RESEARCH ARTICLE

Optimization and Validation of a Real-time PCR Method for the Simultaneous Detection of *Lactococcus garvieae* and *Streptococcus agalactiae* in Fish

Taona R. Zinyakasa*[1,5]
Farisai Chidzwondo[5]
Tatenda Makawa[1]
Sitokozile Sibanda[3]
Exnevia Gomo[4]
Tivapasi Musa[1]
Elizabeth Gori[2]

Authors' addresses:
[1] Veterinary Biosciences, Clinical Science Departments, University of Zimbabwe, 630 Churchill Avenue P.O. Box MP 167, Mount Pleasant, Harare, Zimbabwe
[2] Department of Medical Biochemistry, Molecular Biology & Genetics, College of Medicine and Health Sciences-School of Medicine and Pharmacy, University of Rwanda, P.O. Box 117-Butare, Rwanda
[3] Central Veterinary Laboratory, Department of Livestock and Veterinary Services,18 Borrowdale Road, Harare, Zimbabwe
[4] Faculty of Medicine and Health Sciences, Box A178 Avondale, University of Zimbabwe
[5] Biotechnology and Biochemistry Department, University of Zimbabwe, 630 Churchill Avenue P.O. Box MP 167, Mount Pleasant, Harare, Zimbabwe

Correspondence:
Taona R. Zinyakasa
Veterinary Biosciences, Clinical Science Departments, & Biotechnology and Biochemistry Department, University of Zimbabwe, 630 Churchill Avenue P.O. Box MP 167, Mount Pleasant, Harare, Zimbabwe.

Article info:
Received: 11 June 2024
Accepted: 28 October 2024

ABSTRACT

Background: *Lactococcus garvieae* and *Streptococcus agalactiae* infections contribute to heavy losses in aquaculture farms worldwide. Currently, available pathogen diagnostic tools use biochemical and microbiological methods beleaguered by very low accuracy, reproducibility and specificity.

Aim: To optimize and validate a rapid, sensitive and specific real-time PCR (qPCR) method for detecting *L. garvieae* and *S. agalactiae* in fish.

Methods: Pairs of *Streptococcus*-specific (IGS-s/IGS-a) and *Lactococcus*-specific (CAU12F/CAU15R) primers were tested for specificity and sensitivity in the qPCR. qPCR was carried out at different temperatures and primer concentrations. The optimal conditions were determined to be the temperature and primer concentration with the lowest C_T values.

Results: For both primer sets, the optimal annealing temperature was 60ºC, and the optimal primer concentration was 500 nM. The detection limit for *L. garvieae* was at dilution factor 10^{-3}, with a mean C_T value of 25.0, for *S. agalactiae*, 10^{-4} with a mean C_T value of 29.8. The PCR efficiencies were 97% for *L. garvieae* and 91% for *S. agalactiae*, with linear slopes (R^2 = 0.999). The assay demonstrated high repeatability and reproducibility.

Conclusion: The optimum conditions established for the qPCR method enable rapid, highly sensitive and specific diagnosis of *L. garvieae* and *S. agalactiae* infection in fish

Key words: Diagnostic tool, qPCR, Zoonotic, Aquaculture, Specificity, Sensitivity

Introduction

There has been considerable global increase in lactococcosis and streptococcosis infections in fish over the past ten years (Bwalya *et al.*, 2020; Egger *et al.*, 2023). These zoonotic infections are mainly caused by the bacteria *Streptococcus agalactiae* and *Lactococcus garvieae*. However, recently it has been reported that *L. pauteri* (Kotzamanidis *et al.*, 2020; Altinok and Ture, 2022), closely related to *L. garviae*, and *L. formosensis* (Abraham *et al.*, 2023), are also important agents of lactococcosis. These pathogens have been isolated in a variety of fish species and have caused significant losses in aquaculture farming around the world (Stoppani *et al.*, 2023). Outbreaks reported in India, Brazil, China (Vendrell *et al.*, 2006), Lake Kariba (Zambia) (Bwalya *et al.*, 2020), and other countries (Kotzamanidis *et al.*, 2020; Abraham *et al.*, 2023; Stoppani *et al.*, 2023) demonstrate the growing importance of these infections (Bwalya *et al.*, 2020). In most cases, fish do not exhibit any clear clinical symptoms of infection before they die (Johri *et al.*, 2006). The symptoms like exopthalmia/bulging eyes are

http://www.jbb.uni-plovdiv.bg DOI: 10.69085/jbb20242113

also not distinct between Lactococcus ssp and Streptococcus spp infections, which makes it difficult to distinguish between the two infectious agents (Van Doan *et al.*, 2022).

The techniques traditionally used for diagnosis of lactococcosis and streptococcosis infections in fish are biochemical, histopathological and microbiological assays, partly because of their simplicity (Wen-de *et al.*, 2017; Varadi *et al.*, 2017). However, these methods fall short in accuracy, sensitivity, and specificity (Wen-de *et al.*, 2017). The use of molecular diagnostic methods in the fish industry is growing (Saad *et al.*, 2022; Austin, 2019). This is due to the higher sensitivity and specificity they have over the traditional methods.

Molecular methods are broadly classified into nucleic acid hybridization and amplification (Ali, 2017). Low sensitivity makes hybridization methods less preferable to amplification methods. Thus, amplification methods, which include polymerase chain reaction (PCR), random amplification polymorphism DNA (RAPD), amplified fragment length polymorphism (AFLP), and loop-mediated isothermal amplification (LAMP) have been developed for the detection of pathogens (Roque and Gomez-Gil, 2009; Wen-de *et al.*, 2017; Ador *et al.*, 2022). Superiority of accuracy of molecular methods over conventional bacteriological and phenotypic methods in detecting *S. agalactiae* and *L. garvieae* has been amply demonstrated (Karsidani *et al.*, 2010; Wen-de *et al.*, 2017; Ador *et al.*, 2022). However, the amplification methods are limited by non-specificity, poor reproducibility (RAPD), time and complex procedures (AFLP), or require prior analysis of the variation of many genomic sequences (LAMP) (Wen-de *et al.*, 2017). Several studies highlight the current limitation in this field as the development of optimized and validated protocols that can be easily commercialized and implemented in aquaculture facilities is lagging (de A. and Pilarski, 2015). Real-time PCR (qPCR) is emerging as an optimal method for rapid, sensitive and specific detection of *S. agalactiae* and *L. garvieae* (Leigh *et al.*, 2020).

Several studies support the superiority of qPCR over other molecular methods as a detection and quantification tool. qPCR is rapid, sensitive, highly specific, and reliable in comparison to traditional histopathological, microbiological, and biochemical assays (Sibley and Church, 2012; Harshitha and Arunraj, 2021). Identification and quantification of different bacterial fish pathogens using PCR and qPCR, including *S. agalactiae* (Su *et al.*, 2016), *Yersinia ruckeri* (LaFrentz *et al.*, 2022; de A. and Pilarski 2015), *Flavobacterium psychrophilum* (Chapela *et al.*, 2018), *L. petauri* (Egger *et al.*, 2023; Abraham *et al.*, 2023) and *L. garvieae* (Jung and Kim 2010; Odamaki *et al.*, 2011; Ustaoglu *et al.*, 2024) have been reported.

In Zimbabwe, traditional histopathological, microbiological, and biochemical assays are the most frequently used methods to identify fish pathogens. Gomo *et al.*, (2016) used histopathology examinations to diagnose *Epizootic ulcerative syndrome* in fish. Sichewo *et al.*, (2014), also used biochemical and Gram staining to identify pathogenic bacteria in edible fish. This is because the methods, such as Strep-strips (colloidal gold immunochromatography strips), which are commonly employed for *S. agalactiae* detection, are simple to use (Wen-de *et al.*, 2017).

Traditional methods are generally unable to discriminate *S. agalactiae* and *L. garvieae* from other bacteria, like *S. innae, L. petauri, L. lactis, S. thermophilus*, or *Enterococcus*-like strains (Ogier and Serror, 2008; Egger *et al.*, 2023). In this context, optimising and validating qPCR for simultaneous detection of *S. agalactiae* and *L. garvieae* is critical to facilitating and hastening the implementation of control measures in fish farming. This study aimed to optimize and validate an EvaGreen qPCR assay for the simultaneous detection of *S. agalactiae* and *L. garvieae* in fish. This will allow for rapid diagnosis that is critical for disease management and control.

Materials and Methods

Ethics statement

This study was approved by the National Animal Ethical Committee (NAREC), Zimbabwe Ref: NAREC:017/22.

Bacterial isolates and cultivation conditions

Pure isolates of bacterial strains of *S. agalactiae* and *L. garvieae*, were procured from South Africa (Inqaba Biotechnical Industries (Pty) Ltd. South Africa) and subsequently cultivated in tryptic soy broth (Merck Germany, catalog number 1.05459) at a temperature of 28°C for 24 hours. Pure isolates of phylogenetically related bacterial strains including *A. hydrophila, E. coli, K. pneumoniae, E. faecalis, Proteus, P. syringae pv. Actinidiae*, and *S. aureus* were cultivated as mentioned above and used as negative controls.

Genomic DNA extraction

DNA was extracted from positive controls (*S. agalactiae* and *L. garvieae* DNA isolates) and infected fish samples to obtain the genetic material used in the qPCR assays. Bacterial DNA from infected fish samples was extracted using a spin column DNeasy extraction kit (Qiagen). A nanodrop

spectrophotometer was used for quantification of DNA at a wavelength of 260 nm. The DNA was stored at -20ºC for subsequent PCR amplifications. The positive controls were used to determine the optimum annealing temperature and primer concentration of the selected primers. Once the annealing temperature and primer concentration were optimized, sensitivity, specificity, reproducibility and repeatability were assessed. Validation was done using infected fish samples and sequencing.

Primers

Specific primers previously described by (Su *et al.*, 2016) (IGS-s/IGS-a) and (Jung and Kim, 2010) (CAU12F/CAU15R) for *S. agalactiae* and *L. garvieae*, respectively, were used. The amplicon sizes for *S. agalactiae* and *L. garvieae* were 190 bp and 415 bp respectively.

Annealing temperature optimization

To optimize the annealing temperature, pairs of primers were simultaneously evaluated using qPCR and agarose gel electrophoresis at different annealing temperatures. The PCR mixture (20 µL) contained 4 µL of EvaGreen (Rox) qPCR master mix, 1 µL of each specific primer and 2 µL of template DNA. The PCR was performed in a qTOWER^{3}G analytik jena real-time thermocycler with the PCR cycle program; 95°C for 12 min as the initial activation stage, 40 cycles of amplification (95°C for 15 s, 55°C–65°C for 30 s, 72°C for 30 s). At the end of each cycle, a DNA melting curve analysis of the amplified products was performed between 65ºC and 95ºC to evaluate the melting temperature (T_m) readings taken at 0.5ºC/s increment. The qPCR runs were performed in triplicate for each assay, and post-PCR melt curve analysis was also used to confirm the results. The amplified products were further analyzed by 1.5% agarose gel electrophoresis using SYBR safe DNA gel stain. Optimum temperatures with the lowest cycle threshold (C_T) values were selected.

Primer concentration optimization

The primers were serially diluted from 50 nM to 500 nM and tested at annealing temperatures of 55°C-61°C. The combination of concentrations yielding the lowest cycle threshold (C_T) values with the lowest variation in replicates was chosen. The Taguchi method was used to optimize the primer concentration (Ramakrishna *et al.*, 2013; Cobb and Ciarkson, 1994).

Standard curve generation for sensitivity assay

Ten-fold serial dilutions of the positive controls were used as templates for the qPCR using the optimized annealing temperatures and primer concentrations. Standard curve slopes, C_T values versus log quantity and PCR efficiencies (E) were calculated using the qTOWER^{3}G analytik jena-automated qPCR instrument. The PCR efficiency was calculated from the standard curve as the percentage of template molecules that were doubled during each cycle as shown in Equation 1. This was repeated on three occasions. The detection limit was determined as the lowest concentration (within the linear range) that produced an amplification signal on the qTOWER^{3}G Mastercycler. Triplicate serial dilutions (10^{-1}-10^{-5}) were performed and the dilution factor that gave 95% (24/25) positive amplification was the detection limit. The qPCR amplicons were then confirmed by electrophoresis in 1.5% agarose gel and visualized by SYBR Green staining.

$$(E = [10^{-1}/(\text{slope})^{-1}] \times 100) \quad (1)$$

Repeatability and reproducibility of the qPCR assay

Three separate dilution series were assayed in a single run to evaluate intra-assay variation (repeatability) while interassay variation (reproducibility) was measured by testing each dilution in three separate consecutive runs. The mean, standard deviation (SD) and coefficient of variation (CV) for both intra-assay and inter-assay variation were calculated separately for each standard DNA dilution based on their C_T values using Microsoft Excel software.

Assay specificity

S. agalactiae and *L. garvieae* DNA were used as the positive control. The test samples consisted of DNA from *A. hydrophilla, E. coli, K. pneumoniae, E. faecalis, Proteus, P. syringae pv. Actinidiae* and *S. aureus.* The samples were subjected to the newly optimized assay. Assays of specificity were done using two different approaches: (1) the expected qPCR amplicon size confirmed by gel electrophoresis, and (2) melt curve analysis.

Method validation

Fish sample tests

Healthy fish DNA samples from *Oreochromis niloticus* were collected and spiked with positive controls of *S. agalactiae* and *L. garvieae.* This was done to mimic the natural infection. DNA was extracted from the spiked fish samples and tested using the newly optimized assay. The positive samples were also confirmed by gel electrophoresis using SYBR safe DNA gel stain. Random fish samples of unknown infection status were tested to determine the infection status using the newly optimized assay.

Table 1. *qPCR efficiency with serial dilutions of positive controls at varying annealing temperatures primers (a) IGS-s/IGS-a, (b) CAU12F/CAU15R at 500 nM primer concentration*

	C_T value						C_T value			
Temperature	**55°C**	**58°C**	**59°C**	**60°C**	**61°C**	**Temperature**	**58°C**	**59°C**	**60°C**	**61°C**
Standard dilution						Standard dilution				
1	14.21	14.73	14.63	14.23	14.47	1	17.16	15.31	16.19	15.62
2	18.91	18.51	18.33	18.09	18.2	2	18.55	18.41	19.27	19.03
3	22.41	22.77	22.54	22.14	22.42	3	21.85	21.25	23.05	22.27
4	27.12	26.62	26.04	25.44	26.38	4	23.91	22.73	26.63	26.07
5	31.72	31.92	31.4	28.36	31.09	5	25.14	24.16	30.79	29.83
PCR efficiency	70%	73%	74%	91%	78%	PCR efficiency	77%	70%	97%	90%
Slope	-4.32	-4.24	-4.17	-3.56	-3.99	Slope	-4.05	-4.28	-3.41	-3.6

DNA sequencing

The sequencing of the PCR amplicons produced after fish sample testing was to validate that the optimized protocol is specific in detecting the desired pathogens. PCR positive products (190 bp and 415 bp for *S. agalactiae* and *L. garvieae*, respectively) were directly purified using the GeneClean Turbo Kit, MP Biomedicals, Illkirch, France. Partial sequencing in the 5' direction was done by LGC (Germany). The sequence analyses were done using Staden Package software for sequence contig generation, Basic Local Alignment Search Tool (BLAST) for sequence contig referencing and comparison to bacteria sequences in the GenBank on NCBI to identify the amplified DNA sequence.

Results

Optimization of primers (IGS-s/IGS-a and CAU12F/CAU15R)

Annealing temperature optimization was performed using concentrated positive controls of the bacteria. As shown in Figure 1, with the concentrated primer pair IGS-s/IGS-a, low C_T values, ranging from 12 to 14, were observed between the temperatures 55.0 - 61.8°C. C_T values increased to over 30 at 64.1ºC. With the primer pair CAU12F/CAU15R, low C_T values of 19 and 20 were observed at temperatures 57.1 and 60°C, respectively. Temperatures below 57°C and above 60°C showed higher C_T values.

Annealing was further assessed with serial dilutions at the optimum temperatures observed with the concentrated positive controls, and PCR efficiency calculated. For the IGS primer pair, PCR efficiency was highest at 60.0°C (91%) and lowest (70%) at 55.0ºC, while for CAU12F/CAU15R the values were 97% at 60.0ºC and 70% at 50.0ºC, respectively (Table 1).

Primer concentration optimization

The optimization approach was selected due to the variability of C_T values observed in optimization of annealing temperature. IGS-s/IGS-a primer concentrations were optimized by assessing PCR efficiency of serial dilutions of the positive controls and the primer at 60°C. The highest PCR efficiency was 91% at primer concentration 500 nM (Table 2a). For CAUR12F/CAU15R primer concentration was optimized by assessing C_T values at selected temperatures (Table 2b). The concentration of 500 nM and temperature 60°C had the lowest C_T value (17.59).

Sensitivity test

Reproducibility and repeatability were assessed using C_T values obtained from testing positive control standard serial dilutions in triplicates. Standard deviation (SD) ranged from 0.035-2.37 and coefficient of variation (CV) from 0.015-8.50 for CAU12F/CAU15R and IGS primers. The primer pairs showed a strong relationship between template concentration and the C_T value (Table 3).

The PCR efficiency and R2 were calculated using a standard curve based on the C_T value against DNA concentration. The efficiency was high; 91% and 97%, with the R^2 values >0.99 with slopes of 3.41 and 3.56 for CAU12F/CAU15R and IGS-s/IGS-a, respectively (Figure 3).

The C_T values of serial dilution amplifications were used to determine the limit of detection. The detection limits were DNA concentrations of 0.003 ng/µL and 0.0019 ng/µL (Table 4).

http://www.jbb.uni-plovdiv.bg DOI: 10.69085/jbb20242113

Table 2. *PCR efficiency and C_T values of varying concentrations of primers and positive controls (a) IGS-s/IGS-a and (b) CAU12F/CAU15R*

Primer Conc	Serial dilution	1	2	3	4	5	PCR efficiency	Slope
175nM	CT Values	16.83	21.24	27.82	32.17	no Ct	57%	-5.09
200nM		14.47	18.58	21.93	26.14	29.99	87%	-3.67
225nM		14.12	17.79	22.1	26.4	30.98	77%	-4.05
250nM		14.23	18.63	22.62	25.97	31.42	74%	-4.17
375nM		15.14	18.68	22.88	27.22	31.54	74%	-4.16
500nM		14.23	18.09	22.14	25.44	28.36	91%	-3.56

(a)

Temperature	Primer conc	50nM	80nM	100nM	125nM	150nM	250nM	500nM
					CT Values			
58°C		no Ct	no Ct	no Ct	21.6	no Ct	19.9	19.54
59°C		no Ct	no Ct	24.89	21.75	24.04	20.02	20.91
60°C		no Ct	no Ct	26.35	24.91	24.85	23.44	17.59
61°C		no CT	no CT	32.34	29.06	29.02	26.2	20.52

(b)

Table 3. *Intra- and inter-assay variability tests for (a) CAU12R/CAU15F; (b) IGS-s/IGS-a*
1-interassay; 2-intraassay

Serial dilution	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
mean CT	16.76	19.23	23.21	26.57	31.19	19.94	23.52	26.29	27.85
s.d	0.8	0.035	0.23	0.08	0.57	1.18	1.77	1.87	2.37
c.v (%)	4.77	0.18	0.94	0.32	1.81	5.94	7.52	7.11	8.5
(a1)						(a2)			
Serial dilution	**10^{-1}**	**10^{-2}**	**10^{-3}**	**10^{-4}**	**10^{-5}**	**10^{-2}**	**10^{-3}**	**10^{-4}**	**10^{-5}**
mean CT	20.79	26.47	29.66	32.59	33.57	24.73	29.81	32.77	34.05
s.d	0.48	0.43	0.43	0.64	0.79	0.25	0.49	0.63	0.85
c.v (%)	2.29	1.64	0.015	1.96	2.32	1.01	1.65	1.92	2.49
(b1)						(b2)			

Specificity assay

Different sets of bacterial DNA were used to determine the specificity of the primer pairs. The *S. agalactiae* and *L. garvaeae* bacteria DNA were used as the positive controls. The primers only amplified *S. agalactiae* and *L. garvaeae* positive controls (Figure 3).

Fish DNA test for validation

The validation of the assay was done using electrophoresis and sequencing. All the seven spiked fish samples tested positive for *S. agalactiae* and *L. garvieae* with bands at 190 bp and 415 bp for IGS-s/IGS-a and CAU12F/CAU15R, respectively. Sequencing showed identity of 99.74% to *Lactococcus sp.* strain and 99.80% to *Streptococcus agalactiae* strains with query covers of 92% and 100%.

http://www.jbb.uni-plovdiv.bg DOI: 10.69085/jbb20242113

Table 4a. *CT values of 10-fold serial diluted DNA ranging from 10^{-1}-10^{-5} with primer pairs CAU12F/CAU15R. (Bold shows negative samples). CAU12F/CAU15R*

SERIAL DILUTIONS	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	Replicate
	18.66	22.56	23.52	25.65	**25.32**	**1**
	17.8	19.02	21.75	24.05	**25.37**	**2**
	16.69	19.54	21.59	24.74	**25.41**	**3**
	19.2	20.44	**26.45**	**26.09**	**26.46**	**4**
	16.98	20.36	23.28	**25.63**	**29.17**	**5**
C_T VALUES	19.06	20.16	24.18	29.14	**28.01**	**6**
	18.51	22.17	21.79	24.83	**25.02**	**7**
	16.63	18.45	21.49	24.56	**25.09**	**8**
	15.94	18.66	21.74	24.07	**28.05**	**9**
	15.39	19.24	24.37	**27.79**	**29.05**	**10**
	18.37	20.05	24.87	**28.17**	**32.15**	**11**

Table 4b. *CT values of 10-fold serial diluted DNA ranging from 10^{-1}-10^{-5} with primer pairs IGS-s/IGS-a used to calculate limit of detection*
IGS-s/IGS-a:

SERIAL DILUTIONS	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	Replicate
	21.05	26.9	29.15	32.55	**33.31**	**1**
	20.96	26.43	29.83	31.78	**33.23**	**2**
	20.87	27.13	29.25	32.84	**33.2**	**3**
	20.16	26.57	29.77	32.34	**33.47**	**4**
	21.73	26.61	30.05	**33.26**	**34**	**5**
C_T VALUES	20.55	25.81	29.15	32.63	**no C_T**	**6**
	20.38	26.05	29.73	33.7	**35.19**	**7**
	20.95	26.23	30.31	**33.47**	**no C_T**	**8**
	21.4	25.88	30.4	32.43	**33.12**	**9**
	20.14	25.78	29.35	31.59	**34.45**	**10**
	20.53	25.65	29.31	32.3	**no C_T**	**11**

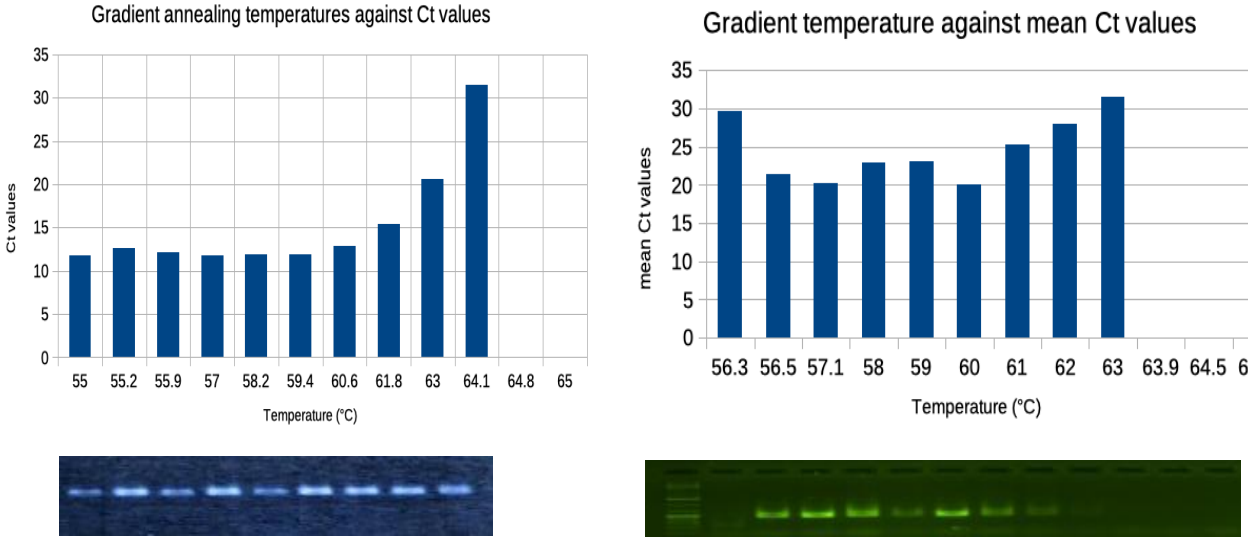
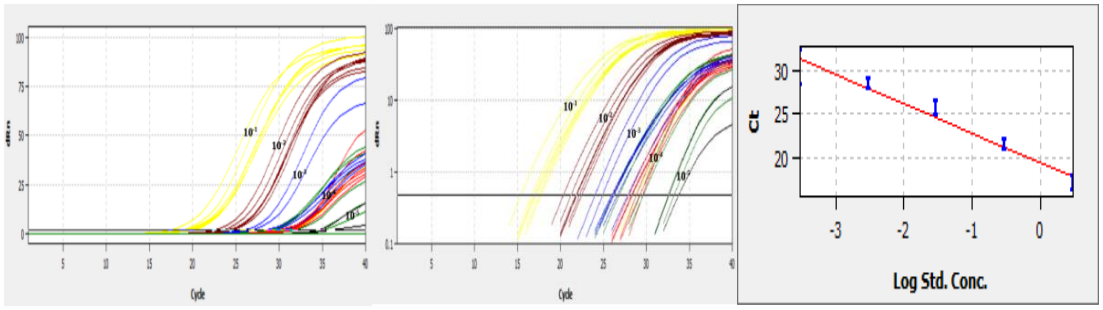
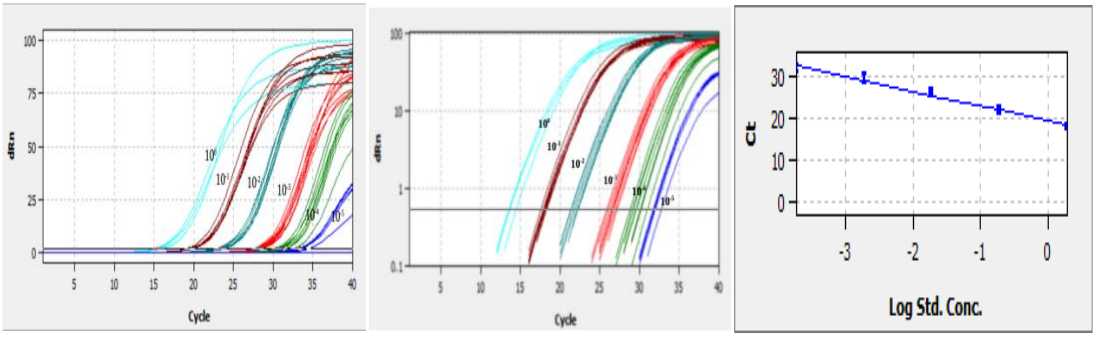


Figure 1. *qPCR cycle threshold (CT) values with the primer pairs (a) IGS-s/IGS-a and (b) CAU12F/CAU15R respectively at different annealing temperatures.*

http://www.jbb.uni-plovdiv.bg DOI: 10.69085/jbb20242113

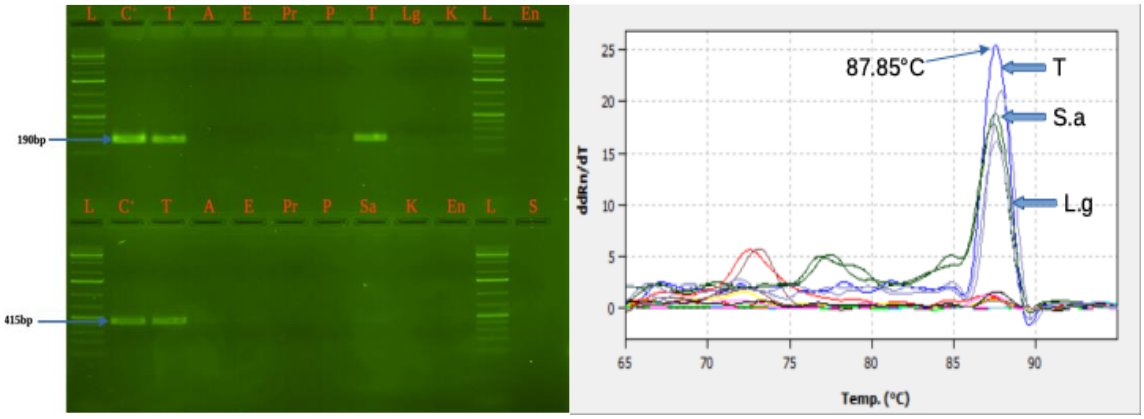


(A) R^2 = 0.999; Slope = -3.41; PCR efficiency = 97%



(B) R^2=0.99; Slope=-3.56; PCR efficiency =91%

Figure 2. *Amplification plots and standard curve of qPCR assay based on CT values against log standard concentration. Amplification plot of 10-fold serial diluted DNA ranging from 10^{-1}-10^{-5} with primer pairs (a) CAU12F/CAU15R and (b) IGS-s/IGS-a respectively; corresponding standard curve (right).*



(A) **(B)**

Figure 3a. *Figure 3a: Corresponding agarose gel electrophoresis of qPCR products showing the specificity of primer pair IGS-s/IGS-a (on top) and CAU12F/CAU15R (below), respectively. Lane L, marker; lane C+, positive control; lane T, positive fish sample; lane A, DNA of A. hydrophilla; lane E, DNA of E. coli; lane Pr, DNA of Proteus; lane P, DNA of P. syringae; lane Lg or Sa, DNA of L. garvieae or S. agalactiae; lane K, DNA of K. pneumoniae; lane En, DNA of E. faecalis; lane S, DNA of S. aureus*

Figure 3b. *Melting curve analysis of qPCR showing specificity of primer pair IGS-s/IGS-a and CAU12F/CAU15R.*

http://www.jbb.uni-plovdiv.bg DOI: 10.69085/jbb20242113

Discussion

This study optimized and validated the real-time PCR (qPCR) for the simultaneous detection of *S. agalactiae* and *L. garvieae*. The optimal temperature of the assay was determined to be 60°C at a primer concentration of 500 nM. The sensitivity and specificity of the primer pairs were very high with a very low detection limit. The applicability of the techniques was demonstrated using fish samples.

Diverse bacterial communities have been reported in fish. These include lactic acid bacteria (Lactobacillus), *S. innae, S. thermophilus*, *Enterococcus*-like strains among others (Mah and Hwang, 2008; Roh *et al.*, 2007). Traditionally, *S. agalactiae* and *L. garvieae* have been detected in fish using microbiological and biochemical tests which, despite being simple, have low accuracy, specificity and are time-consuming (Fortina *et al.*, 2007). qPCR offers more rapid, sensitive and specific detection of the bacteria.

Annealing temperatures between 55-61.8°C amplified the *S. agalactiae* and *L. garvieae* DNA at low C_T values. This range of temperature is narrower than that reported by (Ishii and Fukui, 2001), of between 45-68°C. This shows that the primers had improved specificity. Lower and higher temperatures can result in non-specific and/or no amplification. According to Silalahi and Sasadara, (2021), optimal temperatures show no dimers or non-specific amplification as illustrated in this study. PCR efficiency for *S. agalactiae* and *L. garvieae* DNA amplification were highest at 60°C, indicating that this was the optimal temperature for amplification. Other studies reported similar annealing temperature, despite using different PCR mixes (Jung and Kim, 2010; Su *et al.*, 2016; Escobar *et al.*, 2020; Silalahi and Sasadara, 2021).

The optimized primer concentration for both primer pairs was 500 nM for *S. agalactiae* and *L. garvieae.* Escobar *et al.*, (2020) reported similar concentrations and PCR efficiency for *S. agalactiae* amplification in vaginal samples. The results of the gel electrophoresis and melting curve showed high reproducibility and repeatability further showing that the primer concentration of 500 nM was optimal. According to van Pelt-Verkuil and Hays, (2008), higher primer concentrations are frequently associated with mis-priming and nonspecific amplification, whereas lower primer concentrations can result in low or no amplification of the desired target.

To our knowledge, the current study is the first to report *S. agalactiae* and *L. garvieae* detection by EvaGreen qPCR, doing so in all seven fish samples used. These results show potential for use in the fish industry. The detection limit for *L. garvieae* was 0.003 ng/µl of genomic DNA, with mean C_T values of 24.0, and 0.0019 ng/µl for *S. agalactiae*, with mean C_T value of 29.8. This sensitivity is comparable to that in studies with other pathogenic bacterial species(Yang *et al.*, 2003; Lambertz *et al.*, 2008; Jung and Kim, 2010; Olson *et al.*, 2012; Su *et al.*, 2016) . A low C_T value shows presence of high DNA concentration (McCall *et al.*, 2014). The lower C_T values from this study demonstrate that the assay was more sensitive than reported by (Su *et al.*, 2016) and (Jung and Kim, 2010) who reported the negative samples having mean C_T values of >37, with the positive samples having C_T mean values between 25 and 35.

The standard deviation (SD) and coefficient of variation (CV) values were below 10 for both *S. agalactiae* and *L. garvieae* intra- and interassay variability. Low CV values obtained for intra-assay and inter-assay runs indicate a high level of assay repeatability and reproducibility. This means that the assay can be easily used in different laboratories for precise amplification. It also eliminates the need for sample dilution for many practical applications. Our results are consistent with (Su *et al.*, 2016) and (Jung and Kim, 2010) who reported low variations with the same primer sets.

The standard curves generated on serial dilutions of *S. agalactiae* and *L. garvieae* DNA confirmed the PCR design was optimal as they both showed high corelation between replicates (R^2= 0.99). The PCR efficiency was greater than 90% (91% and 97%, respectively) which also supports high linearity between replicates. Both the qPCRs showed better efficiency compared to those reported by (Su *et al.*, 2016) (106%, with slope -3.195) and (Jung and Kim, 2010) (70%, with slope -4.3). Thus, the assay, master mix performance and sample quality were good. In general, efficiency between 90-110% is considered acceptable (Applied Biosystems, 2008).

In this study, the specificity and quality of the IGS-s/IGS-a and CAU12F/CAUR15 primer pairs was confirmed by testing with other bacteria, thus enabling *S. agalactiae* and *L. garvieae* to be distinguished. There was no cross-reaction between any bacteria DNA templates, confirming that the primer sets were specific for detecting *S. agalactiae* and *L. garvieae* (Agnes and Barnes, 2007). The analyzed melting curves generated consistent peaks of positive samples at 88°C and negative samples at 77°C. (Su *et al.*, 2016) and (Jung and Kim, 2010), reported similar findings. This demonstrated that the IGSs/IGS-a and CAU15R/CAU12F primer pairs were able to distinguish *S. agalactiae* and *L. garvieae* from other samples. Thus, primer pairs IGS-s/IGS-a and

CAU15R/CAU12F proved to be good candidates for the detection of *S. agalactiae* and *L. garvieae*.

Fish samples were successfully spiked with *S. agalactiae* and *L. garvieae* strains. All seven spiked samples were positive for the pathogens, proving that the protocols were optimal and could be used to successfully detect the pathogens in fish samples. The obtained sequences were compared for similarity with reference sequences in the NCBI blast database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The positive samples had high similarity to *S. agalactiae* and *L. garvieae* reference sequences confirming the primers amplified the desired gene. This confirms that the optimized and validated protocols can specifically detect and identify *S. agalctiae* and *L. garvieae* in fish samples. The use of the 16S rRNA gene for the detection of *L. garvieae* has been determined to be insufficient (Shahin *et al.* n.d.; Abraham *et al.*, 2023; Egger *et al.*, 2023; Stoppani *et al.*, 2023). This is due to the close relatedness to recently reclassified species like *L. petauri*. However, the use of other target genes 16S_23S and DNA gyrase subunit B can improve their differentiation (Stoppani *et al*., 2023).

Conclusion

New qPCR assays were successfully optimized and validated in this study using EvaGreen qPCR mix. The assays provide rapid, specific, sensitive and reproducible methods for the identification of *S. agalactiae* and *L.garvieae* in fish tissues. The developed profiles for both pathogens are similar, therefore can be used to detect the pathogens at the same time. The methods are additional tools for the detection of *S. agalactiae* and *L. garvieae* in practical applications, specifically in aquaculture, and may provide an important tool in future studies for the control of these pathogens. The qPCR assays may be improved for the analytical quantification of bacterial load of the two pathogens. This would provide important tools for studying tropism of bacteria in infected fish tissue and for monitoring bacterial colonization in recovering fish. Quantitative analytic tools for bacterial load are valuable for studying of bacterial pathogenesis, host-bacteria interactions and bacterial replication. A multiplex system for the detection of these two pathogens can also be developed with the aid of the findings in this study.

Acknowledgements

We appreciate the support provided by the Department of Biological Sciences, University of Zimbabwe, which supplied bacterial isolates from its culture collection for use as negative controls and Dr Florian Liegeois for laboratory materials used in the study.

Conflict of interest

The authors declare no conflicts of interest in this work.

Author Contributions

The authors confirm contribution to the paper as follows: study conception and design: Elizabeth Gori; Taona Zinyakasa; data collection: Taona Zinyakasa, Tivapasi Mutasa, Tatenda Makava, Sithokozile Sibanda; analysis and interpretation of results: Farisai Chidzwondo, Taona Zinyakasa, Elizabeth Gori, Exnevia Gomo; draft manuscript preparation: Taona Zinyakasa, Elizabeth Gori, Farisai Chidzwondo, Exnevia Gomo. All authors reviewed the results and approved the final version of the manuscript.

Funding

The study had no funding from the university or any external organizations.

Data availability statement

The data that support the findings of this study are available from the corresponding author, [T.Z], upon reasonable request.

Ethics Statement

This study was approved by the National Animal Ethical Committee (NAREC), Zimbabwe Ref: NAREC:017/22.

References

Abraham T, Yazdi Z, Littman E, Shahin K, Heckman T. I, Quijano Cardé E. M, Nguyen D. T, Hu R, Adkison M, Veek T, Mukkatira K, Richey C, Kwak K, Mohammed H. H, Ortega C, Avendaño-Herrera R, Keleher W, LePage V, Gardner I, Soto E. 2023. Detection and virulence of Lactococcus garvieae and L. petauri from four lakes in southern California. *Journal of Aquatic Animal Health*, *35*(3), 187–198.

Ador, M.A.A., Haque, M.S., Paul, S.I., Chakma, J., Ehsan, R., & Rahman, A. (2022). Potential Application of PCR Based Molecular Methods in Fish Pathogen Identification: A Review. Aquaculture Studies, 22, AQUAST621.

Ali S. A. M. 2017. Application of Molecular diagnostics in Microbiology: a Review. *Sudan Medical Laboratory Journal*, *5*(2), 75–84.

Altinok I, Ozturk R. C, Ture M. 2022. NGS analysis revealed that Lactococcus garvieae Lg-Per was Lactococcus petauri in Türkiye. *Journal of Fish Diseases*, *45*(12), 1839–1843.

Applied Biosystems. 2008. Guide to Performing Relative Quantitation of Gene Expression Using Real-Time Quantitative PCR. *Gene Expression*, *2009*, 1–60.

Austin B. 2019. Methods for the diagnosis of bacterial fish diseases. *Marine Life Science and Technology*, *1*(1), 41–49.

Bwalya P, Simukoko C, Hang'ombe B. M, Støre S. C, Støre P, Gamil A. A. A, Evensen Ø, Mutoloki S. 2020. Characterization of streptococcus-like bacteria from diseased Oreochromis niloticus farmed on Lake Kariba in Zambia. *Aquaculture*, *523*(January).

Chapela M. J, Ferreira M, Ruiz-Cruz A, Martin-Varela I, Fernández-Casal J, Garrido-Maestu A. 2018. Application of real-time PCR for early diagnosis of diseases caused by Aeromonas salmonicida, Vibrio anguillarum, and Tenacibaculum

maritimum in turbot: A field study. *Journal of Applied Aquaculture*, *30*(1), 76–89.

Cobb B. D, Ciarkson J. M. 1994. A simple procedure for optimising the polymerase chain reaction (PCR) using modified taguchi methods. *Nucleic Acids Research*, *22*(18), 3801–3805.

de A. Sebastião F, Lemos E. G. M, Pilarski F. 2015. Validation of absolute quantitative real-time PCR for the diagnosis of Streptococcus agalactiae in fish. *Journal of Microbiological Methods*, *119*, 168–175.

Egger R. C, Rosa J. C. C, Resende L. F. L, de Pádua S. B, de Oliveira Barbosa F, Zerbini M. T, Tavares G. C, & Figueiredo H. C. P. 2023. Emerging fish pathogens Lactococcus petauri and L. garvieae in Nile tilapia (Oreochromis niloticus) farmed in Brazil. *Aquaculture*, *565*.

Escobar D. F, Diaz-Dinamarca D. A, Hernández C. F, Soto D. A, Manzo R. A, Alarcón P. I, Pinto C. H, Bastias D. N, Oberg-Bravo C. N, Rojas R, Illanes S. E, Kalergis A. M, Vasquez A. E. 2020. Development and analytical validation of real-time PCR for the detection of Streptococcus agalactiae in pregnant women. *BMC Pregnancy and Childbirth*, *20*(1), 1–10.

Fortina M. G, Ricci G, Foschino R, Picozzi C, Dolci P, Zeppa G, Cocolin L, Manachini P. L. 2007. Phenotypic typing, technological properties and safety aspects of Lactococcus garvieae strains from dairy environments. *Journal of Applied Microbiology*, *103*(2), 445–453.

Gomo C, Hanyire T, Makaya P, & Sibanda S. 2016. Outbreak of epizootic ulcerative syndrome (EUS) in Seranochromis robustus fish spesies in Darwendale dam, Zimbabwe. *African Journal of Fisheries Science*, *4*(1), 204–205.

Haghighi Karsidani S, Soltani M, Nikbakhat-Brojeni G, Ghasemi M, Skall H. F. 2010. Molecular epidemiology of zoonotic streptococcosis/lactococcosis in rainbow trout (Oncorhynchus mykiss) aquaculture in Iran. *Iranian Journal of Microbiology*, *2*(4), 199–210.

Harshitha, R. and Arunraj, D.R., 2021. Real-time quantitative PCR: A tool for absolute and relative quantification. Biochemistry and Molecular Biology Education, 49(5), pp.800-812

Ishii K, & Fukui M. 2001. Optimization of Annealing Temperature to Reduce Bias Caused by a Primer Mismatch in Multitemplate PCR. *Applied and Environmental Microbiology*, *67*(8), 3753–3755.

Johri A. K, Paoletti L. C, Glaser P, Dua M, Sharma P. K, Grandi G, Rappuoli R. 2006. Group B Streptococcus: Global incidence and vaccine development. *Nature Reviews Microbiology*, *4*(12), 932–942.

Jung M. Y, Chang, Y. H, Kim W. 2010. A real-time PCR assay for detection and quantification of Lactococcus garvieae. *Journal of Applied Microbiology*, *108*(5), 1694–1701.

Kotzamanidis C, Malousi A, Bitchava K, Vafeas G, Chatzidimitriou D, Skoura L, Papadimitriou E, Chatzopoulou F, Zdragas A. 2020. First Report of Isolation and Genome Sequence of L. petauri Strain from a Rainbow Trout Lactococcosis Outbreak. *Current Microbiology*, *77*(6), 1089–1096.

LaFrentz B. R, Králová S, Burbick C. R, Alexander T. L, Phillips C. W, Griffin M. J, Waldbieser G. C, García J. C, de Alexandre Sebastião F, Soto E, Loch T. P, Liles M. R, Snekvik K. R. 2022. The fish pathogen Flavobacterium columnare represents four distinct species: Flavobacterium columnare, Flavobacterium covae sp. nov., Flavobacterium davisii sp. nov. and Flavobacterium oreochromis sp. nov., and emended description of Flavobacterium column. *Systematic and Applied Microbiology*, *45*(2), 126293.

Leigh W. J, Zadoks R. N, Costa J. Z, Jaglarz A, Thompson K. D. 2020. Development and evaluation of a quantitative polymerase chain reaction for aquatic Streptococcus agalactiae based on the groEL gene. *Journal of Applied Microbiology*, *129*(1), 63–74.

Mah J. H, Chang Y. H, Hwang H. J. 2008. Paenibacillus tyraminigenes sp. nov. isolated from Myeolchi-jeotgal, a traditional Korean salted and fermented anchovy. *International Journal of Food Microbiology*, *127*(3), 209–214.

McCall M. N, McMurray H. R, Land H, Almudevar A. 2014. On non-detects in qPCR data. *Bioinformatics*, *30*(16), 2310–2316.

Odamaki T, Yonezawa S, Kitahara M, Sugahara Y, Xiao J. Z, Yaeshima T, Iwatsuki K, Ohkuma M. 2011. Novel multiplex polymerase chain reaction primer set for identification of Lactococcus species. *Letters in Applied Microbiology*, *52*(5), 491–496.

Ogier J. C, Serror P. 2008. Safety assessment of dairy microorganisms: The Enterococcus genus. *International Journal of Food Microbiology*, *126*(3), 291–301.

Ramakrishna U. S, Kingston J. J, Sripathi M. H, Batra H. V. 2013. *Taguchi optimization of duplex PCR for simultaneous identification of Staphylococcus aureus and Clostridium perfringens alpha toxins*. *340*, 93–100.

Roh S. W, Nam Y. Do, Chang H. W, Sung Y, Kim K. H, Oh H. M, Bae J. W. 2007. Halalkalicoccus jeotgali sp. nov., a halophilic archaeon from shrimp jeotgal, a traditional Korean fermented seafood. *International Journal of Systematic and Evolutionary Microbiology*, *57*(10), 2296–2298.

Roque A, Soto-Rodriguez S, Gomez-Gil B. 2009. Bacterial Fish Diseases and Molecular Tools for Bacterial Fish Pathogens Detection. *Aquaculture Microbiology and Biotechnology*, *Vol. 1*, *March*, 73–99.

Saad M. Z, Md Yasin I. S, Azmai M. N. A, Salleh A, Mohamad N. 2022. Streptococcosis in fish. *Streptococcosis in Fish*, *May*, 1–242.

Shahin K, Veek T, Heckman T. I, Littman E, Mukkatira K, Welch T. J, Imai D. M, Pastenkos G, Camus A, Soto E, Soto E. n.d.. *Running title : Lactococcus garvieae in California Rainbow trout*.

Sibley C. D, Peirano G, Church D. L. 2012. Molecular methods for pathogen and microbial community detection and characterization: Current and potential application in diagnostic microbiology. *Infection, Genetics and Evolution*, *12*(3), 505–521.

Sichewo P. R, Gono R. K, Muzondiwa J, Mungwadzi W. 2014. Isolation and identification of pathogenic bacteria in edible fish : A case study of rural aquaculture projects feeding livestock manure to fish in Zimbabwe. *Int.J.Curr.Microbiol*, *3*(11), 897–904.

Silalahi D, Wirawan I. G. P, Sasadara M. M. V. 2021. Optimization of annealing temperature for amplification of EhoscnOla locus in pranajiwa (Euchresta horsfieldii) plant collected from mountains, urban and coastal areas in Bali. *IOP Conference Series: Earth and Environmental Science*, *913*(1).

Stoppani N, Colussi S, Pastorino P, Prearo M, Sciuto S, Altinok I, Öztürk R. Ç, Ture M, Vela A. I, Blanco M. del M, Kotzamanidis C, Bitchava K, Malousi A, Fariano L, Volpatti D, Acutis P. L, Fernández-Garayzábal J. F, Sibley C. D, Peirano G, Church D. L. 2023. 16S-23S rRNA Internal Transcribed Spacer Region (ITS) Sequencing: A Potential Molecular Diagnostic Tool for Differentiating Lactococcus garvieae and Lactococcus petauri. *Microorganisms*, *11*(3), 505–521.

Su Y. L, Feng J, Li Y. W, Bai J. S, Li A. X. 2016. Development of a quantitative PCR assay for monitoring Streptococcus agalactiae colonization and tissue tropism in experimentally infected tilapia. *Journal of Fish Diseases*, *39*(2), 229–238.

Thisted Lambertz S, Nilsson C, Hallanvuo S, Lindblad M. 2008. Real-time PCR method for detection of pathogenic Yersinia enterocolitica in food. *Applied and Environmental Microbiology*, *74*(19), 6060–6067.

Ustaoglu D, Öztürk R.Ç, Ture M, Colussi S, Pastorino P, Vela A.I, Kotzamanidis C, Volpatti D, Acutis P.L. Altinok I. 2024.

Multiplex PCR assay for the accurate and rapid detection and differentiation of Lactococcus garvieae and L. petauri. Journal of Fish Diseases, p.e14004.

Van Doan H, Soltani M, Leitão A, Shafiei S, Asadi S, Lymbery A. J, Ringø E. 2022. Streptococcosis a Re-Emerging Disease in Aquaculture: Significance and Phytotherapy. *Animals*, *12*(18), 1–32.

van Pelt-Verkuil E, van Belkum A, Hays J. P. 2008. Analysis of PCR Amplification Products. *Principles and Technical Aspects of PCR Amplification*, 141–182.

Váradi L, Luo J.L, Hibbs D.E, Perry J.D, Anderson R.J, Orenga S. Groundwater P.W. 2017. Methods for the detection and identification of pathogenic bacteria: past, present, and future. Chemical Society Reviews, 46(16), pp.4818-4832.

Vendrell D, Balcázar J. L, Ruiz-Zarzuela I, de Blas I, Gironés O, Múzquiz J. L. (2006). Lactococcus garvieae in fish: A review. *Comparative Immunology, Microbiology and Infectious Diseases*, *29*(4), 177–198.

Wen-de W, Min L, Ming C, Li-ping L, Rui W, Hai-lan C, Fu-Yan C, Qiang M, Wan-wen, L, Han-zhong C. 2017. Development of a colloidal gold immunochromatographic strip for rapid detection of Streptococcus agalactiae in tilapia. *Biosensors and Bioelectronics*, *91*(November 2016), 66–69.

Yang C, Jiang Y, Huang K, Zhu C, Yin Y. 2003. Application of real-time PCR for quantitative detection of Campylobacter jejuni in poultry, milk and environmental water. *FEMS Immunology and Medical Microbiology*, *38*(3), 265–271.