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Optimisation and validation of a PCR to detect viable *Tenacibaculum maritimum* in salmon skin tissue samples

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Abstract

A PCR protocol was optimised and validated for the detection of viable *Tenacibaculum maritimum* cells in salmon skin tissue. Viability conventional (vPCR) and quantitative PCR (v-qPCR) assays both had a limit of detection of 10^3 CFU mL^{-1} viable cells. The v-qPCR assay showed a linear quantification over 4 log units. Conventional vPCR showed complete signal suppression when only dead cells were present at concentrations lower than 10^6 CFU mL^{-1}. While the v-qPCR did not result in complete suppression when only dead cells were present, a method was developed to determine if viable cells were present based on the % Δ in cycle threshold (Ct) value. The procedure was validated for high-throughput processing and an enrichment protocol was validated to reliably detect low concentrations of viable cells both with and without a high background of dead cells. Performing this protocol on naturally infected tissues showed that vPCR and v-qPCR reduced the potential for false positives compared to using conventional PCR and qPCR. The optimised protocol developed for this study provides an efficient, reliable and robust alternative for the detection of viable *T. maritimum* in skin tissue.

Keywords: vPCR, viability, *Tenacibaculum*, salmon, high-throughput, enrichment

Introduction

Tenacibaculum maritimum is a Gram-negative bacteria that is the causal agent of marine tenacibaculosis (also known as marine flexibacteriosis). Tenacibaculosis in finfish typically appears as the development of skin ulcers or erosions of the skin, fins, or within the mouth (Avendaño-Herrera et al 2006). This disease is responsible for severe economic losses in marine aquaculture (Toranzo et al 2005) with impacted species including Atlantic salmon (*Salmo salar*), coho salmon (*Oncorhynchus kisutch*) and sea bass (*Dicentrarchus labrax*) (Frelier et al 1994, Holt et al 1975, Bernardet et al 1994). In 2015, *T. maritimum* was isolated from farmed Chinook salmon (*Oncorhynchus tshawytscha*) experiencing summer mortalities in New Zealand (Brosnahan et al 2017). Since this detection, further testing has shown a widespread distribution of *T. maritimum* in Chinook salmon (*Oncorhynchus tshawytscha*) farms in New Zealand (Brosnahan et al 2018).

During the initial testing of samples collected from the 2015 summer mortalities, only a small number of fish were found to be positive by bacterial culture leading to a perceived low prevalence. Culture of *T. maritimum* from field skin ulcer tissue can be challenging as the slow growth of the bacterium allows overgrowth by environmental bacteria within the sample. Subsequent qPCR testing of these samples and of samples from other salmon farms revealed a higher prevalence of *T. maritimum* (Brosnahan et al 2018). However, as the qPCR results of these samples often revealed a low concentration of target bacteria, it was difficult to determine the significance of the findings. Additionally, it could not be determined whether the qPCR results indicated viable bacteria that were likely to be causing disease or dead bacteria that may have been present incidentally within the lesions.

The viability of bacteria within a sample is traditionally determined by culture methods. However, nucleic acid intercalating dyes applied with PCR have been proposed as viability indicators. These assays, termed viability PCR (vPCR) use dyes that specifically penetrate the compromised membranes of dead cells and bind with their nucleic acid (Nogva et al 2003, Nocker et al 2009). The sample is then subjected to light of a specific wavelength to crosslink the dye with nucleic acid to form covalent bonds. Viability is determined as the presence of the crosslinked dyes within the DNA of a dead cell prevents amplification of the target DNA when PCR is performed.

Since vPCR introduction, a variety of dyes have been proposed including: ethidium monoazide (EMA), a derivative of ethidium bromide, (Nogva et al 2003); propidium monoazide (PMA), a derivative of propidium iodide; and more recently, a mixture of the two (i.e. PEMAX). These dyes will bind with any

nucleic acid present in the sample, therefore the effectiveness of vPCR will be impacted by a range of factors including the abundance of non-target organisms in the sample.

This study was conducted to validate and optimise a method for the use of vPCR to detect viable *T. maritimum* within skin tissue samples collected from naturally-infected fish. Determining the viability of the bacteria is important to understanding the pathogenesis of *T. maritimum* infections in fish. This is the first time this technology has reported to be optimised and validated on an aquatic animal pathogen and the results demonstrate the value of developing these assays for the investigation of infectious disease in aquaculture and wild fisheries.

Materials and methods

Bacterial strain and inoculum preparation. *Tenacibaculum maritimum* previously isolated from a Chinook salmon skin ulcer was used in this study. The bacteria was revived from long-term storage on cryobeads (Cryobank, Copan, CA, USA) stored at -80 °C. Inoculum was prepared from pure culture after aerobic incubation at 22 °C for 48 h in Tryptone, Yeast, Glucose broth with sea salt (TYG-M) (Cipriano et al 1996).

Bacterial concentration was calculated by serial dilution and plating onto TYG-M agar in triplicate. Agar plates were incubated for 3 days at 22 °C prior to colony counts being conducted to determine the colony forming units per mL (CFU mL^{-1}).

***Tenacibaculum maritimum* non-viable (dead) cell stock production**. To prepare dead *T. maritimum* cells for use in experiments, 1 mL of a 48 h culture was heated at 99 °C for 10 min in triplicate. The loss of viability was verified by inoculating the heat treated suspension on to TYG-M agar and into TYG-M broth at 22 °C for 7 days. All heat-treated suspensions were cooled to room temperature prior to PEMAX treatment.

DNA extraction

DNA extractions were performed using the Qiagen QIAcube automated extraction platform using the QIAamp HT kit (Qiagen, Hilden, Germany). Pure bacterial cultures and tissue homogenates greater than 100 µL were processed by centrifugation at 14 000 g for 5 min. The supernatant was then carefully removed and the pellet was used for either immediate DNA extraction or stored at -20°C. DNA was extracted without pelleting for tissue homogenates of 100 µL or less. For both pellets and tissue homogenates, 180 µL of lysis buffer (ATL) and 20 µL proteinase K were added and the sample lysed overnight at 56 °C. Once lysed, samples were then processed following the manufacturer's tissue protocol.

Quantitative (qPCR) and conventional PCR

Both qPCR and nested conventional PCR were performed for all samples (Fringuelli et al 2012, Cepeda et al 2003) targeting an amplicon size of 155 and 400 base pairs (bp) respectively. To visualise amplicons from the conventional PCR, samples were loaded into a 1.5 % agarose gel stained with GelRed (Biotium, Hayward, CA) for gel electrophoresis. Assays used a 2 µL DNA template for samples originating from pure culture, run in duplicate and a 2 and 5 µL DNA template for samples originating from tissue. DNA from a pure culture of *T. maritimum* was used as a positive control and no template controls (NTC) with molecular grade water were run in each PCR.

The limit of detection (LOD) of the viability qPCR (v-qPCR) and viability conventional PCR (vPCR) assay were determined following optimisation of vPCR parameters. Viable cells (10^1 – 10^6 CFU mL^{-1}) were diluted in a background of dead cells (10^6 CFU mL^{-1}) and treated with dye. Viable cells diluted in molecular grade water with no dye treatment were used as controls. All dilution series were carried out in triplicate. For v-qPCR, the LOD was determined to be the lowest bacterial concentration where the target molecules amplified during each replication cycle as shown by the cycle threshold (Ct) value. For vPCR, the LOD was assessed as the lowest dilution that all replicates produced amplicons of the expected size.

For the v-qPCR and qPCR, bacterial concentration was plotted against the corresponding Ct value and standard curves were generated by linear regression of plotted points. The amplification efficiency was assessed using the calculation $E = -1+10^{(-1/slope)}$.

Optimisation of vPCR protocol.

PEMAX (GenIUL, Barcelona, Spain) was dissolved by adding sterile water to create a stock solution of 2 mM. Re-suspended dye was stored at -20 °C. A maximum of five freeze thaw cycles was allowed. The following parameters were optimised: PEMAX concentration (including the use of a double dye exposure); PEMAX treatment time; PEMAX treatment temperature; light exposure time and the use of reaction buffers. All optimisation assays were carried out in triplicate. All samples were agitated during PEMAX treatment on a shaking platform (300 rpm) in the dark.

PEMAX concentration and treatment time

Aliquots (500 µL) of *T. maritimum* cells from a 48 h culture (10^8 CFU mL^{-1}) were heat treated and dead cells harvested by centrifuging at 14 000 g for 5 min in vPCR tubes (GenIUL, Barcelona, Spain). The supernatant was removed and the pellet re-suspended in 245 µL phosphate buffered saline (PBS, pH 7.0-7.3, Gibco, Life Technologies, Grand Island, NY, USA). For optimisation of PEMAX concentration

(10, 25, 50 or 100 µM) and treatment time (15, 20 and 30 min), prepared cells were incubated in the dark at room temperature (~22 °C).

Subsequently, a 15 min photoperiod (PhAST blue LED light, GenIUL, Barcelona, Spain) was applied to all treated samples. The following controls were included in the assay: dead cells with PEMAX treatment at each concentration with no exposure to light (dead + PEMAX, - light); dead cells with no treatment (dead – PEMAX); dead cells with no PEMAX treatment with exposure to light (dead – PEMAX, + light) and live cells with no treatment (live – PEMAX).

PEMAX treatment temperature and photoperiod

Separate suspensions of live and dead cells were prepared as above. For treatment temperature optimisation, samples were treated with PEMAX and incubated in the dark at 0, 4, 22 and 30 °C followed by a 15 min photoperiod. Photoperiod was optimised (5, 10, 15 and 30 min), after the optimal incubation temperature was determined. For both experiments, live and dead cell suspensions without PEMAX treatment were used as controls.

Reaction buffers

Separate pellets of live and dead cells, prepared as above, were re-suspended in the following reaction buffers: broth (TYG-M); sodium deoxycholate (0.01, 0.03, 0.1 and 0.3 %) (Sigma-Aldrich, St Louis, Missouri, USA); Triton-X 100 (0.1, 0.5, and 1 %) (Sigma Aldrich); 1 X Reaction buffer + (GenIUL, Barcelona, Spain); pH (7, 7.5, 8 and 8.5, Gibco, Life Technologies, Grand Island, NY, USA); 0.85 % saline (Fort Richard, Auckland, NZ) and artificial seawater (33 parts per thousand (salt), pH 8.2, Forty Fathoms, USA). Following re-suspension, the samples were treated using the optimal protocol. Live and dead cell suspensions without PEMAX treatment re-suspended in PBS were set up as controls.

Double dye exposure

Separate suspensions of live and dead cells were prepared in the appropriate reaction buffer. The re-suspended cells were treated with 50 µM PEMAX and incubated in the dark for 15 min. The samples were then centrifuged at 14 000 g for 5 min, supernatant removed and the pellet re-suspended in 245 µL PBS. A further 50 µM PEMAX was added to some of these samples. All samples were then re-incubated in the dark for 15 min prior to a 15 min photoperiod. Live and dead cell suspensions without PEMAX treatment were used as controls.

Addition of bacterial cells (live and dead) to skin tissue

Homogenates of Chinook salmon skin tissue were prepared in 0.85 % saline at a dilution of 100 mg mL^{-1}. Aliquots of 245 µL of homogenate were used in the experiment.

Mixtures of live and dead *T. maritimum* cells were used to assess the suitability of PEMAX treatment with mixed cells in tissue samples. All samples contained the same number of cells (i.e. 10^3 and 10^6 CFU mL^{-1}) with the ratio of live to dead cells being adjusted from 100, 80, 60, 40, 20 and 0 % live cells.

Spiked skin tissue homogenates without PEMAX treatment were used as controls in both experiments.

High-throughput format

Homogenates of Chinook salmon skin tissue were prepared as above. Mixtures of live and dead *T. maritimum* cells were prepared for comparison between two formats; individual vPCR tubes and a 96 well plate. All samples contained the same number of cells (i.e. 10^3 and 10^5 CFU mL^{-1}) with the ratio of live to dead cells being adjusted from 100, 50 and 0 % live cells for 10^3 CFU mL^{-1} and 0 and 50% live cells for 10^5 CFU mL^{-1}. Aliquots of 100 µL of spiked tissue homogenates were run in parallel with the two formats. For vPCR tubes, the PhAST blue LED light system was used and for the 96 well plate, the photo activation universal light (PAUL, GenIUL, Barcelona, Spain) was used with 100 % light exposure intensity.

Repeatability

Repeatability was carried out by two users within the same laboratory. Two identical panels of 20 artificially spiked skin tissue samples were prepared by placing 100 mg of tissue in a 2 mL safe-lock tube with known amounts of live and dead cells. Samples were then processed using the optimal protocol by the two users in parallel. A suspension of live and dead *T. maritimum* culture (10^8 CFU mL^{-1}) with no treatment were used as controls.

Determining a cut off value for v-qPCR

A cut-off value was determined for interpretation of the v-qPCR results. All data from experiments performed in this study carried out using the optimised protocol were compiled and the % difference between the change in Ct value of the same sample with and without PEMAX dye treatment (% Δ Ct) was assessed. This was carried out by taking the following steps:

- Δ Ct = Ct treated – Ct not treated
- % Δ Ct treated = (Δ Ct / Ct treated)* 100
- % Δ Ct not treated = (Δ Ct / Ct not treated)* 100
- % Δ Ct = % Δ Ct not treated - % Δ Ct treated

Significant differences of % Δ Ct were analysed between samples containing live and dead cells. A cut-off value was determined for results indicative of samples likely to contain live cells. An accuracy of above 95 % was considered acceptable.

Naturally infected samples

Thirty skin ulcer tissues were sourced from farmed Chinook salmon thought to be infected with *T. maritimum* based on clinical signs. Samples were processed 24 h after collection. Tissue was sub-sampled (~100 mg) and placed into a 2 mL safe-lock Eppendorf tube with a 5 mm sterile stainless steel ball. Saline (1 mL; 0.85 %) was then added to the tube and the contents homogenised. One hundred micro litres of the homogenised sample was used for each of the following treatments: 1) heat treated + PEMAX; 2) + PEMAX; 3) – PEMAX and 4) grown in culture (plated onto agar at neat, 10^{-1}, 10^{-2} dilutions and incubated at 22 °C for 2 weeks).

Enrichment protocol

Enrichment of artificially spiked samples was carried out to increase the sensitivity of the v-qPCR assay. Homogenates of Chinook salmon skin tissue were prepared in 0.85 % saline at a dilution of 100 mg mL^{-1}. The following final concentrations of *T. maritimum* cells were added (cells mL^{-1} (live : dead): $10:10^6$; $10^2:10^6$; $10^3:10^6$; $10^4:10^6$; $0:10^3$; $10^4:0$; $10^5:0$; $0:10^4$). Samples were processed immediately with two aliquots (100 µL each) processed as per the optimised protocol: + PEMAX; - PEMAX. At the same time, 300 µL of the homogenate was inoculated into 3 mL TYG-M broth for 48 h at 22 °C.

After 48 h, 2 mL of the broth was removed and centrifuged at 14 000 g for 5 min. The supernatant was carefully removed and discarded and the pellet was re-suspended in 200 µL 0.85% saline. This 200 µL was then divided into two for processing as above: + PEMAX; - PEMAX.

Statistical analysis

A generalised linear model (GLM) was used to determine any significant differences between dye concentrations, time of incubation, incubation temperature, photoperiod, resuspension buffers, application of high-throughput platform and double dye exposure. This analysis examined the differences between Ct values when a qPCR test was conducted on samples with and without PEMAX treatment (R package *nlme*, Pinheiro et al 2015). The response variable in the model was the Ct value, which was log transformed to meet the assumptions of normality. To test for specific pair-wise differences between the treatment types, a multiple comparison procedure using Tukey contrasts was performed (R package *multcomp*, Hothorn et al 2008). *P-values* of < 0.05 were considered significant.

For reaction buffers, differences were also assessed between dead cells compared with the controls (no dye treatment) and live cells (dye treated) compared with the controls. Pair-wise differences were assessed as above.

Results

Tenacibaculum maritimum dead stock production

Loss of viability by heating at 99 °C for 10 min was confirmed as no growth was observed on either the agar or in the broth after 7 days incubation.

Amplification efficiency and sensitivity of conventional-vPCR and q-vPCR

The LOD for v-qPCR was determined to be 10^3 CFU mL^{-1} (Fig. 1). Below this bacterial concentration the Ct value did not increase (data not shown). The v-qPCR and qPCR was linear over the range of 10^3 – 10^6 CFU mL^{-1}. The corresponding amplification efficiencies were 86 % (v-qPCR) and 107 % (qPCR). As expected, the samples from the v-qPCR dilution that were not treated (live + dead samples – PEMAX) maintained a similar Ct value for all concentrations of live cells.

The LOD of the conventional PCR was 10^3 CFU mL^{-1}. Below this bacterial concentration, production of amplicons was not consistent.

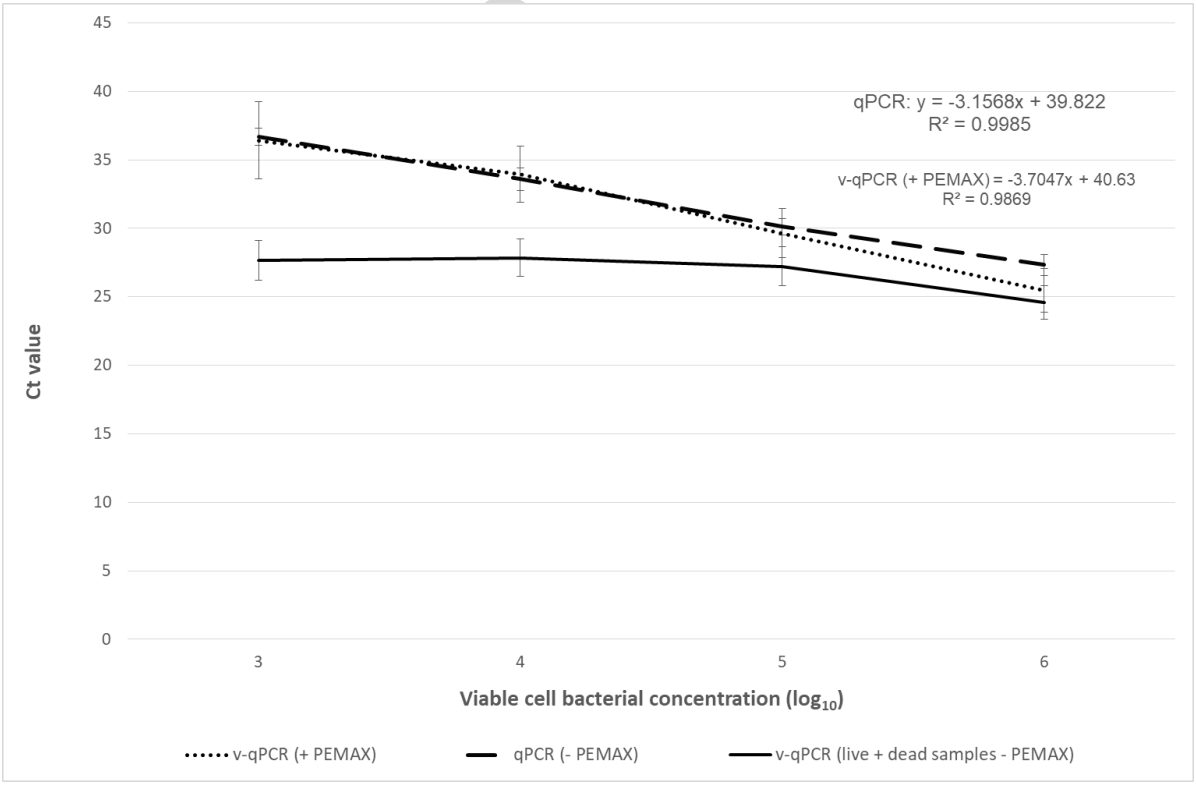

Figure 1. Serial dilutions of live *T. maritimum* cells (10^3 – 10^6 CFU mL^{-1}), n = 3. v-qPCR is diluted in the presence of 10^6 CFU mL^{-1} dead cells with '+PEMAX', treated with dye and 'live + dead samples – PEMAX' not treated with dye. qPCR (-PEMAX) is diluted in molecular grade water and not treated with PEMAX. Error bars represent the SD between the replicates.

Optimisation.

For v-qPCR, a higher dye concentration was more effective as shown by the increasing Ct values of samples containing dead cells. Significant differences were observed between 10 and 100 µM, 10 and 50 µM and also between 25 and 100 µM (p < 0.01, < 0.01 and 0.04 respectively) dye concentrations.

No significant differences were observed in any other parameters tested. These results can be found in the supplementary material (S1). The optimal method used for the rest of this study are summarised in Table 1.

Table 1. Summary of optimised conditions for *T. maritimum* used in this study.

Variable	Optimal parameters
LOD	10^3 CFU mL^{-1}
Inactivation method	99 °C for 10 min
PEMAX concentration	50 µM
PEMAX treatment time	15 min
PEMAX treatment temperature	22 °C
Exposure to light	15 min, 100 % light intensity
Reaction buffer	0.85 % saline

Assessing live cells in artificially spiked tissue samples

Treated samples containing only dead cells (10^3 CFU mL^{-1} in skin tissue), were on average Ct 7.83 higher than samples containing live cells. The number of replicates producing an amplicon via vPCR was lower when the number of live cells in the sample was lower. No amplicons were produced in the conventional vPCR when only dead cells were present.

Treated samples containing only dead cells, (10^6 CFU mL^{-1} in skin tissue), were on average Ct 10.57 higher than samples containing viable cells. The vPCR assay produced amplicons when samples contained live cells and no amplicons were produced in the vPCR when only dead cells were present.

For both bacterial concentrations, the Ct values in the v-qPCR became higher as more dead cells were present. This trend was more evident at the higher bacterial concentration (Table 2, Fig. 2).

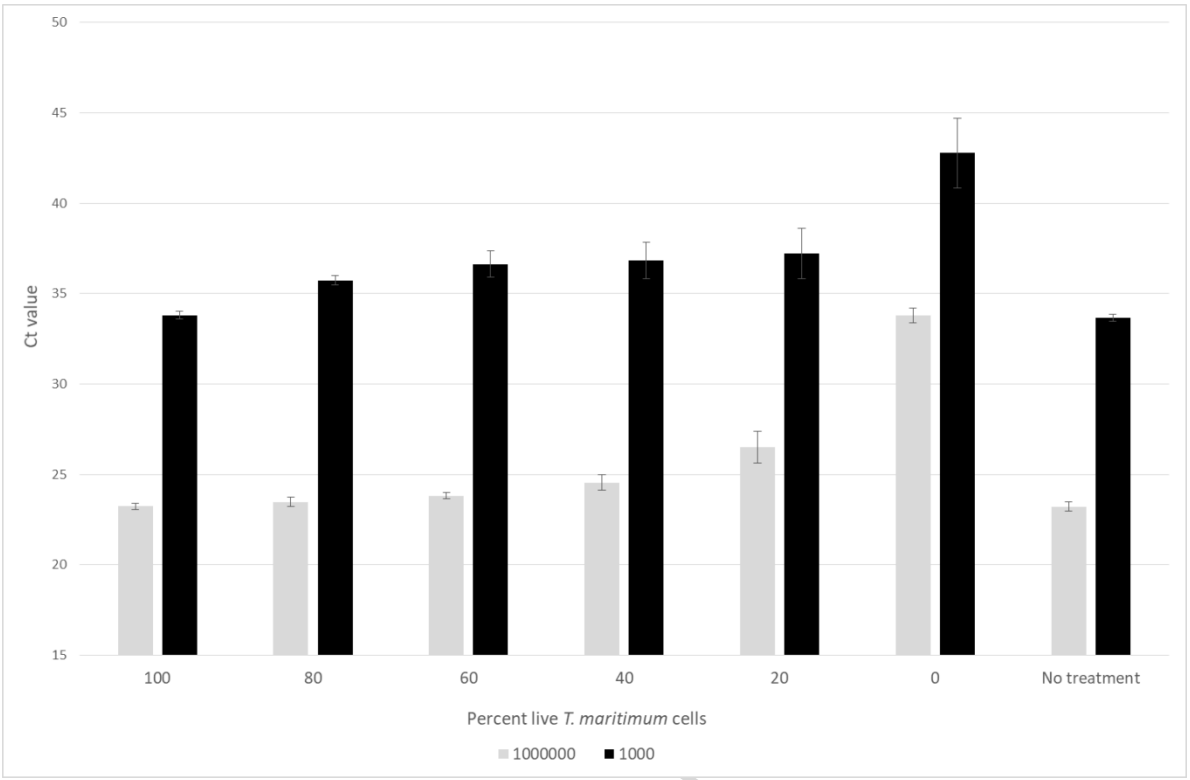

Figure 2. Percentage of live and dead *Tenacibaculum maritimum* cells spiked in skin tissue with a starting concentration of 10^3 and 10^6 CFU mL^{-1}, including controls that contained 100 % live cells (no treatment). n = 3. Error bars represent the SD between the replicates.

Table 2. Conventional vPCR for reaction buffers trialled with PEMAX. The number in last two columns equals the number of replicates that produced an amplicon in the vPCR.

Percent live cells in sample matrix	vPCR result – 10^7 CFU mL^{-1}	vPCR result – 10^4 CFU mL^{-1}
100	3	3
80	3	3
60	3	2
40	3	2
20	3	1
0	0	0

High-throughput testing

No significant differences were observed in the Ct values from the v-qPCR between the two platforms (p = 0.27) or in the vPCR.

Repeatability

No significant difference was seen between the Ct results of the two users (p = 0.71). Sample 20 of the panel contained skin tissue not spiked with *T. maritimum*. This sample gave no signal in the v-qPCR or vPCR in any panel.

Two samples did not give consistent results for the vPCR between the two users. These were both at the lowest bacterial concentration (10^2 CFU mL^{-1}), i.e. lower than the assay LOD (S2).

Interpretation of qPCR results

Results from all tests performed using the optimal protocol were compiled (*n* =103) and the % Δ Ct value determined (Table 3).

Table 3. % Δ Ct between PEMAX treated and PEMAX untreated samples containing live cells, dead cells, or a mix of cells. "Live" = samples that contained a mix of live and dead cells, "dead" = only dead cells were present. † A significant difference was seen between the live and dead samples ($p < 0.0001$).

Sample Type (*n*)	% Δ Ct†
Live (76)	0.37 (0-2.51)
Dead (27)*	5.09 (0.36 - 10.64)

*Six cases showed a Ct value in the PEMAX untreated samples but were not detected in the PEMAX treated samples

A % Δ Ct of 2.5 between PEMAX treated and untreated samples was determined to predict if live cells are present in the sample. For example, a change of ≤ 2.5 % indicates live cells are present and a change of ≥ 2.5 % indicates no live cells are present. Above the LOD of the assay, this value has a 98 % accuracy. Under the LOD of the assay, ≤ 10^3 CFU/mL (Ct 36), the accuracy decreased to 88 %.

Assessing viability of cells in naturally affected tissue samples

Detection of *T. maritimum* in naturally infected tissue was assessed by the following methods: qPCR, PCR, v-qPCR, vPCR and culture.

Of the samples that had no PEMAX treatment, 23/30 produced an amplification curve with an average Ct value of 37.09 in the qPCR and 16/30 samples produced an amplicon in the PCR. For the samples that had PEMAX treatment with no heat treatment, 11/30 produced an amplification curve with an average Ct value of 37.97 in the v-qPCR and 3/30 produced an amplicon in the vPCR. Of the 11 v-qPCR positive samples, 8 were below the LOD of the assay (> Ct 36) thus the results could not be reliably interpreted. For the heat killed and PEMAX treated samples, 11/30 produced an amplification curve with an average Ct value of 37.94 in the v-qPCR and no samples produced an amplicon in the vPCR.

Using the indicative % Δ Ct value of the 11 samples that produced an amplification curve in the v-qPCR, seven samples had a value of ≤ 2.5 % and four had a value of ≥ 2.5 %. This indicates that seven samples are likely to have live cells present and four samples are likely to have no live cells present. However, as only 3 of these samples were above the LOD of the assay, these were the only samples that could be reliably assessed. Of these 3 samples (samples 16, 25 and 27), all values were ≤ 2.5% indicating live cells were present. Two of these (samples 16 and 27) were verified as containing live cells by a positive culture result and also produced a positive amplicon in the vPCR. Sample 25

was not positive by culture or vPCR. As expected, the heat killed PEMAX treated samples all had a value of ≥ 2.5 % in the v-qPCR (Table 4).

Table 5. Naturally infected fish tissue samples processed with and without dye treatment in both conventional and qPCR as well as culture on agar. H = heat killed, N = no fluorescent signal in qPCR. *=below the detection limit of the assay

	qPCR (Ct value)				Conventional PCR amplicon			
Sample	**qPCR**	**v-qPCR**	**H + v-qPCR**	**% Δ Ct**	**PCR**	**v-PCR**	**H+ vPCR**	**Culture confirmation**
1	37.75	39.67*	39.28	0.25	+	-	-	-
2	40.95	N	N		+	-	-	-
3	42.25	N	N		-	-	-	-
4	35.32	37.81*	39.51	0.46	+	-	-	-
5	N	N	N		-	-	-	-
6	N	N	N		-	-	-	-
7	30.43	36.58*	37.16	3.40	+	-	-	-
8	39.12	N	N		+	-	-	-
9	32.02	38.28*	37.2	3.20	+	-	-	-
10	N	N	N		-	-	-	-
11	33.03	36.2*	37.45	0.84	+	+	-	-
12	N	N	N		-	-	-	-
13	40.81	N	N		-	-	-	-
14	35.81	40.54*	42.14	1.54	+	-	-	-
15	39.86	N	N		+	-	-	-
16	30.37	33.02	35.25	0.70	+	+	-	+
17	30.81	39.16*	37.34	5.78	+	-	-	-
18	42.61	N	N		-	-	-	-
19	42.62	N	N		-	-	-	-
20	N	N	N		-	-	-	-
21	39.68	N	N		-	-	-	-
22	41	N	N		-	-	-	-
23	N	N	N		-	-	-	-
24	N	N	N		-	-	-	-
25	29.64	33.05	33.31	1.19	+	-	-	-
26	43.68	N	N		-	-	-	-
27	32.07	35.85	37.87	1.24	+	+	-	+
28	33.13	38.82*	40.83	2.52	+	-	-	-
29	38.65	N	N		+	-	-	-
30	41.5	N	N		+	-	-	-
Average	37.09	37.97	37.94					
SD	4.72	1.63	2.46					

Enrichment protocol

When using the % Δ Ct value on samples processed immediately, samples that contained a low concentration of live cells (< 10^4 CFU mL^{-1}) in a background containing a high concentration of dead cells were analysed as unlikely to contain live cells (≥ 2.5 %, Fig. 3). When these samples underwent enrichment, however analysis showed live cells were likely to be present (% Δ Ct < 2.5 %). Samples that contained only dead cells at a low concentration had a % Δ Ct value of ≥ 2.5 % or no amplification when processed immediately and after enrichment. Compared to the samples processed immediately these samples also had a higher Ct value after enrichment.

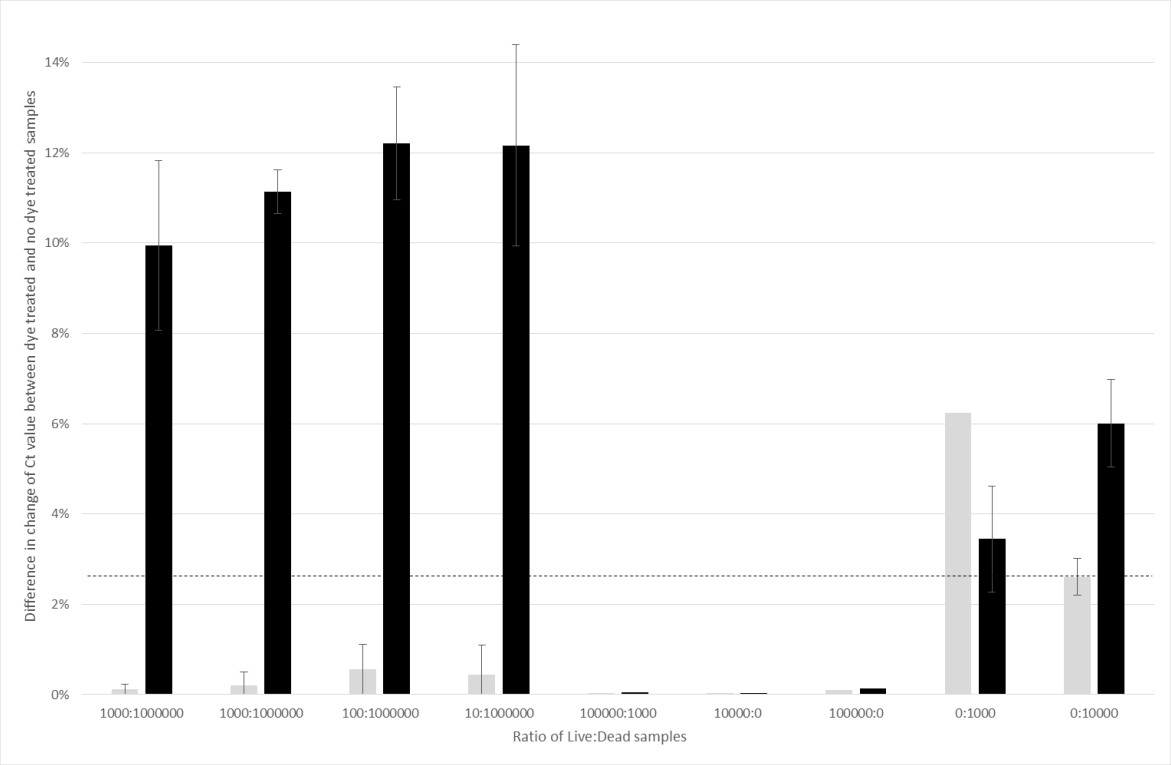

Figure 3. % difference for samples processed immediately (black bars) and processed after enrichment (grey bars). Dashed line = cut off value for cells that are live (2.5 %). *n* = 3. Error bars represent the SD between the replicates. Samples with no error bars only had one replicate amplify in the v-qPCR.

Discussion

The present study represents the first time vPCR has been optimised and validated for an aquatic pathogen. This study further shows the potential of vPCR to be a rapid, sensitive and reliable method to detect live cells in a complex sample matrix. Rapid methods to detect live cells is important in many fields of study, including to inform decisions about a pathogen incursion, differentiate vaccine strains (dead cells) from an infection, and to provide information of the level of live cells present to determine the risk of infection. In the authors' experience, the use of traditional culture methods to answer these questions can be problematic, particularly if the level of infection is low. Importantly, this study

assessed the suitability of v-qPCR and vPCR to identify live cells in a skin tissue matrix where environmental bacteria are expected to be present in relatively high numbers.

The following factors were considered to optimise the viability assays; PEMAX concentration (including a double dye exposure); PEMAX incubation time; PEMAX incubation temperature; photoperiod; and the use of reaction buffers. Dye concentration was the only parameter to have any significant impact on v-qPCR with *T. maritimum.* However, increasing the dye concentration above a certain level did not improve assay effectiveness. Importantly, it was found that none of the concentrations tested were able to penetrate the cell wall of live cells and subsequently impair the amplification of their DNA. One of the three live cell replicates in the vPCR showed complete suppression at the highest dye concentration with the longest incubation time. However, this result was not consistent between all replicates nor was this trend of reduced amplification seen in the v-qPCR.

Incubation temperature was shown to have no effect on dead treated cells, however at 4°C there was a negative impact on live treated cells compared with live treated cells at a lower temperature. However, this trend was not consistent with higher temperatures not showing a significant difference to live cells treated at the lowest temperature.

In contrast to other studies (Codony et al 2015, Takahashi et al 2017), the use of reaction buffers in the present study did not significantly improve the efficiency of the assay for *T. maritimum*. In the present study, reaction buffers either did not improve the assay or resulted in lowered effectiveness, for example when re-suspended in seawater or broth. The effectiveness of ethidium bromide, the chemical EMA is derived from, is known to be influenced by the presence of salt by either competition between the sodium ions and the dye molecules (Graves et al 1981) or due to osmotic shock of the salinity (Shi et al 2011). In the present study, the lowered effectiveness observed is likely due to competition between the dye and the sodium ions in matrices of high salt content, i.e. in seawater and broth. Interestingly, no reductions in efficiency were observed when processing artificially infected skin tissue samples of fish originating from seawater compared to pure culture. However, this would be an important consideration if the sample matrix changed, for example if this protocol was used to detect *T. maritimum* in seawater.

The vPCR showed complete suppression in samples that contained only dead cells when the bacterial concentrations were ≤ 10^6 CFU mL^{-1}. It is documented that longer PCR products result in better or complete suppression of amplicons (Seidel et al 2017, Banihashemi et al 2012). This is due to the dye binding in a certain stoichiometry. That is, the probability that a binding event has occurred and inhibition of amplification during PCR is higher when longer amplicons are amplified. Although, vPCR

has shown to be repeatable and reproducible, it was shown to be less reliable than v-qPCR in artificially spiked samples at low bacterial concentrations. Additionally, conventional PCR is time consuming, does not lend itself to high-throughput processes and with nested conventional PCR, has an inherent risk of contamination which may produce inaccurate results. For these reasons, a v-qPCR protocol is preferred and was optimised in the present study.

The results of the optimised v-qPCR protocol on artificially and naturally infected tissue samples indicated that the tissue matrix was not interfering with assay performance. The protocol was also shown to be robust and transferable with different users producing equivalent results. Testing the v-qPCR on naturally infected tissue demonstrated a lower likelihood of overestimating the infectivity of *T. maritimum* in the sample compared to using qPCR and PCR. The high presence of dead cells within these samples could be an artefact of field sampling leading to cross contamination or it could be an accurate result of dead cells within the skin lesion. Selection of the piece of tissue for testing can affect analysis of pathogen viability. Therefore skin lesions should be sampled from the leading edge as the target pathogen is most likely to be viable and invading the tissue, whereas in the centre of the lesion secondary bacteria are more likely and the target pathogen may no longer be viable (Buller 2014).

Three of the naturally infected tissue samples were within the LOD of the assay (i.e. < Ct 36). These three samples were found to contain viable cells based on the % Δ Ct and two of them were confirmed positive by culture. Detection by culture from these samples proved difficult with heavy mixed environmental growth and as a result it is possible that the culture result for the one negative sample was inaccurate. Repeat processing of naturally infected tissues with an enrichment protocol prior to v-qPCR and culture may help to clarify these results. An enrichment study to detect *Helicobacter pylori* in naturally infected water samples by Santiago et al (2015) produced similar results. Santiago et al (2015) used two methods: v-qPCR and direct viable count combined with fluorescent *in-situ* hybridization (DVC-FISH), on samples that were qPCR positive prior to enrichment and negative after enrichment. This showed that the qPCR positive results were from nonviable cells and that qPCR is not a suitable indicator for determining the infective potential of a sample.

The artificially and naturally infected tissue samples along with the repeatability experiments performed in this study highlight the difficulties of interpretation of lower bacterial concentrations. The enrichment protocol produced a reliable method to detect viable cells at low bacterial concentrations in artificially spiked skin tissue. Samples that contained only dead cells were either not amplified or had a much higher Ct value after enrichment. This effect is unlikely due to the dilution of the sample as the process ensured the same amount of homogenate was used after enrichment. This effect is more likely due to

DNA degradation of the heat treated samples. This degradation of DNA during the heat process was demonstrated occasionally in the present study between the Ct results of the live and dead control samples and it is unknown if this same rate of degradation would occur in naturally infected tissue samples. Although the additional enrichment step adds extra time to the process, it remains more efficient than traditional culture methods.

The present study observed a reduced amplification efficiency of the v-qPCR with live cells diluted in a background of dead cells. This could be due to the carryover of dye or dead cell debris from the sample interfering with the PCR reaction. However this is unlikely to affect the results of v-qPCR for detection of viable *T. maritimum* if the concentration of bacteria is above the LOD. When the concentration of the sample is below the LOD, the enrichment protocol should be employed to improve the effectiveness of v-qPCR. As the LOD is unknown prior to testing the sample, it is recommended to use the enrichment protocol as a standard method. The linear range and LOD for v-qPCR and vPCR of *T. maritimum* in the present study is consistent with previous studies (Dinu et al 2012, Maće et al 2013, Thanh et al 2017, Daranas et al 2018). Similarly, Fittipaldi et al (2012) found that v-qPCR for live cell concentrations < 10^3 CFU mL^{-1} in the presence of a high number of dead cells was unreliable.

The present study has shown the importance of a thorough validation process for both the target pathogen and sample matrix with clearly defined limitations. Live *T. maritimum* in skin tissue, can be detected using vPCR or q-vPCR by calculating the % Δ Ct. This has been shown to be a reproducible and reliable method when the amount of cells present are above the LOD of the assay. This sensitivity is expected to be within the range of an infectious dose of *T. maritimum*. The detection limit reported in the present study is below the lowest published infectious doses for this pathogen of 6.36 x 10^5 CFU mL^{-1} (Frisch et al 2018, Rahman et al 2015, van Gelderen et al 2010). Moreover, the natural load on the gills of fish infected with *T. maritimum* has been reported to be equivalent of 10^{10} CFU mL^{-1} by qPCR (Downes et al 2018).

Conclusion

The developed protocol has shown reliable detection of live *T. maritimum* cells from skin tissue above 10^3 CFU mL^{-1} in artificially spiked samples. The detection of live *T. maritimum* cells in naturally infected samples shows the utility of this assay to investigate disease pathogenesis. However, further samples will need to be tested to improve v-qPCR assay confidence. Additionally, as the method can be transferred to a 96 well plate format, high-throughput processing to increase efficiency is possible. An enrichment protocol is recommended to allow detection of low numbers of live cells with and without a background of high concentration of dead cells.

Competing interests

The authors declare that they have no competing interests.

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Highlights

- PCR method to detect live *Tenacibaculum maritimum* cells in skin tissue samples.
- Enrichment protocol can reliably determine low concentrations of live *T. maritimum* cells by PCR.
- Method is adapted to high-throughput to allow efficient processing of samples.