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Development and application of a sensitive droplet digital PCR for the detection of red mark syndrome infection in rainbow trout (*Oncorhynchus mykiss*).

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Abstract

Red Mark Syndrome (RMS) is a skin disease of uncertain aetiology affecting farmed rainbow trout (*Oncorhynchus mykiss*). It consists of single or multiple skin lesions usually localized on the trunk of fish approaching market size.
Studies showed as rickettsia-like organism (RLO), currently referred as *Midichloria*-like organism (MLO), is supposed to be involved in RMS.
Midichloria-like has been identified consistently in RMS-affected fish. Its identification within skin and spleen has been possible through the use of PCR methods, including standard PCR, nested PCR and quantitative PCR (qPCR).
Droplet digital PCR (ddPCR) is a relatively novel, sensitive, accurate and absolute quantification technique. Recent studies applied successfully this technique to infectious spleen and kidney necrosis virus (ISKNV) in teleosts, proving its superiority when compared to qPCR.
The goal of the present study is to establish a sensitive ddPCR method to rapidly detect and quantify MLO DNA.
We explored the feasibility of ddPCR to detect MLO from 40 rainbow trout spleen samples and compared the data with qPCR. The detection limit of the ddPCR was found to be 2.25 copy number, which is lower than the 36 copy number determined for TaqMan real-time PCR (qPCR). This indicated that the sensitivity of the ddPCR assay was one order of magnitude higher than the sensitivity of the qPCR assay. The detection results for fish samples showed that the positive detection rate of ddPCR (65%) was higher than that of qPCR (52.5%).
The method used has revealed to be specific to MLO and does not cross-react with other fish bacterial DNA. The ddPCR method established in this study shows superiority for detection in minimal volume samples with low bacterial loads and may be used both as surveillance of possible transmission routes and potential sources.

Keywords: red mark syndrome, *Midichloria*-like organism, droplet digital PCR, real-time PCR.

Introduction

Red mark syndrome is a skin condition of uncertain etiology characterised by multiple skin lesions and affecting market size rainbow trout, usually over 100 grams. Morbidity can reach up to 60% and, by affecting the fish's external appearance, this disease can eventually have a significant impact due to downgrading of the commercial product.
RMS has been diagnosed for the first time in Scotland, in late 2003, and has rapidly spread throughout Europe but also in the Middle East, and the Americas (Metselaar et al, 2022).
Case definition studies for this syndrome (Oidtman et al. 2013, Metselaar et al. 2022) has been described in details, including specific features such as histological scoring systems and ultrastructural features (Galeotti et al, 2017; Orioles et al, 2019 and Galeotti et al, 2021), experimental infections through cohabitation studies (Verner-Jeffreys et al., 2008; Jørgenson et al, 2019; Orioles et al., 2021; Pasqualetti et al., 2021) and treatment options and efficacy (Schmidt et al, 2021).
Two of the most important etiological agents that have been associated with this disease are RLO and *Flavobacterium psychrophilum (*Metselaar et al, 2022). No association were found between the latter bacteria and RMS by recent studies (Metselaar et al, 2020).
The RLO, belonging to the Midichloriaceae family and thus referred as MLO (Montagna et al, 2013; Cafiso et al. 2016), is currently considered the strongest etiological candidate for RMS (Metselaar et al, 2020). It has been consistently detected by IHC (immunohistochemistry), PCR (polymerase chain reaction), nested PCR and qPCR (quantitative real-time PCR), in at least three different geographical areas (Metselaar et al, 2020).

PCR and nested PCR techniques have some disadvantages including relatively low sensitivity and the need to use agarose gel electrophoresis to visualize the amplification products. This can generate DNA products contamination. Real time PCR is a quantitative detection method with an higher sensitivity and specificity. It does not require the use of gel electrophoresis. To produce relative quantification a reference gene is required, while for absolute quantification, a standard curve is needed. Moreover, qPCR could display low accuracy in samples with low bacterial loads, such as water samples, eggs, larvae and fry (Lin et al. 2020).

When compared with the above PCR methods, droplet digital polymerase chain reaction (ddPCR) has been shown to generate accurate results in low copy number quantification and is less susceptible to PCR inhibitors (Gutiérrez-Aguirre et al., 2015; Li et al., 2018). This technique has been used in the detection of bacteria, such as *Flavobacterium psychrophilum* and *Yersinia ruckeri* in Norwegian aquaculture systems water (Lewin et al., 2020), virus, such as spleen and kidney necrosis virus (Lin et al., 2020) and Singapore Grouper Iridovirus (Yuan et al., 2016) and parasites, such as *Gyrodactylus salaris* in Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*) (Rusch et al., 2018).

To the authors' knowledge, this is the first report of its use for the detection of MLO in rainbow trout.

Materials and methods

Primers and probe

The primers and probe selected derived from Lloyd et al., 2011 and recognize the sequence registered in GenBank as accession number EU555284 (Lloyd et al., 2008) (Table 1).
The probe was labeled with Fluorescein (FAM) as fluorescent reporter and Black Hole as fluorescence quencher (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA). The

primers were synthetized by Invitrogen (Thermo Fisher Scientific). The same primers and probe were used in both ddPCR and qPCR reactions.

Optimization of the ddPCR method

The ddPCR method was performed in a 20 µL reaction volume, in accordance with the manufacturer's instructions (Bio-Rad, USA). Briefly, the reactions included 10 µL of 2× ddPCR™ Supermix No-UTP for Probes (Bio-Rad, USA), 1 µL of each forward and reverse primer (10 µM), 1 µL of probe, 7 µL of DNase/RNase-free H_2O and 1 µL of DNA template (corresponding to 50 ng of total DNA). The reactions were optimized for probe concentration (125, 250, 500, 750, 1000 and 1500nM). The reaction mixture (20 µL) for each sample was loaded into a well of a disposable DG8™ cartridge (Bio-Rad, USA) and 70 µL of Droplet Generation Oil (Bio-Rad, USA) were placed into each of the adjacent oil wells in the cartridge (Bio-Rad, USA). Droplets were produced in each well using a QX200™ droplet generator (Bio-Rad, USA). The droplets were then transferred to a 96-well PCR plate (Bio-Rad, USA). To differentiate the amplitude between the negative and positive droplets and to reduce the background of the negative droplets, we performed a temperature gradient in the annealing step. The PCR amplifications were performed with an initial step of 95 °C for 10 min, followed by 40 cycles of 94 °C for 30 s, 64.5 °C for 60 s and 1 cycle of 98 °C for 10 min, with a final hold at 4 °C. To achieve the best results for the method, a range of annealing temperatures (58, 58.5, 59.4, 60.8, 62.5, 63.9, 64.5 and 65 °C) was tested. After PCR amplification of the DNA target in the droplets the microdroplets from each well were read individually using a QX200 Droplet Reader (Bio-Rad, CA, USA). A threshold was set between the positive and negative microdroplet clusters and the copy number of each well was evaluated automatically by QuantaSoft™ version 1.7 (Bio-Rad, CA, USA).

A post-run analysis was adopted to evaluate the quality of the data produced by the ddPCR reaction. The preliminary quality control was the count of total number of droplets generated with ≥ 7,000 droplets/well. To ensure an accurate classification of compartments and thus a reliable quantification of positives and negatives droplets, three different criteria were adopted.The first requirement was to set a quantification threshold that would allow the production of a single amplicon per signal. The second aspect was the definition of the best peak resolution; in order to produce the widest signal separation. The third criteria was based on the removal of stragglers or 'rain' droplets that have an intermediate fluorescence and do not belong to either the positive or negative population.

Real-time PCR method

The qPCR amplification reactions were performed in a final volume of 20 µL, which contained 1× iQ Supermix (Bio-Rad, Hercules, CA, USA), 400 nM each primer, 250 nM probe, DNA (1 µL) and H_2O (6.9 µL). The qPCR reactions were performed using the CFX96 real-time PCR System (Bio-Rad) as follows: denaturation at 95°C for 3 min, 40 cycles of 95°C for 30 s and 64.5 °C for 60 s. Fluorescent signals were collected during the 64.5°C step of each cycle.

Specificity of the ddPCR method

To evaluate the specificity of the MLO-specific ddPCR method, DNA from the fish affected with RMS and DNA from bacteria strains of *Lactococcus garvieae*, *Yersinia ruckeri*, *Photobacterium damselae piscicida* and *Vibrio anguillarum* were tested.

Sterilized water was used as negative control. These bacteria strains were stored at the Veterinary Pathology laboratory of Udine University. The authors cultivated the bacteria using standard media and conditions, and performed the DNA extraction by QIAamp DNA Mini Kit (Qiagen, Hilden, Germany), following their standard protocol for bacteria.

Analytical sensitivity of ddPCR and qPCR methods

To evaluate the sensitivity of the two methods, a serial diluition (1:1; 1:2; 1:5; 1:10; 1:50; 1:100) was prepared starting from a MLO positive samples derived from the RMS experimental infection model established at the DTU-Aqua, National institute of Aquatic Resources and European reference laboratory for fish and shellfish diseases, Denmark. The same sample serial dilution was tested by qPCR and ddPCR to determine the detection limit of the two methods. Each dilution was tested in triplicate, in three independent runs, to evaluate inter-assay reproducibility.
The standard curve for the qPCR quantification was generated using a 10-fold serial dilution of the DNA of another MLO positive sample derived from DTU aqua institute. Fluorescent signals from the FAM-labeled MLO probe in the qPCR assay were analysed by the CFX96 real-time PCR System (Bio-Rad). The qPCRs were performed in triplicate. The amplified DNA of the dilutions was purified, the concentration was determined, the number of copies was calculated, and the standard curve was constructed by plotting log of the number of copies (Log starting quantity) against the values of Ct.

Comparison of efficacy of ddPCR and qPCR methods

To compare the efficacy of the ddPCR method for MLO detection with that of qPCR, we collected spleen from 40 healthy and infected rainbow trout spleens obtained from the RMS experimental infection unit at the DTU-Aqua. Total DNA was extracted from tissue samples using the QIAamp DNA Mini Kit (Qiagen), in accordance with the manufacturer's instructions. The DNA was tested by ddPCR using the optimized protocol described above. The results were compared with those obtained from the qPCR method, which was performed in parallel.

Statistical analysis

All statistical analyses and data plotting were performed using GraphPad Prism software (Version 5.0; La Jolla, CA, USA). Kappa statistics were used to compare the detection results from the ddPCR and qPCR methods and to determine their level of agreement.

Results

Development of an MLO-specific ddPCR method

Initially, we assessed different probe concentrations (125, 250, 500, 750, 1000 and 1500 nM) using the ddPCR method. The results showed that the optimal concentration was 250 nM, which generated the largest number of positive droplets (Figure 1 and 2). Next, we identified the optimal annealing temperature for the ddPCR method by testing temperatures of 58, 58.5, 59.4, 60.8, 62.5, 63.9, 64.5 and 65°C. As shown in Figure 3, as the annealing temperature rose, the distinction between the intensity signals of the positive (blue) and negative (grey) droplets became bigger. The annealing temperature of 64.5°C gave the greatest distinction between the positive and negative signals, the largest number of positive droplets and the lower dispersion among the positive

droplets. Therefore, 64.5°C was chosen as the optimal annealing temperature for the ddPCR method.
The ddPCR software reported the copy number concentration of each sample automatically after applying the positivity threshold that allows the production of a single amplification signal. The minimum number of droplet accepted was 7'000 droplets/well.

The Poisson error and total error were calculated by QuantaSoft software and indicate the Poisson 95% confidence intervals for each copy number determination. Results obtained were accepted when relative standard deviation values were below the 5%.

Specificity of the ddPCR method

To evaluate the specificity of the ddPCR method, DNA from the fish affected with RMS and DNA from four bacteria strains: *Lactococcus garvieae*, *Yersinia ruckeri*, *Photobacterium damselae piscicida* and *Vibrio anguillarum* were tested. As shown in Figure 4, only MLO samples tested positive, whilst samples containing all the other pathogens tested negative. The results showed that the ddPCR method was specific for the detection of MLO.

Analysis of standard curves using the qPCR method

Fluorescent signals from the FAM-labeled MLO probe in the qPCR assay were analysed by the CFX96 real-time PCR System (Bio-Rad) and a standard curve was drawn (Figure 5). The slope of the standard curve and the PCR efficiency (99.2%) value were in the appropriate range. The equation for the regression line was $Y = -3.23 x + 41.459$. The R^2 value of the standard curve was 0.9943 and indicated good linearity.

Detection limits of ddPCR and qPCR methods

A serial dilution of the DNA of a positive sample supplied by DTU Aqua, was used to measure the detection limits of the qPCR and ddPCR methods. In the sensitivity tests, the lowest detectable concentration was 2.2 copy number for ddPCR, corresponding to 1:50 dilution of the sample and 6 copy number input, instead, the lowest detectable concentration for qPCR was 36 copy number, corresponding to 1:10 dilution of the sample and 30 copy number input (Table 2, Figure 6). The results indicated that the established MLO-specific ddPCR method was able to detect very low concentrations of template and was considerably more sensitive (an order of magnitude higher) than the qPCR method used in our study.

Detection of MLO in rainbow trout spleen samples using ddPCR and qPCR methods

The 40 samples of rainbow trout (healthy and infected) spleen were obtained from DTU aqua institute. The MLO presence in these samples was detected using both the qPCR and ddPCR methods. The detection rates were calculated, and the results are shown in Table 3. The ddPCR method gave more positive samples (65%) when compared to the qPCR method (52.5 %), which indicated that ddPCR was a more effective method for the detection of the bacteria in fish with low bacterial loads. The percentage of agreement between the two analyses was 87.5%, with a Cohen's k-coefficient of 0.75 (substantial agreement). Table 3 also shows that the bacterial load range detected by ddPCR was 2 to 606 copies whilst the bacterial load range detected by qPCR was 2 to 378 copies.

Positive droplets ranged from 0 to 285, with a mean of 13.53 and a standard deviation of 47.51. Copy number of detected DNA using ddPCR ranged from 0 to 606, with a mean value of 26.03 and standard deviation of 97.66. The ratio between detected DNA and number of positive droplets is 1.92.

Discussion

The ddPCR method has been used as a specific and sensitive molecular detection system in fish pathology for some bacteria, virus and parasites (Yuan et al, 2016; Rusch et al., 2018; Lewin et al., 2020; Lin et al., 2020). When compared with other detection techniques, ddPCR is advantageous as it is sensitive, accurate and does not require an external standard curve (Gutiérrez-Aguirre et al., 2015). In this study, we established a ddPCR method to detect and quantify MLO DNA loads associated with RMS in fish spleen samples. This showed high specificity and sensitivity.
The sensitivity experiments demonstrated that the ddPCR was one order of magnitude more sensitive than the qPCR. The limit of detection was 2.2 copy number (6 input copy number, 1:50 dilution of the positive sample), whilst the limit of detection for the qPCR was 36 copy number (30 input copy number, 1:10 dilution of the positive sample). When compared with the previously reported MLO detection techniques, the sensitivity in this study was higher when compared to PCR (0.1 pg/µL) (Lloyd et al., 2011; Cecchini et al., 2017). Other studies have also reported similar sensitivity results for ddPCR detection of *Flavobacterium psychrophilum* (1-240 gene copies/ml water; Lewin et al., 2020), spleen and kidney necrosis virus (1.5 copies/µL; Lin et al., 2020), and *Gyrodactylus salaris* parasite (7.8-8.8 copy/µL; Rusch et al., 2018).
Our ddPCR system showed a higher positive predictive value when compared to the qPCR, which indicated that ddPCR is a more effective method for bacteria detection, and showed a better reproducibility than the qPCR, both true especially when the bacterial DNA is at a low concentration (Lewin et al., 2020). All these features could be extremely useful in future RMS studies to investigate the presence of MLO in possible vectors such invertebrates and parasites (MLO seems to be able to be transiently and carried by *Ichthyophthirius multifiliis* – Pasqualetti et al., 2021), water, eggs and environment in general where DNA can be scarce. Monitoring pathogen levels in water samples could aid early detection and surveillance especially in RAS systems and for research purposes.

The ddPCR data were direct measurements taken by a digital mechanism, without the need for standard curves (Cao et al., 2017). The digital system provided a total number of negative and positive droplets, which were used in the calculation of the absolute quantification by the Poisson distribution (Basu, 2017).
However, when compared to qPCR, the ddPCR exhibited a narrower range of detection because the ddPCR system generates approximately 20,000 droplets per reaction and counts the number of positive and negative droplets. When the template copy number is higher 20,000, the ddPCR droplets become completely saturated. This may be a limitation to the ddPCR method (Hindson et al., 2011), but it can be easily resolved by diluting the samples.
In summary, a new methodology for detection and absolute quantification of MLO has been established and applied in this study. The ddPCR method is repeatable and can detect extremely low concentrations of MLO DNA without the need for standard curves. This methodology might support further research on RMS by providing a powerful tool to detect the presence of MLO in the environment (through eDNA detection and quantification). Further work is needed to evaluate the repeatability and stability within and among laboratories.

In order to detect MLO in fish tissue samples with a high level of sensitivity, we established a ddPCR method for the detection and absolute quantification of MLO and compared the sensitivity and accuracy with qPCR. Our study has provided a powerful, accurate, sensitive and precise tool for the surveillance of RMS in fish samples and for use in general fish bacteria research.

Declaration of Competing Interest

The authors declare they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Tables and Figures

Table 1. Primers and probe used in the study.

Gene	Sequence name	Sequence (5'-3')
MLO	Forward	GGCTCAACCCAAGAACTGCTT
	Reverse	GTGCAACAGCGTCAGTGACT
	Probe	CCCAGATAACCGCCTTCGCCTCCG

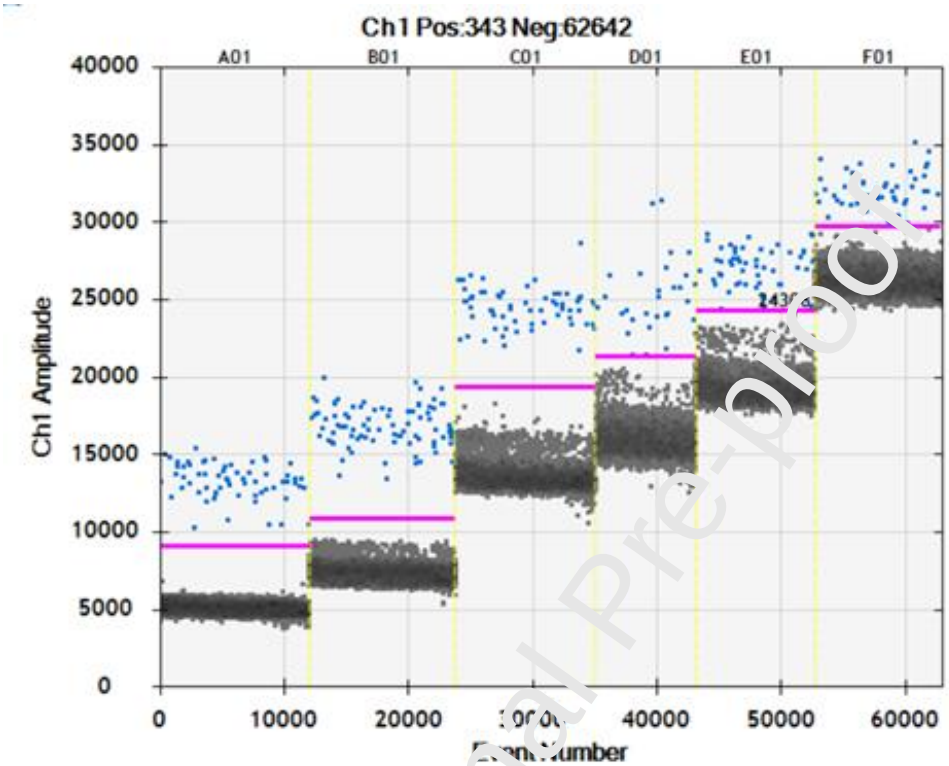

Figure 1. Optimization of the probe concentration for the ddPCR method. Lanes A01, B01, C01, D01, E01 and F01, which are divided by vertical yellow lines, represent the gradient of the following probe concentrations: 125, 250, 500, 750, 1000 and 1500 nM. Lane B01 (probe concentration 250 nM) showed the lower positive events dispersion. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

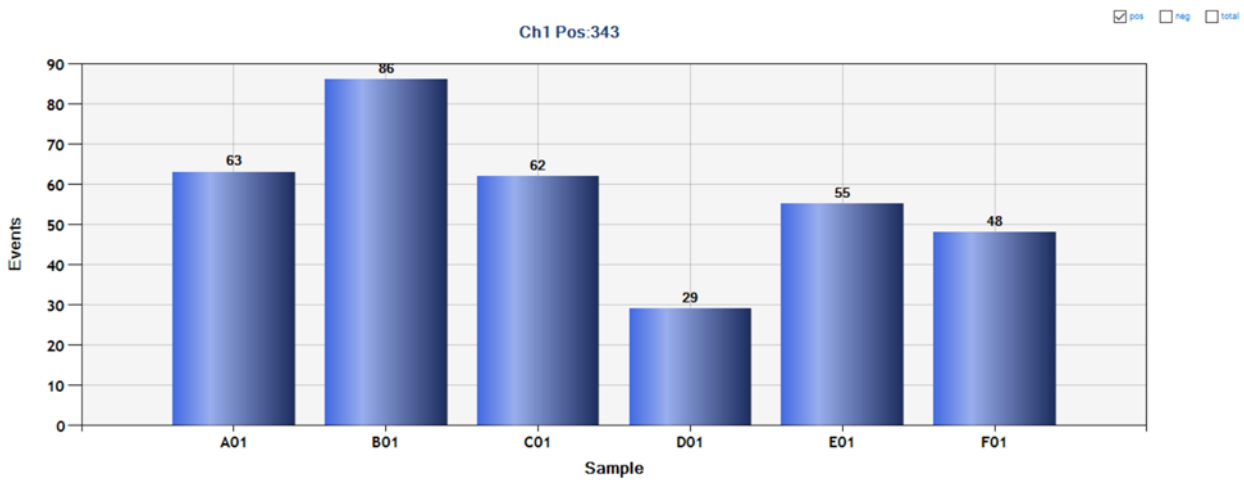

Figure 2. Optimization of the probe concentration for the ddPCR method. Columns A01 to F01 represent the gradient of the following probe concentrations: 125, 250, 500, 750, 1000 and 1500 nM. Lane B01 (probe concentration 250 nM) showed the higher positive events count.

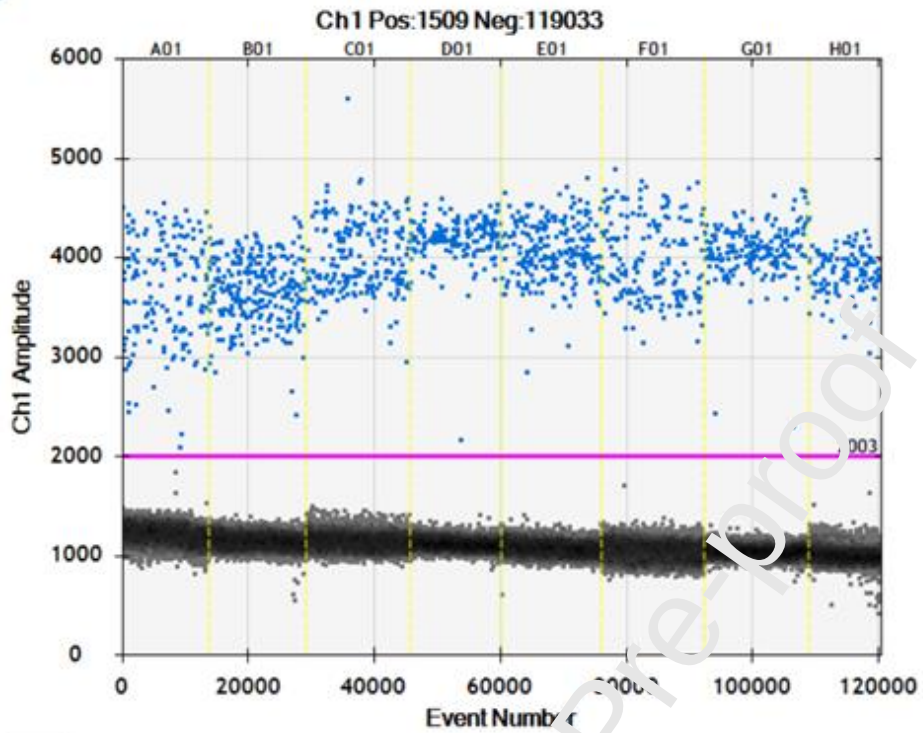

Figure 3. Optimization of the annealing temperature for the ddPCR method. Lanes A01 to H01, which are divided by vertical yellow lines, represent the gradient of the following annealing temperatures: 58, 58.5, 59.4, 60.8, 62.5, 63.9, 64.5 and 65°C. Lane G01 (annealing temperature 64.5°C) showed the lower positive events dispersion. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

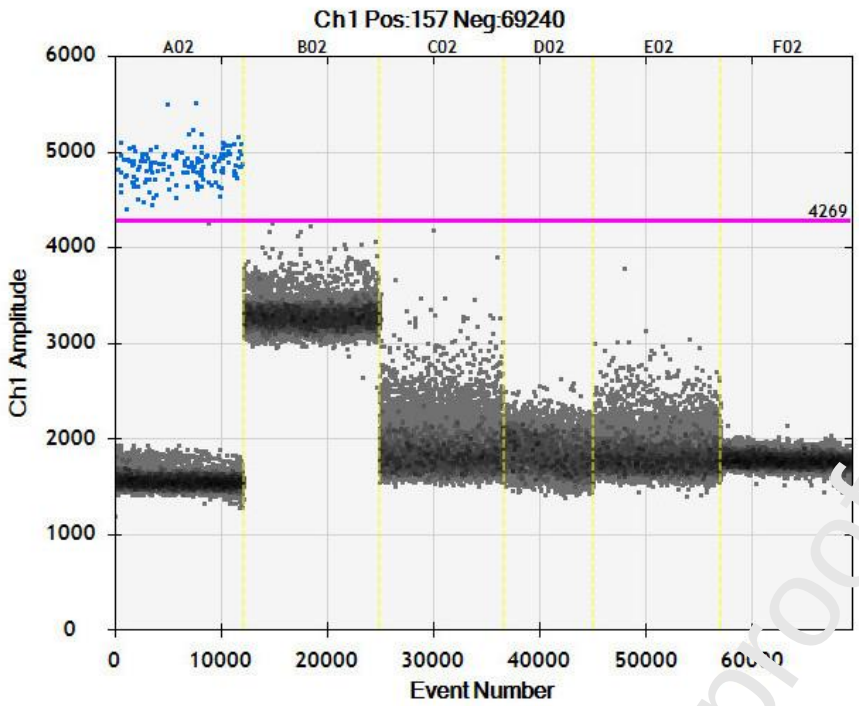

Figure 4. The specificity of the ddPCR method. Lanes A02, B02, C02, D02, E02 and F02, which are divided by vertical yellow lines, represent the fluorescence for MLO, *Photobacterium damselae piscicida*, *Yersinia ruckeri*, *Lactococcus garvieae*, *Vibrio anguillarum* and water (negative control) samples, respectively. Only MLO samples tested positive, whilst samples containing the other pathogens or water tested negative. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

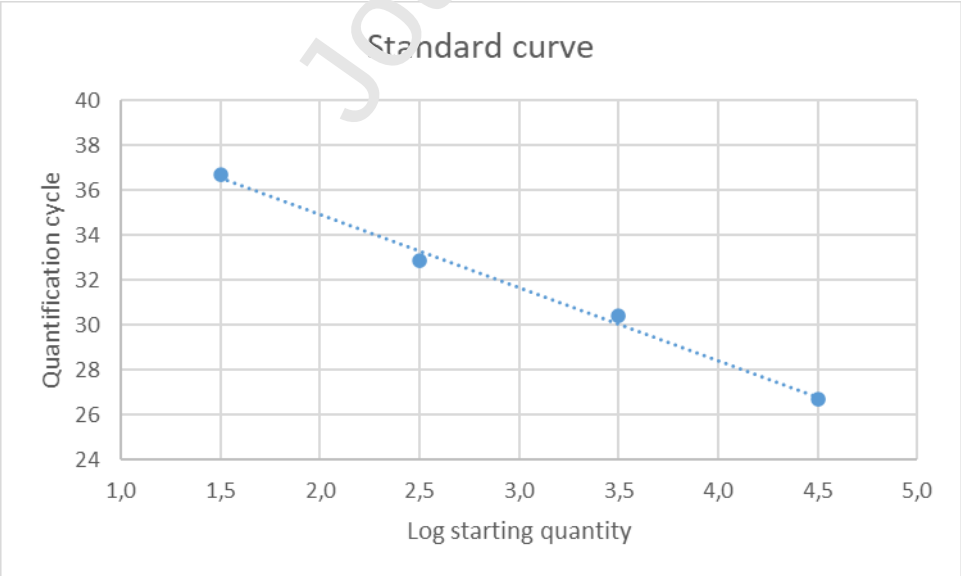

Figure 5. Standard curve from the qPCR method. Ten-fold serial dilutions of the DNA amplified and purified of a MLO positive sample were assessed in triplicate by the qPCR method. The quantification correlation

was obtained by plotting the quantification cycle (mean of the triplicate) against the log starting concentration. The R^2 value was 0.9943.

Table 2. Sensitivity of the ddPCR and qPCR methods, obtained by the serial dilution of a positive sample DNA.

Sample dilution	Input DNA copy number	ddPCR			qPCR		
		1	2	3	1	2	3
1:1	300	302	304	272	272	252	261
1:2	150	144	156	138	144	145	138
1:5	60	50	55	38	80	83	82
1:10	30	20	28	24	36	36	36
1:50	6	2.5	2	2	ND	8	ND
1:100	3	ND	ND	ND	ND	ND	ND

ND = non detected.

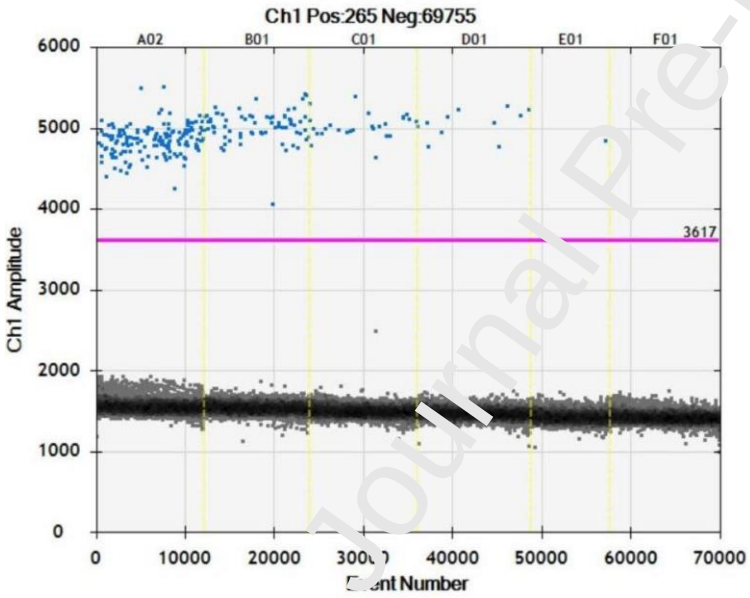

Figure 6. Determination of the limit of detection for the ddPCR method, one of three test. Lanes A02 to F01, which are divided by vertical yellow lines, represent the following observed copy number: 302, 144, 50, 20, 2.5 and 0, respectively, corresponding to the following dilutions of the positive sample: 1:1, 1:2, 1:5, 1:10, 1:50 and 1:100 respectively. The lowest detectable concentration was 2.5 copy number. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Table 3. RLO DNA copy number in rainbow trout spleen determined by using ddPCR and qPCR.

Sample n.	ddPCR			qPCR	
	Number of droplets	Positive droplets	Detected DNA	Mean Cq value	Detected DNA
1	14034	6	18	37.8	13
2	10886	1	2	ND	0
3	13904	1	2	ND	0
4	10883	4	14	38.8	6
5	12973	7	11	39.6	4
6	10898	1	2	ND	0
7	11385	0	0	ND	0
8	13326	2	4	39.2	5
9	14788	0	0	ND	0
10	15279	0	0	ND	0
11	16679	0	0	ND	0
12	15645	0	0	ND	0
13	12839	0	0	ND	0
14	13603	0	0	ND	0
15	14470	3	4	39.7	3
16	15038	3	17	38.4	8
17	12333	2	6	ND	0
18	15330	2	44	37.2	18
19	14584	3	19	37.3	18
20	14949	2	4	40.4	2
21	16686	6	18	39.3	4
22	10943	0	0	ND	0
23	10295	0	0	ND	0
24	11576	2	4	39.6	4
25	10438	1	2	40.2	2
26	11738	5	10	39.4	4
27	9847	7	14	38.1	10
28	8706	3	8	39.7	3
29	14226	5	10	39.0	5
30	14390	86	192	36.1	40
31	14542	4	10	39.4	4
32	10777	1	2	39.4	4
33	11097	6	16	37.7	14
34	9054	0	0	ND	0
35	11035	285	606	32.9	378
36	7186	0	0	ND	0

37	9802	0	0	ND	0
38	7695	0	0	ND	0
39	9241	0	0	ND	0
40	10516	93	2	ND	0

ND = non detected.

Declaration of interests

☐The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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☒The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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Highlights for

Development and application of a sensitive droplet digital PCR (ddPCR) for the detection of Red Mark Syndrome infection in Rainbow trout (*Oncorhynchus mykiss*)

. RMS is wide spread in rainbow trout industry worldwide

. First time reported development and validation of ddPCR for detection of RMS

. Comparison of this technique with standard PCR techniques currently used

. Digital droplet PCR could be used for surveillance studies on RMS