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Tenacibaculum ovolyticum 16S rDNA Quantitative-PCR Assay Development and Field Testing

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Abstract: In British Columbia (BC; Canada) Atlantic salmon (*Salmo salar* L.) production, *Tenacibaculum* members are associated with 'mouthrot' and disease identification is based on gross observation and clinical data. Genomic similarities (i.e., putative virulence factors) between *T. ovolyticum* and other better-characterized agents of mouthrot could imply potential pathogenicity. While *T. ovolyticum* has not been directly linked to salmon mortality events in BC, it has been isolated from diseased marine fish. To investigate *T. ovolyticum*'s pathogenicity in situ, a *T. ovolyticum* 16S rDNA qPCR assay targeting a ~155 bp amplicon was developed. The assay was used to screen 67 biotic and 33 abiotic samples collected from a BC Atlantic salmon (*Salmo salar* L.) net-pen site before, during, and after a mouthrot outbreak. The assay was specific, quantifiable and detectable for *T. ovolyticum* over 6-log and 8-log units, respectively. However, cycle quotients differed between the BC isolate and type strain of *T. ovolyticum*, suggesting that qualitative use of the qPCR assay in field samples would be more accurate. Only two out of 100 samples were *T. ovolyticum*-positive, indicating limited involvement in this particular outbreak. However, the ecological role of *T. ovolyticum* and its involvement in the pathogenesis of other mouthrot outbreaks in Atlantic salmon is unknown.

Keywords: *Tenacibaculum*; tenacibaculosis; mouthrot; bacterial stomatitis; yellow mouth; aquaculture; qPCR; TaqMan

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1. Introduction

Members of the bacterial genus *Tenacibaculum* (e.g., *T. maritimum*, *T. dicentrarchi*, and *T. finnmarkense*) are putative agents responsible for tenacibaculosis in mariculture fishes; a disease characterized by epidermal ulcerations, which can be accompanied by yellow plaques, and changes in fish behaviour [1–4]. Mouthrot, a regional variant of tenacibaculosis in British Columbia (BC; Canada), can induce mortality in Atlantic salmon (*Salmo salar* L.) post-smolts after transfer into saltwater [5,6] to a size of ~500 g [7] and presents with variably sized, yellow, oral plaques and ulcerations. Mitigating these mortalities necessitates antimicrobial treatments [5] incurring greater associated production costs and the potential for bacteria to develop antibiotic resistance [8]. In 2022, mouthrot outbreaks in BC have been treated using *per os* antibiotics (e.g., florfenicol, trimethoprim, and sulfadiazine) based on gross pathological findings and increased daily mortality. Since several *Tenacibaculum* species and isolates, including *T. ovolyitucm* (T.ovo), can be cultured from diseased BC Atlantic salmon, it is unclear which species or isolates are the causative agent(s) for individual mouthrot outbreaks.

Although characterization of T.ovo as a pathogen is limited, clinically, T.ovo has been found in lesions of sardine (*Sardina pilchardus* W.) eggs [9], American lobster (*Homarus americanus* H.) [10], halibut (*Hippoglossus* L.) fry [11], and Atlantic salmon [12]. In vivo exposure trials also identified that T.ovo could dissolve the chorion and zona radiata

of halibut eggs through exoproteolytic activity when the bacteria comprised over 30% of the epiflora [13]. Phylogenetically, T.ovo is similar to other putative pathogens (i.e., *T. dicentrarchi* and *T. finnmarkense*) using 16S rDNA sequencing and multi-locus sequence analysis [11,14]. Beyond short amplicon comparisons, whole-genome sequencing identified that T.ovo encoded potential virulence factors [12,15] similar to other potentially pathogenic *Tenacibaculum* species (e.g., *T. maritimum* [16], *T. dicentrarchi*, and *T. finnmarkense* [17,18]). Overall, T.ovo's genomic similarity to pathogenic *Tenacibaculum* species and correlations to disease in other fish indicates that more research is needed to understand the potential of T.ovo as a pathogen to cultured Atlantic salmon and other aquatic organisms. Thus, the primary objectives of this study were to develop a quantitative-PCR (qPCR) assay to identify and quantify T.ovo, and subsequently use the assay on samples [4] collected before, during, and after a mouthrot outbreak at an Atlantic salmon net-pen site.

2. Materials and Methods

2.1. Assay Development

2.1.1. Isolates and DNA Used

A T.ovo isolate (20-4135-2 [in-house name]) was obtained by swabbing a mouth lesion on an Atlantic salmon that exhibited mouthrot at a BC marine net-pen site in the Broughton Archipelago, and was cultured on *Flexibacter maritimus* media supplemented with kanamycin (50 $\mu g \cdot mL^{-1}$) at 12 °C. T.ovo 20-4135-2 (Genbank Accession #: OP629685) was most similar to T.ovo da5A-8 based on 16S rDNA sequencing using universal 27F (5′- AGAGTTTGATCATGGCTCAG -3′) and 1492R (5′- GGTTACCTTGTTACGACTT -3′) primers (Table 1). T.ovo 20-4135-2 was also sequenced using MinION nanopore long-read technologies [19]. A FastANI [20] comparison against the National Center for Biotechnology Information (NCBI, https://www.ncbi.nlm.nih.gov [accessed on 1 August 2022]) sequences of T.ovo da5A-8 (GCF_001641405.1_ASM164140v1), T.ovo EKD 002^T (DSM 18103) (GCF_000430545.1_ASM43054v1), and T.ovo To-7Br (GCF_021852385.1_ASM2185238v1) were over 95% similar, passing the threshold for *Tenacibaculum* species-level identification [19,21,22]. Recent work on the complete genome of T.ovo 20-4135-2 identified six copies of 16S rDNA and a single nucleotide polymorphism [19]. Additional bacterial species, including T.ovo EKD 002^T (DSM 18103), and Atlantic salmon DNA were also used in this study (Table 1). The DNA extraction of samples was accomplished using the Omega E.Z.N.A. Tissue extraction kit (Omega Bio-tek, Inc., Norcross, GA, USA) according to manufacturer's guidelines and DNA was stored at −20 °C. All extractions had an $A_{260/280}$ of 1.8–2.0 and an $A_{260/230}$ of 2.0–2.3.

2.1.2. Alignments and Oligonucleotide Generation

Tenacibaculum spp. 16S rDNA sequences were obtained from the NCBI database before being aligned using MUSCLE in MEGAX [23]. The resulting alignment, in conjunction with the primer (MAR-4 Forward [Fw], MAR Reverse [Rv]) and probe (MAR) placement [24], were used to design the primers and probe for a T.ovo-specific qPCR assay (Table 2).

Table 1. Bacterial isolates and DNA used. 'NA' indicates the data are not available; '-' indicates a negative OVO assay qPCR reaction using 100 ng of bacterial DNA. Bacterial designation is the identifier provided by the supplier, or the most similar NCBI BLAST comparison describing the gene used, *—length of the amplicon, query cover (%), similarity (%), and E-value.

In-House Sample Name	Bacterial Designation or Most Similar NCBI BLAST Comparison	NCBI BLAST Comparison				Obtained from	Grown on	Grown at °C
		16S rDNA Amplicon Length (bp) *	Query Cover, Similarity, E-Value					
20-4135-2	*Tenacibaculum ovolyticum* da5A-8	1368	100	99.9	0	BC Atlantic salmon	FMM+K	12
T.mar 2.1C	*Tenacibaculum maritimum* NLF-15	1366	99	100	0	BC Atlantic salmon	FMM+K	12
20-4116-9	*Tenacibaculum dicentrarchi* TdChD04	1409	99	98.8	0	BC Atlantic salmon	FMM+K	12
20-4106-2	*Tenacibaculum finnmarkense* Tsp.2	1426	100	99	0	BC Atlantic salmon	FMM+K	12
DSM 17995	*Tenacibaculum maritimum* R-2^{T}	NA		NA		DSMZ	MA	30
DSM 18103	*Tenacibaculum ovolyticum* EKD 002 ^{T}	NA		NA		DSMZ	MA	15
DSM 18841	*Tenacibaculum gallaicum* A37.1^{T}	NA		NA		DSMZ	MA	28
ATCC BAA-459™	*Tenacibaculum skagerrakense* D30^{T}	NA		NA		ATCC	MA	12
DSM 18842	*Tenacibaculum discolor* LL04 11.1.1^{T}	NA		NA		DSMZ	MA	28
FP	*Flavobacterium* sp.	1317	99	>95	0	Environmental sample	CA	12
ATCC 43844™	*Polaribacter glomeratus* UQM 3055^{T}	NA		NA		ATCC	MA	30
ATCC 23079™	*Flexibacter flexilis* CR-63^{T}	NA		NA		ATCC	CA	21
F.flex Contam	*Dermacoccus* sp.	589	>91	>88	$<1 \times 10^{-78}$	Culture contaminate	CA	21
Pcocus	*Paracoccus* sp.	1184	100	>96	0	Culture contaminate	FMM+K	12
Beluga HI TSA 1	*Pseudomonas* sp. CC11J	1434	99	99.8	0	BC White Sturgeon	TSA/CA	12
Beluga HI TSA 2	*Flavobacterium* sp. T69L.09.B.RBT.MI.W. Kidney	1386	99	99.9	0	BC White Sturgeon	TSA/CA	12
LI C4 P1	*Vibrio splendidus* BST398	1375	100	99.7	0	Environmental sample	FMM+K	12
LI C3 PCB	*Pseudoalteromonas* sp. NBRC 107703	1357	100	99.7	0	Environmental sample	FMM+K	12
MS7 F1	*Celluphaga* sp. W5B	1366	100	98.7	0	BC Atlantic salmon	FMM+K	12
MS5 M2	*Dokdonia* sp. 6a	1358	100	99.1	0	BC Atlantic salmon	FMM+K	12
MS5 F3	*Cellulophaga baltica* NN015840	1349	100	99.8	0	BC Atlantic salmon	FMM+K	12

Table 1. *Cont.*

In-House Sample Name	Bacterial Designation or Most Similar NCBI BLAST Comparison	NCBI BLAST Comparison		Obtained from	Grown on	Grown at °C
		16S rDNA Amplicon Length (bp) *	Query Cover, Similarity, E-Value			
Aero sp. kida	*Aermonas* sp.	NA	NA	BC Atlantic salmon	FMM+K	12
V.anguill	*Vibrio anguillarum* 155 5RH	NA	NA	BC Atlantic salmon	FMM+K	12
V.aest	*Vibrio aestuarianus*	NA	NA	Dr. Tim Green	NA	NA
Shewn.sp	*Shewnanella* sp.	NA	NA	Dr. Tim Green	NA	NA
P.unid	*Pseudoalternomonas udina*	NA	NA	Dr. Tim Green	NA	NA
E.coliTop10	Similar to *Escherichia coli* DH10B™	NA	NA	Invitrogen Topo TA Cloning Kit	LB	37

^{T} = Type strain; FMM+K = *Flexibacter martimus* media plus kanamycin (50 μg·mL^{-1}), CA = *Cytophaga* agar, TSA = Tryptic soy agar, LB = Luria-Bertani media, MA = Marine agar, DSMZ = Deutsche Sammlung von Mikroorganismen und Zellkulturen, ATCC = American Type Culture Collection, BC = British Columbia (Canada), NCBI BLAST—National Center for Biotechnology Information Basic Local Alignment Search Tool. Amplicons for identification were generated using universal 16S rDNA primers (27F, 1492R).

Table 2. Primers and probes used for the OVO assay based on 16S rDNA sequences.

Primer or Probe Name	Sequence	T_m (°C)	Length (bp)	Amplicon Length (bp)
Tenaci-G Fw	TRC CTT STA CAK RRG GAT ARC C	49.7	22	~155
Tenaci-G Rv	CTA TCG THG CCA TGG TAA GCC G	65.9	22	
OVO Probe (FAM Fluorophore)	TGT TAA TTA GAG GCA TCT	49.2	18	NA

The developed primers denoted as Tenaci-G forward (Fw) and Tenaci-G reverse (Rv), are in the same genomic location as the MAR primers [24] (Figure 1). Degenerate base pairs were used for the primers to allow a 100% match to all compared *Tenacibaculum* species (Figure 1), and the probe denoted as 'OVO probe' was designed to be 100% homologous to all 16S rDNA sequences described as T.ovo on NCBI, including isolates da5A-8, NCIMB 13127, To7 Br, NBRC 15947, IFO 15947, and IAM14318 (Figure 1). The probe was also 100% similar to T.ovo EKD 002^T [15]. Primers and the TaqMan hydrolysis probe were obtained from Sigma-Aldrich and Eurofins Genomics. Primers and the probe used together (Table 2) will be collectively termed the OVO assay.

2.1.3. Temperature Gradient Test and Amplicon Sequencing

A temperature gradient test (TGT) was completed using two separate PCR master-mixes. In the first, each reaction (i.e., well) had a 10 µL Light-Cycler SYBR-Green Master kit (Roche Diagnostics, Laval, QC, Canada), 1 µL of 10 µM Tenaci-G Fw, 1 µL of 10 µM Tenaci-G Rv, 7 µL of 20-4135-2 isolate DNA (14.5 ng·µL^{-1} [~100 ng per reaction]), and 1 µL of PCR grade water (Roche Diagnostics, Laval, QC, Canada). The second was identical, except that SYBR-Green was substituted with the Probes Master kit (Roche Diagnostics, Laval, QC, Canada), and 1 µL of 2.5 µM OVO probe replaced the water. Both TGTs used a CFX96 thermocycler (BIO-RAD, Hercules, CA, USA) with the following thermal profile: 95 °C (5 min), 40 cycles of 95 °C (30 s), variable annealing temperatures (60, 59.4, 58.3, 56.3, 53.9, 52, 50.7, and 50 °C; 30 s) and 72 °C (30 s). All temperatures for each master-mix were run in triplicate, including no-template controls, with a cut-off cycle quotient (Cq) of 35.

All qPCR products were cleaned (MinElute® Reaction Cleanup Kit, QIAGEN, Hilden, Germany) and subjected to bidirectional Sanger sequencing (University of Alberta, Molecular Biology Facility) using the Tenaci-G Fw and Rv primers. Sequences were aligned in MEGAX, and the resulting consensus sequence was used for NCBI BLAST comparison.

After the temperature gradient tests, all qPCR tests used the following, unless mentioned otherwise, and will be referred to as the optimized master-mix: each reaction (well) had a volume of 20 µL comprising 10 µL of probes master solution (Roche Diagnostics, Laval, QC, Canada); 1 µL of the OVO probe (final concentration 0.125 µM); 1 µL each of the Tenaci-G Fw and Rv primers (final concentration 0.5 µM each); 7 µL of template DNA (100 ng total, final concentration 5 ng·µL^{-1}); 52 °C was selected as the annealing temperature; and no-template controls and positive controls were included. All samples were run in triplicate, and the cut-off Cq was set to 35.

2.1.4. Primer and Probe Optimization

The Tenaci-G Fw and Rv primers underwent optimization in factorial using final concentrations of 1, 0.75, 0.5, and 0.25 µM while the final probe (0.125 µM) and template concentration (20-4135-2, 5 ng·µL^{-1}) remained constant. Similarly, the OVO probe was optimized at several final concentrations (0.25, 0.125, 0.05, and 0.025 µM) while the final primer (0.5 µM) and template concentration (20-4135-2, 5 ng·µL^{-1}) remained constant. ANOVAs and Tukey HSD tests [25] compared Cq values from the primer and probe optimizations. Prior tests were used to interpret reagent efficiency.

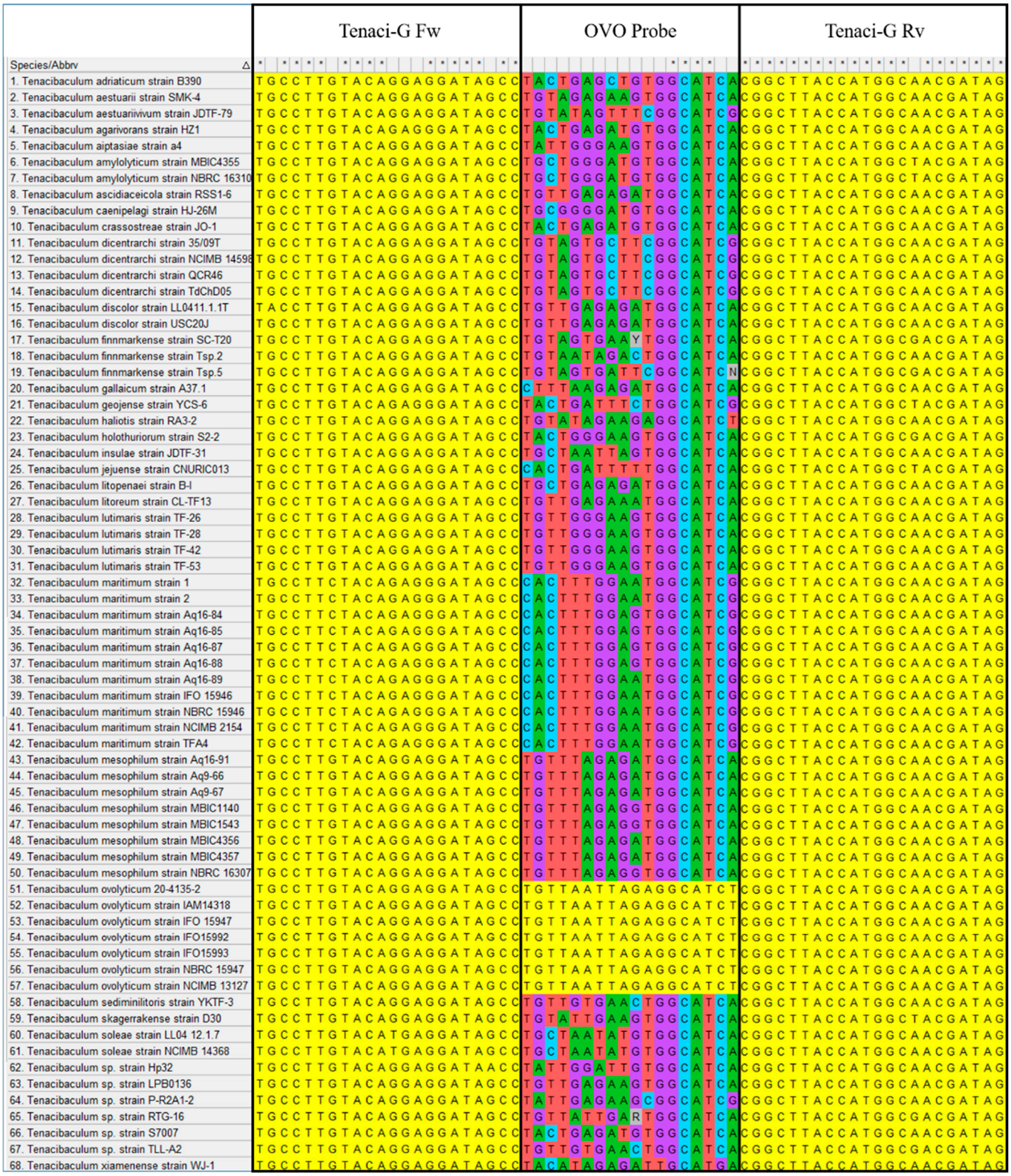

Figure 1. In silico 16S rDNA alignment of several *Tenacibaculum* species from the NCBI website and *T. ovolyticum* 20-4135-2 on MEGAX using MUSCLE for the Tenaci-G forward primer, OVO probe, and Tenaci-G reverse primer. Yellow highlighted boxes represent sequences that match the oligonucleotide.

2.1.5. Standard Curves (Sensitivity and Amplification Efficiency Testing)

Standard curves to determine the OVO assay's sensitivity and amplification efficiency were performed using 8-log units (1000, 100, 10, 1, 0.1, 0.01, 0.001, and 0.0001 ng) of T.ovo 20-4135-2 with or without 100 ng of *S. salar* DNA (muscle or kidney).

Rough estimates for the limit of detection (LOD), were based on the complete genome length (~4.1–4.2 Mb) of T.ovo EKD 002^{T} (DSM 18103) (NCBI: GCF_000430545.1_ASM43054v1), da5A-8 (NCBI: GCF_001641405.1_ASM164140v1), and 20-4135-2 (19), and the DNA Copy Number, and Dilution Calculator (https://www.thermofisher.com/ca/en/home/brands/thermo-scientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/thermo-scientific-web-tools/dna-copy-number-calculator.html [accessed on 10 September 2021]) using a 'Custom DNA Fragment' and 'Genome Length' of 4.15 Mb, indicating ~223,345 copies of the genome per·ng^{-1}.

2.1.6. Outgroup Testing and Fluorescence in Mixed Cultures

To assess the OVO assay's specificity, outgroup testing using 100 ng of the bacterial species genomic DNA mentioned in Table 1 was conducted.

To assess the possibility of reduced Cq values from shifted fluorescence in mixed cultures of *Tenacibauclum* species, samples of T.ovo 20-4135-2 at 100 ng, 50 ng, and T.ovo 20-4135-2 at 50 ng mixed with 50 ng of either *T. ovolyticum* EKD 002^{T} (DSM 18103), *T. dicentrarchi* 20-4116-9, *T. finnmarkense* 20-4106-2, *T. maritimum* R-2^{T} (DSM 17995), *T. gallaicum* A37.1^{T} (DSM 18841), or *T. discolor* LL04 11.1.1^{T} (DSM 18842) isolate were prepared and run under the same aforementioned conditions. An ANOVA and Tukey HSD test [25] compared Cq values between samples.

2.2. Net-Pen Sample Screening

2.2.1. Sample Descriptions

Samples were previously collected and provided [4] (Table 3). Selected samples were from the Midsummer (MS) commercial net-pen site located in the Broughton Archipelago, BC. Samples included collections before the introduction of Atlantic salmon (collection 1 [C1]), one week after smolt entry (collection 2 [C2]), and both during and after treatments for mouthrot (collections 4 [C4] and 6 [C6], respectively). Outbreak status was previously defined [4]. Triplicates of water (0 m, 5 m, 10 m), invertebrate (*Mytilus* sp.), and fish tissues (euthanized and dead) were selected for each stage of a single outbreak (Table 3). Eight bacterial isolates that previously tested negative for *T. maritimum* and *T. dicentrarchi* [4] were also chosen to attempt to fill diagnostic gaps. There were 100 samples: 67 biotic samples consisting of 59 samples from the net-pen and 8 isolates; and 33 abiotic net-pen samples.

2.2.2. DNA Extractions

DNA was previously extracted [4] (Table 3) and stored at −20 °C. Frozen DNA outside acceptable parameters (i.e., $A_{260/280}$ and an $A_{260/230}$ of 1.8–2.0 and 2.0–2.3, respectively) or lacking frozen DNA were re-extracted from samples stored in RNALater (Invitrogen™, Waltham, MA, USA) at −20 °C using the Omega E.Z.N.A. Tissue extraction kit (Omega Bio-tek, Inc., Norcross, GA, USA) with the following modifications: DNA was eluted using 100 µL of provided elution buffer; and water samples collected on 0.22 µm filters were homogenized using a Fisherbrand™ Bead Mill 24 (Fisherbrand, Pittsburgh, PA, USA).

2.2.3. qPCR Application

All net-pen DNA samples were normalized to 14.5 ng·µL^{-1} if possible; in several instances, water samples were below target values and were directly used for qPCR. The optimized master-mix and thermal profile as described in 'Assay Development: Temperature Gradient Test and Amplicon Sequencing' was used. Detection of T.ovo was reported as the mean Cq ± standard deviation.

Table 3. Net-pen sample summary from the Midsummer site [4] used for qPCR analysis with number of biological replicates in parentheses. 'C#' refers to the collection. Water samples are described by sampling depth (m). Fish tissue samples were collected from euthanized (Euth.) and dead or moribund specimens (Dead).

Sample Type:	C1—Pre-Fish Entry	C2—One Week Post-Fish Entry	C4—During Treatment	C6—After Treatment
Water	0 m (2)	0 m (3)	0 m (3)	0 m (3)
	5 m (2)	5 m (3)	5 m (3)	5 m (3)
	10 m (2)	10 m (3)	10 m (3)	10 m (3)
Fish Tissues (Euth.)	NA	Skin (3)	Skin (3)	Skin (3)
		Gill (3)	Gill (3)	Gill (3)
		Upper Jaw (3)	Upper Jaw (3)	Upper Jaw (3)
		Kidney (3)	Kidney (3)	Kidney (3)
Fish Tissues (Dead)	NA	Skin (3)	Skin (3)	Skin (3)
		Kidney (3)	Kidney (3)	Kidney (3)
Invertebrate	*Mytilus* sp. (1)	*Mytilus* sp. (1)	*Mytilus* sp. (2)	*Mytilus* sp. (1)

3. Results

3.1. Assay Development

3.1.1. Temperature Gradient Test and Amplicon Sequencing

All tested temperatures resulted with amplification and fluorescence for both the SYBR-Green and the probes master PCR mix. Melt curve analysis from the SYBR-Green TGT showed a single product with a T_m of 75.5–76 °C. An NCBI BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi [accessed on 1 September 2022]) comparison of a 127 bp section of the 16S rDNA amplicon sequence from the Tenaci-G primers indicated the closest match was *T. ovolyticum* da5A-8 (max and total score: 235, query cover: 100%, E-value: 6e-58, Percent Identity: 100%).

3.1.2. Primer and Probe Optimization

An ANOVA indicated that the Fw primer and Rv primer explained the variation between resultant Cq values (Fw: $F_{3,23} = 311.4$, $p < 2 \times 10^{-16}$; Rv: $F_{3,23} = 8.5$, $p = 5.5 \times 10^{-4}$); however, there was no interactive influence (Fw·Rv: $F_{9,23} = 1.8$, $p < 1.3 \times 10^{-1}$). An ANOVA also indicated that the probe also explained variation in Cq values ($F_{3,7} = 23{,}496$, $p = 2.3 \times 10^{-14}$). The largest concentrations for the final forward (1 µM) and reverse primer (1 µM), and the probe (2 µM) provided the lowest Cq values (Table 4). However, given other Cq values, fluorescence, reagent efficiency, and protocol simplicity, the final concentrations of 0.5, 0.5, and 0.125 µM were used for the forward and reverse primer, and probe, respectively, in further testing (Table 4).

Table 4. *T. ovolyticum* primer and probe assay optimization with the cycle quotient (Cq) and Cq standard deviation (SD). Only the results from the same forward and reverse primer concentration are displayed. Significant differences ($p < 0.05$) between Cq are denoted by different superscript letters; different letter cases indicate separate statistical comparisons.

Assay	Tenaci-G Fw Primer (µM)	Tenaci-G Rv Primer (µM)	OVO Probe (µM)	Cq Mean	Cq SD
OVO	0.25	0.25	0.125	16.99 ^{a}	0.07
OVO	0.5	0.5	0.125	14.84 ^{b}	0.30
OVO	0.75	0.75	0.125	13.97 ^{c}	0.28
OVO	1	1	0.125	13.58 ^{d}	0.06

Table 4. *Cont.*

Assay	Tenaci-G Fw Primer (µM)	Tenaci-G Rv Primer (µM)	OVO Probe (µM)	Cq Mean	Cq SD
OVO	0.5	0.5	0.025	NA [A]	NA
OVO	0.5	0.5	0.05	19.9 [B]	0.11
OVO	0.5	0.5	0.125	15.22 [C]	0.11
OVO	0.5	0.5	0.25	13.61 [D]	0.12

3.1.3. Standard Curves (Sensitivity and Amplification Efficiency Testing)

T.ovo 20-4135-2 could be identified over 8-log units; however, only 6-log units could be used for quantification (0.001–100 ng) based on deviations in the slope (Table 5). From the standard curves, using the limit of quantifiable detection (LOQD), correlation coefficients were above 0.99 and amplification efficiencies were above 88% (Figure 2, Table 5). The minimum LOD and LOQD were estimated to be 22.3 and 223 bacteria, respectively (Table 5).

Table 5. Standard curves generated using *T. ovolyticum* 20-4135-2 DNA using the OVO assay. The limit of detection (LOD) and limit of quantifiable detection (LOQD) show the number of bacteria when a cut-off cycle of 35 is used. Correlation coefficients, slope, and amplification efficiencies are based on the LOQD.

Standard Curve	LOD	LOQD	R^2	Slope	Amplification Efficiency (%)
Genomic	2.23×10^1–2.23×10^8	2.23×10^2–2.23×10^7	0.9995	−3.56	90.78
Spiked (*S. salar* muscle DNA)	2.23×10^1–2.23×10^8	2.23×10^2–2.23×10^7	0.9995	−3.64	88.23
Spiked (*S. salar* head kidney DNA)	2.23×10^1–2.23×10^8	2.23×10^2–2.23×10^7	0.9995	−3.59	89.84

2.23×10^1 (1×10^4 ng)–2.23×10^8 (1×10^3 ng).

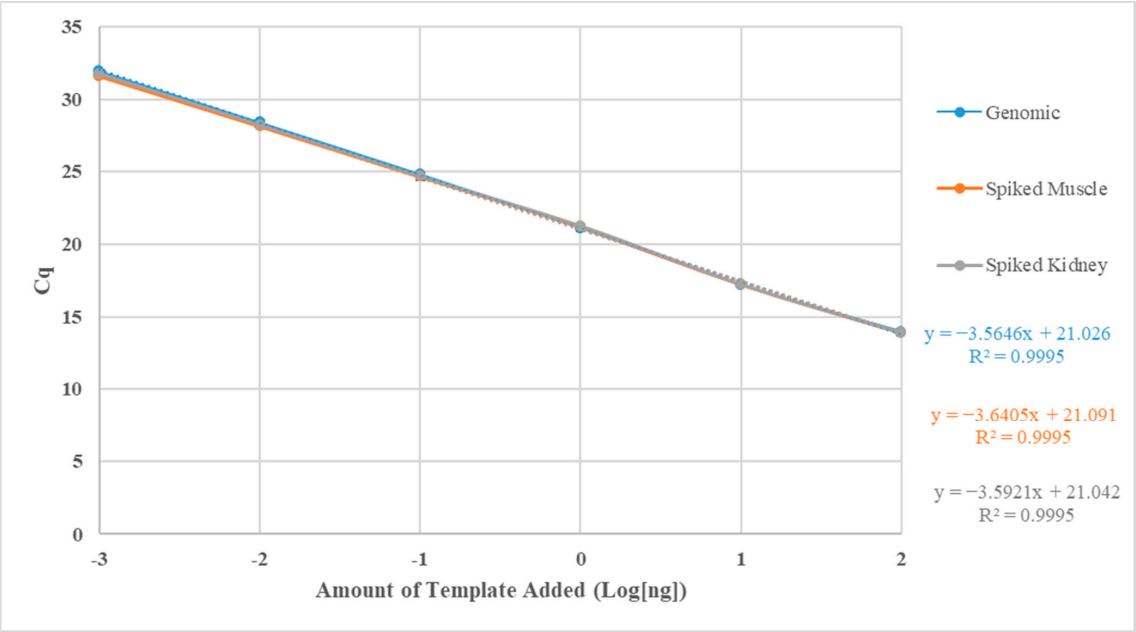

Figure 2. OVO assay qPCR standard curves using *T. ovolyticum* (T.ovo) 20-4135-2 template DNA. All standard curves display the limit of quantifiable detection using the cycle quotient (Cq) against the log-transformed amount of template DNA added (ng). Blue is the standard curve generated using only template T.ovo DNA. Orange and gray standard curves are generated using template T.ovo DNA spiked with 100 ng of Atlantic salmon muscle or kidney DNA. Standard deviation is displayed for each point and was below 0.3.

3.1.4. Outgroup Testing and Fluorescence in Mixed Cultures

Outgroup testing indicated no false positives with the bacteria tested and only the positive control (T.ovo 20-4135-2 [mean Cq = 15.3 ± 0.25]) and the T.ovo EKD 002^T (mean Cq = 20.40 ± 0.043) amplified.

An ANOVA on mixed *Tenacibaculum* cultures identified differences in Cq values ($F_{7,13}$ = 16.39, $p = 1.64 \times 10^{-5}$). An ANOVA identified that the 100 ng T.ovo sample was different from all other tested samples ($p < 4.19 \times 10^{-2}$), with a Cq one less than 50 ng of the same isolate. The sample with two isolates of T.ovo (i.e., 50 ng of 20-4135 and 50 ng of EKD 002^T) was marginally different than 100 ng of T.ovo 20-4135-2 ($p = 4.19 \times 10^{-2}$). The sample with only 50 ng of T.ovo 20-4135-2 was not significantly different from all mixed cultures ($p > 0.05$). The sample with two isolates of T.ovo (i.e., 20-4135 and EKD 002^T) had a lower mean Cq than the 50 ng of T.ovo 20-4135-2; however, it was statistically similar ($p = 6.6 \times 10^{-1}$). The only recorded difference when using only 50 ng of T.ovo 20-4135-2 was when it was combined with *T. dicentrarchi* and *T. maritimum* ($p = 0.04$). The only differences when using two isolates of T.ovo (i.e., 20-4135 and EKD 002^T), aside from what was mentioned, was between the 50 ng of T.ovo with 50 ng of *T. maritimum* ($p = 5.23 \times 10^{-3}$) isolate.

3.2. Net-Pen Sample Screening

All qPCR plates were successfully completed as identified by statistically similar Cq's between T.ovo-positive controls. T.ovo was detected in two out of 100 samples: a water sample at 10 m from before the introduction of fish (water, mean Cq = 25.85 ± 0.083) and in a gill tissue sample taken one week after smolt entry (euthanized, mean Cq = 32.88 ± 0.044).

4. Discussion

4.1. Assay Development

The OVO assay is the first qPCR assay to identify and quantify T.ovo from both biotic and abiotic substances, and the first assay to attempt to quantify an amount of T.ovo in situ. The tested assay was sensitive (LOD and LOQD of 22.3 and 223 bacteria, respectively), specific (only positive for known T.ovo cultures), and had amplification efficiencies (88–91%) that are described in other qPCR studies [26–28]. Amplification efficiencies are within ranges described by standard guidelines [29–31]; however, with amplification efficiencies on the low end of the acceptable range, qualitative results may be more accurate.

Previous research has demonstrated that 16S rDNA sequencing can lead to misinterpreted bacterial species-level designations [18], and 16S rDNA qPCR can lead to false positives when nucleotide polymorphisms occur on the targeted section of the bacteria [28]. This indicates that other genomic targets may bear more utility. For qPCR, however, when fluorescence caused by the target region is distinct from other species and few to no polymorphisms between copies occur (as interpreted by complete genome sequencing on T.ovo 20-4135-2 [19]), it could indicate that 16S rDNA may still be situationally useful [24].

Genomic studies with incomplete de novo T.ovo sequences describe only a single copy of 16S rDNA [12,15]; however, full genome investigations with T.ovo 20-4135-2 describe six copies of 16S rDNA [19]. A potential reason for the discrepancy between copy numbers could be based on the sequencing technologies used; short-read sequencing assembly [12,15] versus long-read sequencing assembly [19]. When using short-read sequencing technologies, highly similar DNA sequences could be aggregated as one sequence [32]. However, with the Cq difference between *T. ovolyticum* 20-4135-2 and EKD 002^T, it would support that different isolates have different copy numbers of 16S rDNA. Varying extraction efficiencies could also lead to further differences between the number of 16S copies within a sample when normalized to a set concentration. With this discrepancy between copy numbers and Cq, the qualitative use of the OVO assay would be more appropriate.

This work is currently based on two T.ovo isolates, and ideally, more isolates would be utilized as with previous work on *T. dicentrarchi* [22]. However, few T.ovo isolates are commercially available or have been identified. Traditional methods to identify isolates include but are not limited to Sanger sequencing and MLSA; however, both methods are

time consuming and can be costly when screening multiple isolates. An economically feasible tool (i.e., qPCR) to identify T.ovo isolates may help overcome this issue and provide a larger set of isolates to test.

4.2. Net-Pen Sample Screening

The abiotic and biotic factors influencing microbial communities associated with mouthrot are multifaceted [33]. Variations in environmental conditions, such as salinity and temperature, may have the potential to influence T.ovo distributions and ecological niches. The single environmental water sample positive for T.ovo (1 out of 100) was collected before the introduction of fish (C1) at 10 m in spring/early summer when the water column had a salinity of 30 ppt and was ~8.25 °C [4]. Greater salinity concentrations have been shown to promote the growth of T.ovo (>50% seawater media [13]; 70-100% seawater media [34]) and T.ovo can grow at cooler temperatures in contrast to other *Tenacibaculum* species (i.e., 4–25 °C [34]). T.ovo was also isolated from deep seawater (i.e., 344 m), which grew optimally at cooler temperatures (10–20 °C) [15]. During subsequent collections (C2, C4, C6) at the net-pen site, the water was not only higher in salinity but also warmer suggesting that growth and presence could be multifaceted.

While T.ovo has been speculated to be a fish pathogen [12,13,15], the absence of T.ovo in 98 out of 100 samples tested in this study suggests that it was not directly responsible for the reported mouthrot outbreak in Atlantic salmon production in BC [4]. Another study described T.ovo with low mean abundance (<0.1%) in the oral cavity of BC produced Atlantic salmon that were deemed healthy even though affected with mouthrot [33]. Reasons for the lack of detection could be due to the number of target bacteria being below the OVO assay's LOD, DNA degradation in samples experiencing multiple freeze–thaw cycles since their collection, and sample selection bias where target bacteria could be present in other samples collected during other mouthrot outbreaks [4]. More work is needed to interpret if T.ovo is an opportunistic pathogen or has an important role in the microbiome of marine fish, which could be accomplished using experimental infection trials with shedders and cohabitants as with *T. maritimum* and *T. dicentrarchi* [5,6].

Fish health status and life stage can also influence microbial distribution [11,33]. Mouthrot is commonly associated with smolts that have recently transferred to saltwater [5,6,33]. The only other detected presence of T.ovo in this study was in the gill tissue of a euthanized fish one week after smolt entry (C2). Stressful events, such as saltwater transfer post-smoltification, result in novel microbiomes being established and increased susceptibility to environmental pathogens. During this environmental transition, the skin and mucosal microbiota of Atlantic salmon have been known to experience increased proportions of *Tenacibaculum* species on healthy and diseased salmon [33,35,36]. Exposing Atlantic salmon in vivo and tracking the change in T.ovo using qPCR or high-throughput sequencing could help clarify if T.ovo is commensal to the microbiome or if it is an opportunistic pathogen.

The absence of T.ovo during and after outbreaks could be related to dysbiosis in the skin and external gill tissue of the Atlantic salmon microbiome. Similar niche requirements within the genus *Tenacibaculum* can lead to a sole species outcompeting others. Members of the *Tenacibaculum* genus can competitively influence the abundance of other species in healthy and diseased Atlantic salmon [33,35], but co-infections with multiple *Tenacibaculum* genotypes have also been reported [11]. In this study, *T. dicentrarchi* was presumed to be a contributing agent to the recorded mouthrot outbreaks [4], where an increase in the abundance of *T. dicentrarchi* may have reduced the abundance of T.ovo. Previous research exposing Atlantic salmon to salmonid alphavirus identified a dysbiotic event, where an increased abundance of T.ovo was recorded [37]. This increase in T.ovo could have occurred as there were no competitors within the genus that were identified that have the same niche requirements. Understanding the relationship between T.ovo and dysbiotic events (whether it is purely opportunistic or contributes to pathogenicity) is important for future research. More work is needed to understand the microbial ecology associated

with T.ovo as there are still gaps in understanding its role in the environment, in hosts, and mouthrot outbreaks.

5. Conclusions

A 16S rDNA qPCR assay for T.ovo was developed for research purposes, where qualitative results appear to be more accurate than quantitative results. Upon application of the assay, two out of 100 samples were positive for T.ovo before an outbreak at a marine net-pen site; and it was concluded that T.ovo was not an agent contributing to the specific outbreak. A multispecies approach might be the next best step in understanding environmental influences and *Tenacibaculum* spp. community dynamics.

Future studies could include testing the non-specific Tenaci-G primers with *T. maritimum* [24] and *T. dicentrarchi*, and the *T. finnmarkense* [28]-specific 16S rDNA probes for a multiplex-PCR screening of BC samples similar to previous work with *Tenacibaculum* spp. in Australia [38]. Studies screening the remaining samples from [4] and other net-pen sites to quantify the fluctuation of T.ovo in the ecosystem during the early stages of mouthrot outbreaks would also be helpful to understand this bacteria's role in future disease outbreaks.

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