stocks. Conceivably, sampling during such treatments or other stressful situations may also represent a relevant source of eDNA for detection of other infectious pathogens in marine aquaculture.

DATA AVAILABILITY STATEMENT

This work was based on previously published genome sequences referenced in the text. Data regarding details on sites and samples areconfidential or deemed not relevant.

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