## Title

A highly sensitive, non-invasive qPCR-based strategy for direct quantification of *Yersinia ruckeri* in fish faeces

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## Abstract

Finfish with asymptomatic *Yersinia ruckeri* infections pose a major risk as they can transmit the pathogen and cause clinical outbreaks in stock populations. Current tools have insufficient quantitative ability for accurately detecting the trace levels of *Y. ruckeri* typically associated with asymptomatic infection, necessitate invasive or lethal sampling, or require long processing times. This study presents a highly sensitive qPCR-based method, targeting part of the *Y. ruckeri* 16S rRNA sequence, that is capable of detecting extremely low levels of *Y. ruckeri* in non-invasively collected faecal samples. Quantitative precision and accuracy of faecal sample analysis was consistent, despite the complexity of the faecal matrix. The assay demonstrated linearity over a 6 log-wide dynamic range. Its limit of detection (LOD) and limit of quantification (LOQ) were 4 and 10 copies of the target sequence, respectively. Sensitivity of the assay was comparable to other qPCR-based methods without requiring invasive or lethal sampling. Applicability as a screening strategy was tested using passively collected faecal samples. Asymptomatic *Y. ruckeri* infection was detected in all samples, though none of the fish exhibited overt infection. This method will be beneficial for finfish disease management if developed further as a non-invasive, screening tool against asymptomatic *Y. ruckeri* infection.

# 1 Introduction

*Yersinia ruckeri,* a Gram-negative member of the family *Enterobacteriaceae*, is a major pathogen impacting aquaculture. First identified as the cause of yersiniosis or Enteric Redmouth disease (ERM) from rainbow trout, *Oncorhynchus mykiss* Walbaum, in the Hagerman Valley, USA (Ross et al. 1966), *Y. ruckeri* causes disease in various species of farmed and wild fish (Davies and Frerichs 1989). It is now considered a ubiquitous pathogen that has been isolated from diseased fish, as well as other taxa and the environment, in locations around the world (Davies and Frerichs 1989, Wheeler et al. 2009). Salmonids are known to be particularly susceptible to *Y. ruckeri*, which is capable of causing mass mortalities, and the pathogen significantly impacts global salmonid culture.

Clinical infections typically present as haemorrhaging in and around the oral cavity, vent and the base of fins, as well as blood spots in the eye and exophthalmia (Busch 1978, Carson and Wilson 2009). *Yersinia ruckeri* can establish subclinical infection in the distal fish intestine, resulting in asymptomatic carriers. These carriers act as pathogen 'reservoirs', transmitting infection horizontally through the water column via faeces and periodic intestinal sloughing, particularly when stressed (Tobback et al. 2007). Research has indicated that infection may be detectable in intestine and faeces prior to onset of acute systemic infection (Rodgers 1992).

While vaccination has managed to protect fish to varying degrees against the effects of acute yersiniosis and subsequent mortality, it has been unable to prevent the establishment of asymptomatic carriers within surviving populations (Austin et al. 2003). Clinical expression from pre-existing subclinical infection has been reported in different asymptomatic salmonid populations up to two months after a clinical outbreak (Rodgers 1992, Tobback *et al.* 2007). A method for accurate detection and precise quantification of subclinical infections would therefore benefit disease management strategies.

Pathogen detection has traditionally been achieved using culture-based methods in combination with serological or biochemical identification techniques. Results may require several days or even weeks

to obtain, and may not always be ambiguous as the phenotypic characteristics by which the bacteria are identified may be difficult to interpret and classify, or may not always be expressed (Tholozan et al. 1999). Molecular techniques constitute an important advance in the detection of pathogens, offering rapid, highly specific and straightforward alternatives to traditional microbiological analyses.

The 16S rRNA gene has been widely used as a phylogenetic marker in molecular assays investigating bacterial diversity in natural ecosystems (Ward et al. 1990). It is a conserved, common but distinctive, cellular component that varies in an orderly fashion across phylogenetic lines and is present as a multicopy gene in the prokaryotic genome, making it an ideal target for specific and highly sensitive detection of a target microbial species. Consequently, it has been employed widely in detection and identification of bacterial species (Ward *et al.* 1990, Head et al. 1998), and represents an excellent candidate for use in molecular pathogen detection assays.

Among the numerous molecular techniques available, quantitative real-time PCR (qPCR) is one the most widely used techniques for detection of pathogens. The ability to monitor PCR product accumulation through the early exponential stage of amplification allows qPCR assays to both detect and quantify target DNA. This is particularly advantageous in enabling simultaneous detection of pathogen presence and pathogen loads in samples. Consequently, qPCR has been applied to the diagnosis of a wide range of pathogens from various sources including food products (Postollec et al. 2011), faecal and environmental samples (Bélanger et al. 2003, Rinttilä et al. 2004), and infected plant and animal tissues (Osman and Rowhani 2006, Marancik and Wiens 2013). These applications include assays for detection of *Y. ruckeri* (Argenton et al. 1996, Bastardo et al. 2012), though commercial applicability of these particular methods and their value as a screening tool are limited, as they involve invasive or destructive sampling of putatively infected fish.

Use of faecal samples represents a feasible, non-invasive means of sampling putative carrier fish for asymptomatic *Y. ruckeri* infection. A non-invasive method offers the possibility of screening each individual of a population where necessary while minimising sampling impacts on stock. Such sampling

51 strategies would offer a far more accurate understanding of asymptomatic infection prevalence within
52 populations than possible through random representative sampling. However, faeces represent
53 complex biological samples not only due to the presence of a variety of bacteria in addition to the
54 target species, but also due to a range of inhibitors such as complex polysaccharides, polyphenolic
55 compounds, and food degradation products (Monteiro et al. 1997, Koonjul et al. 1999). The complexity
56 of the faecal matrix impedes extraction of target material and inhibitory constituents can hamper PCR
57 assay efficiency if not effectively eliminated from the reaction, resulting in reduced analytical
58 resolution and reliability (Monteiro *et al.* 1997, Pontiroli et al. 2011). Previous recommendations for
59 obviating these issues have included specialised commercial extraction kits (Pontiroli *et al.* 2011) and
60 multi-stage purification protocols such as the Griffiths method (Griffiths et al. 2000) .

61 Studies have previously attempted detection of *Y. ruckeri* in faeces, and initial efforts focused on the
62 development of novel selective media met with some success, though the method could not
63 consistently distinguish target bacteria from competing microbes (Rodgers 1992). More recently,
64 selective enrichment culture media were developed and used successfully in conjunction with PCR for
65 *Y. ruckeri* detection (Wilson and Carson 2001). However, relying on culture of bacteria from initial
66 samples, these methods are intrinsically unsuitable for accurate and precise quantitative analysis.

67 The objective of this study was to develop a direct method for the detection of *Y. ruckeri* in an organic
68 matrix such as faeces, which can be collected non-invasively from finfish, and for the accurate
69 quantification of pathogen present at extremely low levels, using a quantitative real time PCR assay.
70 To provide a measure of accuracy and precision in an applied context, the results were also validated
71 against outcomes using fish spleen, which is a more common target for finfish pathogen detection
72 assays.

## 2 Materials and Methods

### 2.1 *Yersinia ruckeri* culture

Tryptone soy broth (TSB; Oxoid, UK) was inoculated from frozen *Y. ruckeri* stock (serotype 01b, strain UTYR001) and incubated at 18°C for 18 h. Cells were concentrated by centrifuging at 8000 x*g* for 10 min. Recovered cells were washed twice by resuspending in 1M phosphate-buffered saline (PBS; pH 7.2) and centrifuging as before to eliminate any extraneous nucleic acid from lysed cells in culture. Washed cells were resuspended in 2 mL PBS that had been pre-cooled to 4°C to produce a stock suspension. Aliquots of 100 µL cell suspension diluted 1:100 in PBS were prepared in triplicate, and cells inactivated by addition of 0.03% (v/v) neutral buffered formalin (NBF) followed by incubation at 18°C for 2 h, for bacterial enumeration. Following inactivation, cell concentration in aliquots was enumerated optically using a haemocytometer and used to determine mean cell concentration in stock suspension. Stock cell suspension was diluted accordingly in PBS to achieve a final concentration of $1 \times 10^6$ cells $mL^{-1}$.

### 2.2 Organic matrices: spleen and faeces

All organic matrix samples were obtained either through non-invasive collection (faeces) or from mortalities unrelated to this study (faeces and spleens), obviating the need for animal ethics considerations.

Fish spleens were used to determine the comparative effectiveness of analysing faecal samples to determine *Y. ruckeri*-infection status of fish. Confirmed *Y. ruckeri*-free spleens were obtained from barramundi, *Lates calcarifer* (Bloch), fry mortalities weighing approximately 13 g, which had been maintained in a pathogen-free environment and sacrificed as part of a non-disease related study. Spleens were excised aseptically, fixed in 1.5 mL RNA preservation solution (4M ammonium sulfate, 25 mM sodium citrate, 10mM EDTA, pH 5.2) over 24 h at 18°C, and then stored at -20°C.

Faeces for assay validation were obtained from a fresh Atlantic salmon, *Salmo salar* L., mortality weighing approximately 1.1 kg. To eliminate possible environmental contamination during assay validation, the entire intestine was excised, cut open longitudinally, and faeces gently scraped off and collected aseptically. Faeces were centrifuged at 100 x*g* for 10 min and the supernatant discarded. Concentrated faecal solids were combined with an equal weight of PBS, agitated vigorously, and used immediately to prepare samples for analysis as described in Section 2.3.

Faeces used to substantiate applicability of the assay as a non-invasive tool were collected from five 500 L freshwater tanks, each containing approximately 25 rainbow trout (≈250 g) showing no signs of disease, 3 h post-prandium. Mortalities collected from these populations several months previously had exhibited typical signs of yersiniosis but had not been analysed for positive pathogen identification. Faeces samples were recovered from 1mm mesh collectors appended to the tank outlets. Faecal solids were centrifugally concentrated at 100 x*g* for 10 min and supernatant, which comprised approximately 50% of the initial sample weight, was discarded.

### 2.3 *Yersinia ruckeri* dilution series and spiked sample preparation

A 10-fold serial dilution of the *Y. ruckeri* cell suspension in PBS was prepared, ranging from $1 \times 10^6$ cells $mL^{-1}$ to $1 \times 10^0$ cells $mL^{-1}$. From each dilution, five sets of duplicate 1 mL aliquots were prepared, producing five series of seven duplicated 10-fold dilutions. Two of these dilution series were used to spike faecal samples, one was used to spike spleen samples, and two maintained as pure-culture dilutions for use as standards (Fig. 1). In addition to molecular analysis, spiked faeces and pure-culture standards were analysed using culture-based techniques to provide comparison of assay performance.

*Yersinia ruckeri* serial dilution aliquots were combined with equal volumes of faeces suspension (≈500 mg faecal solids), producing two series of seven duplicated 10-fold serial dilutions of *Y. ruckeri* cells in faeces suspension. Spiked samples were mixed thoroughly and incubated at 4°C for 12 h, with 30 s vortex-agitation every hour. Following incubation, as per a modified technique from Rodrigues-Szulc et al. (1996) each sample was vortex-agitated for 30 s following addition of 0.1% (v/v) Tween 20

(Sigma-Aldrich, USA), faecal solids precipitated at 100 x*g* for five min and supernatant transferred to new 1.7 mL tubes. One replicate series was analysed through culture-based methods, while the other was prepared for assessment by molecular techniques. For molecular analysis, bacteria and any remaining solids were precipitated at 8000 x*g* for 10 min. Pellet and bottom 100 µL of supernatant was retained for analysis, with remaining supernatant discarded. For microbiological analysis, supernatant was used as obtained.

Spleens (Mean weight: 25.2 ± 3.1 mg) were removed from fixative and rinsed lightly in PBS. Individual spleens were placed in 14 separate 2.0 mL tubes containing 200 µL PBS, and homogenised aseptically using a micro-pestle. From each serial dilution of *Y. ruckeri* 1 mL volumes were added to two spleen samples, producing seven duplicated 10-fold serial dilutions of *Y. ruckeri* cells combined with homogenised spleen. Aliquots were thoroughly mixed and incubated at 4°C for 12 h, with 30 s vortex-agitation every hour. Following incubation, bacteria and splenic solids were precipitated at 8000 x*g* for 10 min. The pellet and bottom 100 µL of the supernatant was retained for analysis, with the remaining supernatant discarded.

One standard (pure-culture) dilution series was prepared for molecular analysis. Briefly, bacteria were harvested by centrifuging at 8000 x*g* for 10 min, and pellet and partial supernatant retained as with other samples. The second standard dilution series was used for microbiological analysis without further modification.

### 2.4 Preparation of non-invasively collected faeces for molecular analysis

For detection and quantification of *Y. ruckeri* in non-invasively obtained faecal samples, 500 mg of faecal solids from each sample were combined with equal volumes of PBS containing 0.1% (v/v) Tween 20 and vigorously vortex-agitated for 30 s. Faecal solids were precipitated at 100 x*g* for five min and supernatant transferred to new 1.7 mL tubes for analysis.

## 2.5 Detection and quantification of *Y. ruckeri* in faeces using conventional microbiological techniques

For detection and quantification of *Y. ruckeri* in samples, supernatant from spiked faeces was applied to blood agar plates (Remel, USA) for enumeration of colony forming units (CFU) using a simultaneous colony counting technique modified from Chen et al. (2003). Briefly, from each replicate of every dilution, three 10 µL volumes were applied (six droplets in total per dilution), with a maximum of four dilutions per plate. Plates were prepared in triplicate, dried in laminar air flow for 15 min and incubated at 18°C for 24 h. *Y. ruckeri* dilutions prepared as standards (only PBS, no organic matrix) were also similarly plated and incubated. CFU were identified post-incubation based on colony morphology (Rodgers 1992, Carson and Wilson 2009) and optically enumerated to determine *Y. ruckeri* presence.

Limit of detection (LOD) for the CFU-based assay was defined as being the minimum nominal *Y. ruckeri* cell concentration of samples, at which *Y. ruckeri* presence was detected in at least 95% of replicates, irrespective of whether the number of CFU fell within the countable range of 3 – 30 per droplet (Chen et al. 2003). As far as the authors are aware, prior literature has not included Limit of Quantification (LOQ) estimations for a microbiological quantitation assay using this format. For the purposes of this study, the LoQ of the assay was defined as the minimum *Y. ruckeri* cell concentration (with CFU per droplet within the countable range) at which the Coefficient of Variation across all replicates for that concentration was less than 35%.

## 2.6 Detection and quantification of *Y. ruckeri* in faeces and spleen using quantitative real-time polymerase chain reaction (qPCR)

### 2.6.1 Extraction of total nucleic acid

All samples were incubated at 37°C for 30 min after the addition of 395 µL extraction buffer (4 M Urea, 0.2 M sodium chloride, 1 mM sodium citrate, 1% SDS) supplemented with 5 µL Proteinase K to lyse

cells. The resulting suspension was cooled on ice for 5 min and protein, cellular debris, and detergent were removed by centrifugation in 7.5 M ammonium acetate at 14000 x*g* for 5 min at 18°C. Nucleic acids were recovered from the supernatant by isopropanol precipitation at 14000 x*g* for 10 min at room temperature. The nucleic acid pellet obtained was washed twice with ethanol and eluted in 200 µL elution buffer [molecular-grade water containing 10 µM TRIS-HCL and 0.05% (v/v) Triton X100 (Sigma-Aldrich)].

### 2.6.2 Quantitative real-time PCR

All qPCR analyses were conducted on a CFX Connect Real-Time PCR detection system (Bio-Rad) with efficiency and linearity ($R^2$) of standard curves held to between 85-110% and 0.98-1.00 respectively. A hydrolysis (TaqMan) probe-based PCR assay was developed for detection of *Y. ruckeri* (Ghosh et al. 2016). Primers used to amplify a 247-bp region of the *Y. ruckeri* 16S rRNA gene were previously developed and tested exhaustively to establish intra- and inter-species specificity (Carson 1998, Wilson and Carson 2001). Primer and probe sequences used are presented in Table 1. Each PCR reaction consisted of 5 µL 2X MyTaq HS Mix (Bioline), forward and reverse primers (400 nM each), *Y. ruckeri* 16S ribosomal gene-specific hydrolysis probe (100 nM) and 2 µL template in molecular grade water to a final volume of 10 µL. No-template controls (NTC) using molecular grade water instead of template, extraction controls and sample controls (unspiked) were included in each run. Cycling conditions consisted of an initial activation of DNA polymerase at 95°C for 3 min, followed by 40 cycles of 5 s at 95°C and 30 s at 60°C. Assay results were quantified by analysis of raw fluorescent unit (RFU) data from cycles 5 to 40 using the CM3 mechanistic model developed by Carr and Moore (2012) included in the qPCR package for RStudio statistical computing software (R Core Team 2013), and verified empirically.

### 2.6.3 Establishment of Limit of Detection (LOD), Limit of Quantification (LOQ) and dynamic range of qPCR assay

Based on 16S gene abundance as reported by Johnson et al. (2015), *Y. ruckeri* was estimated to have seven 16S rRNA gene copies per cell.

Limit of detection for the qPCR assay used was defined as being the minimum concentration at which the target gene fragment could be amplified with 95% certainty. Limit of quantification was defined as the minimum concentration at which the assay accurately identified an expected number of 16S gene copies in 100% of samples with a mean coefficient of variation (CV) of less than 35%.

For LOD and LOQ determination 100 µL aliquots of eluted nucleic acid were pooled from each replicate of the highest concentration *Y. ruckeri*-PBS samples. Pooled nucleic acid suspension was diluted in elution buffer to a concentration equivalent to approximately $5 \times 10^8$ 16S gene copies $mL^{-1}$. From this suspension, 10 further dilutions ($5 \times 10^7$, $5 \times 10^6$, $5 \times 10^5$, $5 \times 10^4$, $5 \times 10^3$, $4 \times 10^3$, $2 \times 10^3$, $1 \times 10^3$, $5 \times 10^2$, and $2.5 \times 10^2$ 16S gene copies $mL^{-1}$) were prepared. All replicates for each dilution were independently prepared, with 16 independent replicates prepared for each of the five lowest concentrations ($4 \times 10^3$ to $2.5 \times 10^2$ gene copies $mL^{-1}$) to account for Poisson variability intrinsic to low-copy dilutions.

Replicate dilutions were used at 2 µL $reaction^{-1}$ in qPCR, producing mean estimated copy-concentrations of $1x10^6$, $1x10^5$, $1x10^4$, $1x10^3$, $1x10^2$, $1x10^1$, 8, 4, 1 and 0.5 copies $reaction^{-1}$. Assay conditions, and reagent concentrations were identical to those previously used. Results were quantified by analysing RFU data from cycles 5 to 40 as before, and compared to expected copy number distributions as determined by Rutledge and Stewart (2010).

211 # 3 Results

212 ## 3.1 Microbiological detection and quantification of *Y. ruckeri* in spiked faeces

213 No CFU were detected in dilutions corresponding to fewer than 100 cells $mL^{-1}$ using culture-based
214 methods in either faecal samples or standards (pure culture). The LOD of the assay, where at least
215 95% of replicates showed presence of *Y. ruckeri*, was ≥ 1 x $10^2$ cells $mL^{-1}$. The LOQ of the assay,
216 determined using standards, was 983.33 cells $mL^{-1}$ (CV=28.4%) of *Y. ruckeri*. The concentration of *Y.*
217 *ruckeri* in spiked faecal samples was 1044.44 cells $mL^{-1}$ (CV=32.9%) at the greatest detectable dilution.

218 ## 3.2 Detection and quantification of *Y. ruckeri* in faeces and spleen using qPCR

219 When validating the assay using decimal dilutions of pure culture (standards) and spleen and faecal
220 samples spiked with *Y. ruckeri*, there was no amplification in any of the negative controls. *Yersinia*
221 *ruckeri* was successfully detected in all samples analysed by qPCR in standards, demonstrating linearity
222 from $10^0$ to $10^6$ cells $mL^{-1}$ (Efficiency=99.9%; $R^2$=0.998; Slope=3.325). Amplification in all spiked faecal
223 samples was delayed in comparison with standards and spiked spleen samples, exhibiting
224 approximately 4-fold loss in sensitivity (Fig. 2).

225 Assay applicability as a non-invasive screening tool was evaluated using non-invasively collected faecal
226 samples, which were all positive for *Y. ruckeri*. Concentrations of *Y ruckeri* detected ranged from 4 x
227 $10^3$ to 5.5 x $10^3$ copies per sample, equivalent to approximately 85 to 134 cells $mL^{-1}$ faecal suspension
228 (≈500 mg faecal solids).

229 ## 3.3 Limit of Detection (LOD) and Limit of Quantification (LOQ) for qPCR assay

230 Limit of Detection for the assay was established at 4 copies per reaction, at which dilution 100% of
231 replicates were amplified successfully. Limit of Quantification was determined to be 10 copies per
232 reaction, at which dilution the mean detected copy number was 7.37 (CV=31.54%).

## 4 Discussion

Successful detection and direct quantification of *Yersinia ruckeri* in spiked barramundi spleen and Atlantic salmon faeces was achieved in this study using qPCR-based detection of 16S ribosomal DNA. The method presented here provides an effective alternative to conventional microbiological methods for detection and quantification of *Y. ruckeri*, offering rapid and highly sensitive analytical ability over a 6 log-wide linear dynamic range using non-invasively acquired samples.

The assay was able to detect and quantify low levels of *Y. ruckeri* in spiked faecal samples, supporting the viability of this method of faecal testing as a straightforward, non-invasive strategy for screening fish populations. For the purposes of assay validation, faeces were obtained from excised Atlantic salmon intestine to minimise environmental contamination. However, aseptic collection of manually stripped faeces, preceded by topical decontamination of the vent area, would likely be sufficient for field applications. Applicability as a potential screening tool was corroborated by the successful detection and quantification of *Y. ruckeri* in faecal samples collected non-invasively from rainbow trout that showed no external signs of yersiniosis. Analytical success achieved here using samples from different finfish species also suggests broad applicability of the method. The molecular approach used here provides a distinct advantage in sensitivity, specificity and speed of analysis compared to outcomes from culture-based methods, which typically take a minimum of 2 to 3 days to achieve. In contrast, the qPCR assay developed here can provide highly specific, direct quantification of *Y. ruckeri* in infected samples within approximately 2.5 h. Commercial multisubstrate identification systems commonly used in culture-based detection may result in multiple result profiles due to variable expression or masking of bacterial phenotypic characteristics, often making interpretation subjective and therefore of limited reliability (Rodgers 1992). Besides the advantage in processing time compared to culture-based detection strategies, PCR methods provide considerably greater specificity and PCR assays have previously been developed successfully for rapid detection of *Y. ruckeri*. However, these assays were reliant on analysis of blood or organs obtained through invasive or lethal sampling of potentially infected fish (Argenton *et al.* 1996, Altinok et al. 2001, Bastardo *et al.* 2012). The potential

259 negative impact of such sampling methods on stock make these strategies less feasible as screening
260 tools than the method developed in this study.

261 Sensitivity of the current method was comparable to the most sensitive assays used in other studies
262 developed for bacterial detection in faecal samples, where lower limits of detection have ranged from
263 10 to $1x10^4$ copies of the target gene sequence (Bélanger *et al.* 2003, Inglis and Kalischuk 2004, Rinttilä
264 *et al.* 2004). Analysis of faeces and spleen samples spiked with serial decimal dilutions of *Y. ruckeri*
265 cells demonstrated this assay could detect single cells of *Y. ruckeri*, equivalent to approximately 7
266 copies of the target gene sequence, in 500 mg of suspended faecal solids or in 25 mg of splenic tissue.
267 The lower limit of detection in previous studies using PCR and qPCR assays to detect *Y. ruckeri* in fish
268 tissues such as spleen ranged from 3.4 to $2x10^4$ cell-equivalents $g^{-1}$ (Gibello et al. 1999, Bastardo *et al.*
269 2012, Keeling et al. 2012). In comparison, the assay developed here demonstrated greater sensitivity
270 (2 cell-equivalents $g^{-1}$) even when analysing faecal samples, despite the difficulties associated with
271 molecular analysis of faeces (Monteiro *et al.* 1997). The successful detection and quantification of *Y.*
272 *ruckeri* in non-invasively collected faecal samples substantiated the general premise of this assay's
273 applicability as a non-invasive tool for screening of stock. While *Y. ruckeri* present in the water may
274 have contributed to the level of pathogen detected in these non-invasively collected samples,
275 attempted measurement of pathogen load in water following previous *Y. ruckeri* challenges have
276 indicated *Y. ruckeri* presence was below detectable limits and therefore unlikely to have significantly
277 impacted outcomes in this study. In general, given the demonstrated sensitivity of this method to even
278 trace amounts of the target pathogen, its ability to detect minute quantities of *Y. ruckeri* even in the
279 culture water make the outcomes of this study particularly relevant to contemporary aquaculture
280 environments. Further development of this assay could include a separate assessment of pathogen
281 levels in the water associated with non-invasively collected faecal samples to improve precision and
282 accuracy of faecal pathogen quantification.

283 The sensitivity of qPCR may be affected by a variety of factors, including the quantity and quality of
284 the extracted nucleic acid, and the presence of inhibitory substances (Chen et al. 1997). The reliability
285 of a qPCR assay must therefore be assessed in the context of its lower limit of detection (LOD) and
286 limit of quantification (LOQ) (Bustin et al. 2009). The detection limit of this assay (LOD) was 4 copies
287 of the target gene sequence and the LOQ was determined to be 10 copies. Estimating 7 copies of the
288 16S gene per cell based on numbers reported for *Y. ruckeri* (Johnson *et al.* 2015), the LOQ and LOD of
289 the assay were approximately 1 and 0.5 cell-equivalent respectively, or the equivalent of 2.65 ag and
290 1.07 ag of the target gene transcript respectively, per qPCR reaction. In comparison, using bacterial
291 culture-based methods, the LOQ and LOD were approximately $10^3$ and $10^2$ CFU $mL^{-1}$ respectively,
292 indicating the deficiency in sensitivity compared to the qPCR assay developed here and rendering
293 further detailed investigation, such as accurate identification and quantification of all isolated
294 bacteria, superfluous in the context of this study.

295 To address analytical difficulties due to faecal matrix complexity in this study, protocols for extraction
296 of bacteria from food matrices were adapted to minimise faecal pathogen retention through the
297 addition of Tween 20, a polysorbate surfactant (Rodrigues-Szulc *et al.* 1996). This was supplemented
298 with a nucleic acid extraction protocol optimised empirically for use in this assay. Some PCR inhibition
299 was apparent in faecal samples examined in this study despite the optimisation measures used. In
300 utilising a selective enrichment culture, as previously used in combination with PCR to alleviate faecal
301 complexity and increase assay sensitivity (Rodgers 1992, Carson and Wilson 2003), precision and
302 accuracy is potentially compromised by non-uniform replication of bacterial cells within a sample due
303 to a number of reasons. For instance, target distribution is heterogeneous at low concentrations in
304 samples and does not conform to stochastic variation. Cells may also be non-culturable despite being
305 viable (Tholozan *et al.* 1999). As a result, there is an elevated possibility for Type II error using such
306 methods, as demonstrated by the false negative results obtained at low dilutions through culture-
307 based methods in this study. In contrast, the outcomes from the qPCR assay were consistent and

capable of reliably detecting single-cell quantities of *Y. ruckeri*. Further optimisation of the extraction protocol could reduce PCR inhibition and improve the current assay.

Quantification of amplified product in qPCR has traditionally been achieved by comparison of relative amplification with a standard of known starting target quantity. The inherent difficulty in constructing target-specific standard curves that are consistent across multiple assays has impeded broad adoption of direct quantification, particularly for large-scale applications (Rutledge and Cote 2003). A primary assumption in standard analysis of qPCR data is that of equal amplification efficiency, with any variation from this being interpreted as a variation in amplifiable template quantity, as opposed to intrinsic variation due to differences in thermal cycler performance or reagent formulations (Diaco 1995). In an attempt to account for intrinsic variation, studies have included co-amplified internal controls in qPCR assays. However, evidence suggests that preferential amplification of one template over another may be common in such a scenario (Suzuki and Giovannoni 1996). Quantification independent of standard-curve comparison, as used in this assay, obviates the need for constructing standards, as well as issues arising from intrinsic variations in amplification efficiency, by applying a mechanistic model that accurately predicts product accumulation through an entire reaction, thereby determining initial template quantities (Rutledge and Stewart 2010, Carr and Moore 2012). In this study, a mechanistic model developed by Carr and Moore (2012) was used to quantify detected amounts of the *Y. ruckeri* 16S rRNA gene transcript. The method was consistent through the entire dynamic range of the assay for each type of sample analysed.

At low template concentrations, the intrinsic variation in target quantity between samples at very low copy numbers is an important consideration, which does not conform to stochastic distributions but can be modelled using a Poisson distribution (Bustin *et al.* 2009, Rutledge and Stewart 2010). This explains the difference between LOD and LOQ observed for the qPCR assay developed here. However, LOD in this assay was close to the stochastic qPCR sensitivity limit of 3 copies, which assumes a Poisson distribution, a 95% chance of including at least 1 copy in the reaction, and the ability to detect single

copies (Wittwer and Kusakawa 2004). Attempting to reliably detect lower concentrations would extend into digital PCR strategies that are not practicable for the intended scope and application of this assay (Bustin *et al.* 2009).

The ubiquity of *Y. ruckeri*, and its ability to establish and maintain asymptomatic infection in fish, makes it a particularly problematic challenge for finfish aquaculture. This study provides the ability to detect and quantify *Y. ruckeri* infection, even at extremely low levels and without the need for invasive or lethal sampling, which will be of benefit to the aquaculture industry. It can be argued that the methods presented here do not differentiate between live and dead cells. However, the exquisite sensitivity of this assay provides a more conservative approach to disease management in commercial aquaculture, where a false positive-detection may be preferable to the risk of undetected infection at the initial screening stage. Supplementary assays, such as viable colony counts or RT-qPCR, could be combined with the strategy presented here to provide secondary confirmation of pathogen viability if required. With further optimisation of extraction protocols to minimise inhibition, the qPCR assay presented here offers an effective screening tool for future epidemiological surveys. It could form an important part of aquaculture health management strategies targeting *Y. ruckeri*, especially in the context of investigating asymptomatic infection.

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