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SHORT COMMUNICATION

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A comparison of the use of different swab materials for optimal diagnosis of amoebic gill disease (AGD) in Atlantic salmon (*Salmo salar* L.)

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Abstract

Routine gill swabbing is a non-destructive sampling method used for the downstream qPCR detection and quantitation of the pathogen *Neoparamoeba perurans*, a causative agent of amoebic gill disease (AGD). Three commercially available swabs were compared aiming their application for timelier AGD diagnosis (Calgiswab® (calcium alginate fibre-tipped), Isohelix® DNA buccal and cotton wool-tipped). Calcium alginate is soluble in most sodium salts, which potentially allows the total recovery of biological material, hence a better extraction of target organisms' DNA. Thus, this study consisted of (a) an in vitro assessment involving spiking of the swabs with known amounts of amoebae and additional assessment of retrieval efficiency of amoebae from agar plates; (b) in vivo testing by swabbing of gill arches (second, third and fourth) of AGD-infected fish. Both in vitro and in vivo experiments identified an enhanced amoeba retrieval with Calgiswab® and Isohelix® swabs in comparison with cotton swabs. Additionally, the third and fourth gill arches presented significantly higher amoebic loads compared to the second gill arch. Results suggest that limiting routine gill swabbing to one or two arches, instead of all, could likely lead to reduced stress-related effects incurred by handling and sampling and a timelier diagnosis of AGD.

KEYWORDS

amoeba, calcium alginate, diagnostics, sodium citrate

1 | INTRODUCTION

Amoebic gill disease (AGD), caused by *Neoparamoeba perurans*, is one of the main health challenges for the global Atlantic salmon (*Salmo salar* Linnaeus, 1758) farming industry (Oldham, Rodger, & Nowak., 2016; Rodger, 2014). This parasite's presence in a number of other marine fish species (Oldham et al., 2016), including cleaner fish species used for the biological control of sea lice in Atlantic salmon farms (Haugland, Olsen, Rønneseth & Andersen, 2017), has resulted in the emergence of new challenges for the industry especially as high mortalities can result if AGD is left untreated (Munday, Zilberg, & Findlay, 2001).

Current approaches for controlling AGD are resource-demanding and labour-intensive, involving numerous treatments throughout

a production cycle. Freshwater bathing has been established as the standard method of treating the disease in Tasmania but is limited by restricted access to freshwater (Nowak, Vadenegro-Vega, Crosbie, & Bridle, 2014). Another recognized treatment is the use of hydrogen peroxide in cooler production areas. However, this latter treatment has a reduced safety margin at higher temperatures (Adams, Crosbie & Nowak, 2012) or where fish are compromised by advanced-stage AGD (McCarthy et al., 2015). Overall, AGD-related mortality is increasing, causing major economic losses in regions of Atlantic salmon aquaculture, including Tasmania, Norway and Scotland (Martinsen, Thorisdottir, & Lillehammer, 2018; Oldham et al., 2016; Shinn et al., 2015).

The use of routine diagnosis procedures for AGD is critical for the timely treatment of AGD-infected fish. Although histopathology remains one of the preferred techniques for the case definition of AGD (Clark & Nowak, 1999; Rodger, 2014), the monitoring of gross gill scores (Taylor, Muller, Cook, Kube, & Elliott, 2009) is by far the most extensively used and practical method for establishing AGD severity and is used as a key prompt for intervention using available treatments. Both of the above techniques are commonly used together, with microscopic analysis employed to confirm the presence of lesion-associated amoebae within the gills. Since the identification of *N. perurans* as the causal agent of AGD (Crosbie, Bridle, Cadoret, & Nowak, 2012; Young, Crosbie, Adams, Nowak, & Morrison, 2007), specific DNA-based molecular diagnostic assays for the detection of the amoebae have been developed in different studies (Bridle, Crosbie, Cadoret, & Nowak, 2010; Downes et al., 2015; Fringuelli, Gordon, Rodger, Welsh, & Graham, 2012).

Even though histopathology of gill filaments enables confirmation of both the presence of the pathogen and the resultant localized and systemic host response, it requires destructive sampling, which could mean the killing of valued stock during epidemiological studies (Adams & Nowak, 2004; Douglas-Helders, Saksida, Raverty, & Nowak, 2001). Therefore, the use of non-destructive tools to confirm the presence of *N. perurans* was studied by Downes et al. (2017). When non-destructive gill swabbing was performed, results showed a great improvement on the sensitivity of diagnosis in comparison with gill filament biopsies. However, the type and physical structure of swab fibres influence the uptake of target organisms from the site of swabbing and the subsequent release of target organisms from the swab for downstream DNA extraction and qPCR quantification (Turner, Harry, Lofland, & Madhusudhan, 2010). Currently, Isohelix® DNA buccal and cotton wool-tipped swabs are the most commonly used swabs in a commercial setting. However, it is hypothesized, for the purpose of this study, that calcium alginate fibre-tipped swabs could offer an advantage, as they can be dissolved in most sodium salts to give soluble sodium alginate, potentially enhancing collection of targeted organisms. This material has multiple uses in the area of bioengineering for cell encapsulation, surgical sponges, polymer films or wound dressings (Boateng, Matthews, Stevens, & Eccleston, 2008; Klöck et al., 1994; Kneafsey, O'Shaughnessy, & Condon, 1996). The polymer's simple structure and highly hydrophilic nature allow the diffusion of biological fluids into the polymer. This can translate into a better recovery and subsequent extraction of target organisms' DNA, and these properties have been exploited for detection of pathogens in bacterial infections from skin and nose (Panpradist et al., 2014), potentially improving diagnostic sensitivity. However, this material has never been investigated in the context of the aquaculture industry.

Whilst the type and physical structure of swab fibres are important, the method and area of swabbing are also relevant for successful diagnosis of AGD. Although the universal swabbing method is based on sampling of the second gill arch within research (Adams & Nowak, 2003, 2004a; Chalmers et al., 2017; Munday et al., 2001; Taylor et al., 2009; Wynne, Cook, Nowak, & Elliott, 2007; Young, Dyková, Nowak, & Morrison, 2008), worldwide aquaculture industry performs swabbing of all four gill arches. Although the swabbing of all gill arches increases the swabbing area and therefore detection of *N. perurans* is more likely to be successful, irritation of the gills could be greater. Even though the effect of gill swabbing has not been yet investigated, it is logical to assume that the more you inflict physical changes on such a sensitive organ, the higher are the probabilities of causing additional stress on the fish (Mallat, 1985).

The work undertaken in this study focused on determining whether there was a difference of diagnostic sensitivity for AGD between sampling swab types in vitro and in vivo. Additionally, the study aimed to verify whether there was a difference between samples taken from different gill arches (2–4) culminating in a potential enhancement of diagnostic sensitivity for AGD. The outcomes of applying different swab materials were also assessed to establish if the scoring method correlated with the detection of *N. perurans* through the molecular techniques used with this sampling regime.

2 | MATERIALS AND METHODS

2.1 | Clinical swabs

Three different commercially available swabs, Calgiswab®, a standard calcium alginate swab (Puritan®, USA), and two swabs currently used for pathogen detection in the aquaculture industry, namely Isohelix® DNA buccal swabs (Isohelix, UK) and cotton wool-tipped swabs (Shintop, UK), were tested.

2.2 | Clonal development and culture conditions of *Neoparamoeba perurans*

Amoebae were obtained from AGD-infected fish, which had previously been humanely killed using an overdose of the anaesthetic MS-222 (100 ppm) and destruction of the brain according to UK Home Office Schedule 1 methods at the Institute of Aquaculture Marine Environmental Research Laboratory (MERL), Machrihanish, Scotland. Work was conducted under University of Stirling ethical approval, reference number AWERB/1617/173/New ASPA. For isolation of amoebae, AGD-affected gills were first pathology-scored

according to the gill scoring protocol of Taylor et al. (2009). All the gill arches from the left side were excised and placed in 50-mL tubes with 35 ppt filtered sea water (using a 0.2-μm-size filter) (FSW) from MERL, shaken for 30s, and the gills were discarded, with the liquid potentially containing amoebae being transferred to 25-cm^2 tissue culture flasks (Sarstedt AG & Co. KG, Germany). Monitoring of the flasks was performed daily, and bacterial contamination was limited through repeated washes with filtered sea water (FSW) with a 0.22-μm Millipore membrane filter (Sigma-Aldrich, UK). Isolates were routinely maintained in 75-cm^2 cell culture flasks supplemented with malt yeast broth (MYB; 0.1% yeast (product number: Y1625; Sigma-Aldrich) and 0.1% malt (product number: 70,146; Sigma-Aldrich) per litre of FSW) at 15°C.

Amoebae were maintained and regularly observed under a compound light microscope, with different morphologies, including the attached pseudocyst and floating trophozoites being visible. To limit bacterial growth, flasks were washed with FSW every 2 days and supplemented with fresh MYB or FSW depending on the level of bacterial contamination. Sub-culturing was performed every 7–10 days, depending on amoeba growth, by transporting from smaller flasks to bigger flasks according to cell growth. Flasks were shaken for no longer than 30s, and this mechanical disruption culminated in the detachment of the amoeba which was then transferred to 125-cm^2 cell culture flasks (Sarstedt AG & Co. KG, Germany) for the generation of a high-yield amoeba culture.

For the development of clonal cultures, amoebae were isolated through a manual single-picking technique (with a flame-drawn glass pipette) in 96-well plates (Corning®, USA) supplemented with 100 μl of MYB. After approximately 14 days, amoebae were transferred to 75-cm^2 cell culture flasks (Sarstedt AG & Co. KG, Germany) supplemented with 10 ml of MYB. When a monoclonal culture had developed and grown, cells were then harvested and centrifuged at 800 × g for 10 min. The supernatant was discarded by slowly pipetting it out, and the pellet was resuspended in 2.5 ml of FSW. The number of cells was quantified using a haemocytometer (Neubauer Improved, Marienfeld, Germany). Replicates of five counts were performed in four large squares of the whole grid. Cell density was adjusted to the desired quantity by dilution with FSW.

For confirmation of the presence of *N. perurans*, a DNA extraction was performed with the DNeasy Tissue Kit (Qiagen, Doncaster, Vic., Australia) followed by a diagnostic PCR with specific primers for the 18S rRNA gene of *P. branchiphila* (F: '5-GACCCTTTTGGGAAGAGATG-3'; R: '5-CAGCCTTGCGACCATACTC-3'), *P. pemaquidensis* (F: '5-GACCCTTTTGGGAAGAGATG-3'; R: '5-CAGCCTTGCGACCATACTC-3') and *N. perurans* (F: '5-CTGGTTGATCCTGCCAGTAGTC-3'; R: '5-CAGCCTTGCGACCATACTC-3') used by Young et al. (2007). Amplification of the 18S rRNA gene was performed in volumes of 20μl containing between 10 and 20 ng of DNA, myTaq polymerase (Bioline, UK) and a set of primers (10 μM) for the previous mentioned 18S rRNA sequences described in Young et al. (2007).

The PCR cycle conditions comprised 95°C for 5 min; 95°C for 30 s, 58°C for 30 s and 73°C for 2 min, for 35 cycles; and 73°C for 8 min. Full-length 18S rRNA gene of *N. perurans* (637 bp) was used as a positive control and a sample with only ddH_2O as the negative (no template DNA; NTC) control. The PCR products were subjected to electrophoresis through 1% agarose/Tris–borate EDTA buffer, and bands were visualized by staining with a final concentration of 0.5 μg/ml from a 10 mg/ml ethidium bromide stock.

2.3 | Evaluation of the potential inhibition of *Neoparamoeba perurans* PCR detection by sodium citrate

In order to determine whether sodium citrate inhibited subsequent molecular analysis, amoebae were harvested from a one-week-old 75-cm^2 flask tissue culture, centrifuged at 800 × g for 10 min and collected in Eppendorf tubes to give a final concentration of 1×10^3 cells ml^{-1}. Finally, these were resuspended in 1 ml of 0.2 M sodium citrate solution. Samples were stored at 4°C for 7 and 14 days. Following DNA extraction, PCR detection was performed as described in the previous section.

2.4 | In vitro testing of the three clinical swabs

2.4.1 | Spiking of swabs with N. perurans

Swabs were inoculated with a sample volume of 15 μl, which was less than the fluid capacity for all swabs, as described in Panpradist et al. (2014). Each swab type ($n = 10$) was spiked with 15 μl containing different numbers of amoebae: low (10 amoebae), medium (100 amoebae) and high (1,000 amoebae). Calcium alginate-tipped swab tips were immersed in a 1.5-ml centrifuge tube with 0.2 M sodium citrate and manually shaken for 30s before being discarded. Isohelix® and cotton swab tips were immersed in 1.5-mL centrifuge tubes with 95% ethanol. Ethanol samples were stored in the freezer at −20°C and sodium citrate samples were stored in the fridge at 4°C until molecular analyses were carried out.

2.4.2 | Retrieval/recovery of N. perurans from agar plates

Each seawater agar plate (SWA; FSW at 35 ppt salinity, filtered through 0.22 μm, and 10 g agar) ($n = 10$ per group) was spiked with 50 μl containing different numbers of amoebae: low (10 amoebae), medium (100 amoebae) and high (1,000 amoebae), and the volume was made up to 5 ml with FSW. Plates were incubated for 2 hr at 15°C in order to allow the attachment of amoebae to the agar surface. The overlay was then removed prior to the immediate standardized swabbing. This method followed the swabbing of the plate first in a vertical angle. Then, the plate was rotated 90 degrees to the right and final swabbing was done to cover the whole surface of the plate.

The treatment of the swab tips for molecular analysis was carried out as described in previous sections.

2.5 | In vivo testing of the three clinical swabs

2.5.1 | Experimental fish and swabbing method for AGD-infected gills

The in vivo experiments were carried out at Marine Environmental Research Laboratory (MERL), Institute of Aquaculture, Machrihanish (Scotland, UK, 55.4°N 5.7°W). The experimental facility was supplied with flow-through sea water (35 ‰), filtered at 100 μm. Fish were maintained under ambient temperatures (min: 11°C; max 13°C) and fed with commercial salmon pellets equivalent to 1% of their body weight per day.

For the gill swabbing, a total of 60 fish were sampled over the course of two sampling events (December 2018 and April 2019). Fish were infected with AGD by cohabitation challenge. The cohabitation challenge was undertaken according to methods developed at the Institute of Aquaculture, Stirling, Scotland, and was carried out as follows. Challenge cohabitants were produced using a stock of infected Atlantic salmon held at the MERL facility as part of an in vivo amoeba culture. Four of these pre-infected fish were added to a separate stock of 40 naïve Atlantic salmon smolts with an average weight of 0.162 kg (± 0.007) and average length of 25.83 cm (± 0.864). Gills were grossly assessed until the appropriate gill score for cohabitation infection (approximately 1.5–2 gill score after 3 weeks of cohabitation) was achieved. These cohabitants were adipose fin-clipped, marked with Panjet (0.0652 g alcian blue ml^{-1}, Sigma-Aldrich, UK) and added to the appropriate challenge groups (6 cohabitants $tank^{-1}$). Gills were scored according to the conventional gill scoring method (Taylor et al., 2009). Swabbing was carried out on both sides of the hemibranchs of the second, third and fourth gill arches in replicates of ten for each gill arch and swab type (90 samples in total). The treatment of the swab tips for molecular analysis was carried out as described in previous sections.

2.6 | Swab processing, DNA extraction and qPCR quantification

Prior to the DNA extraction, a pretreatment of the ethanol-preserved swab tips was first needed. They were removed from storage, vigorous agitation was performed with a Top Mix FB15024 Vortexer (Fisher Scientific, UK) for 60s at a maximum frequency setting, and, ultimately, the tip of the swabs was discarded. For the sodium citrate tubes, no swab tip was longer inside the tube so they were directly centrifuged.

To pellet the amoebae, tubes were centrifuged at 14,000 x *g* for 10 min. Ethanol was carefully discarded, and the remaining liquid was pipetted off. Tubes were left open to dry for up to 1 hr in a heat cabinet at 60°C. The same procedure was followed for the sodium citrate-preserved swabs; however, no drying step was needed for the alginate swabs.

After centrifugation of the tubes, DNA extraction was then performed following the manufacturer's instructions for the Wizard® SV Genomic DNA Purification (Promega) with a few variations. A volume of 100 μl of nuclei lysis buffer, 25 μl EDTA (both included in the DNA Extraction Kit) and 10 μl of proteinase K (New England Biolabs, USA) was added to each tube, and tubes were incubated for 3 hr or overnight in a heat cabinet at 60°C. Once the incubation was finished, a volume of 250 μl of preheated (at 60°C) SV buffer was added to the tubes and the contents were transferred to the columns. Columns were then centrifuged at 12,000 x *g* for 1 min. A column wash was performed with 500 μl of the column wash buffer, and columns were centrifuged for 3 min at 14,000 x *g*. Lastly, DNA was eluted in 50 μl of distilled water.

NanoDrop results from the spiked samples showed DNA yields ranging from 0.54 to 4.90 ng μl^{-1}. Plate swabs showed a higher range from 0.75 to 8.35 ng μl^{-1}. From the in vivo trials, similar ranges were observed (0.55 to 7.65 ng μl^{-1}). For the detection of the pathogen, a standard volume of DNA solution was used from each sample (5 μl/qPCR).

The qPCR quantification was carried out using the qTOWER^3 (Analytik Jena, Germany) with a set of primers designed in Mowi Laboratories, Fort William, UK (FW: 5' GTT CTT TCG GGA GCT GGG AG 3'; RV: 5' GAA CTA TCG CCG GCA CAA AAG 3'), and a probe (FAM) (5' CAA TGC CAT TCT TTT CGG A 3'). Primer and probe concentrations for each well were 0.3 μM and 0.15 μM, respectively. Every reaction volume was set to 20 μl. A volume of 15 μl was set for the primers, probe and master mix (Luna® Universal Probe qPCR Master Mix, New England Lab, USA), and the remaining was the 5 μl of DNA sample.

DNA extracted from cultured amoebae was used as a positive control, whilst Milli-Q water was used as a negative control (NTC). All samples were analysed in duplicate.

The standard curve was performed from a stock solution of plasmid DNA (PCR2.1-AGD) (provided by Mowi Laboratories, Fort William, UK) at 320 ng μl^{-1} followed by a set of standard dilutions (from 1×10^1 copies to 1×10^{10}). PCR conditions comprised a pre-denaturation step at 95°C for 60 s, followed by 45 cycles of a denaturation step at 95°C for 15 s, an extension step at 56°C for 60 s and a last step of melting curve.

2.7 | Statistical analysis

All results obtained from the in vitro and in vivo testing were exported to IBM SPSS statistical analysis software (v23, IBM Corporation) and were processed and tested to determine significant differences between type of swabs, amoebic loads and gill arches. Shapiro–Wilk test was conducted to verify normality, followed by Levene's test to determine homogeneity of variance. Two-way ANOVA was then performed on the data to examine the significance between means followed by post hoc Tukey HSD test to discriminate between experimental groups. A Pearson correlation test was also performed to assess the correlation (R^2) between *Ct* values and observed gill scores.

3 | RESULTS

3.1 | Evaluation of the potential inhibition of PCR by sodium citrate

After the incubation period, a subsequent PCR of amoeba samples stored for 7 ($n = 3$) and 14 days ($n = 3$) in 0.2 M sodium citrate was carried out. Results showed that sodium citrate did not affect PCR chemistry, demonstrated by the presence of specific bands for *N. perurans* 18S rRNA sequence (637 bp) (Figure 1).

3.2 | Detection of *N. perurans* from the in vitro and in vivo testing

3.2.1 | In vitro *experiments*

A two-way ANOVA was conducted that examined the effect of swab type and amoeba load on DNA quantity. Although there were statistically significant differences between swab types and the amoebae load ($p < .001$), there was not a statistically significant interaction between the swab type and spiked amoeba dose on the *Ct* values for the spikes and for the swabbing of plates ($p > .001$).

When a *post hoc* Tukey HSD test was performed to examine the differences between groups, significantly lower mean *Ct* values were observed when Isohelix® DNA buccal swabs were used for the spiked swabs versus other two swab types (ANOVA and post hoc Tukey HSD test; $p < .05$) when higher concentrations of amoebae were used (100 and 1,000).

When swabbing was performed on the agar plates, there were no significant differences in means among swab or amoeba numbers (post hoc Tukey HSD test; $p > .05$) (Figure 2).

3.2.2 | In vivo *experiments*

During this study, two experiments were performed (30 fish per experiment; 60 fish in total). Although the experimental conditions were kept the same throughout the trials, AGD gill scores were different. During the first trial in December, there were more fish presenting lower scores (16 fish: scores ≤ 3, and the rest presented higher scores) compared to the latter trial where a greater number of fish presented higher scores (20 fish: scores ≥ 3, and the rest presented lower scores). A higher number of negatives were found from the first trial in December (Calgiswab: 40%; Isohelix: 3.33%; cotton: 43.33%), in comparison with the low number of negatives from cotton and calcium alginate swabs during the second trial in April (Calgiswab: 0%; Isohelix: 3.33%; cotton: 3.33%) (Table 1).

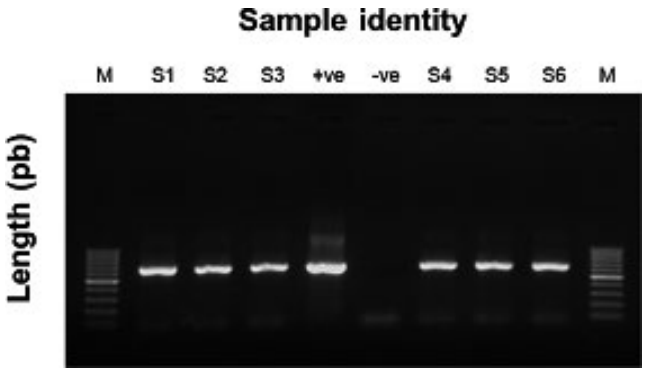

FIGURE 1 PCR results after preservation of amoebae in 0.2 M sodium citrate for 7 days (lanes S1–S3) and 14 days (S4–S6). M: 100-bp DNA ladder. +ve control: *N. perurans* 18S rRNA sequence. –ve control: ddH_2O

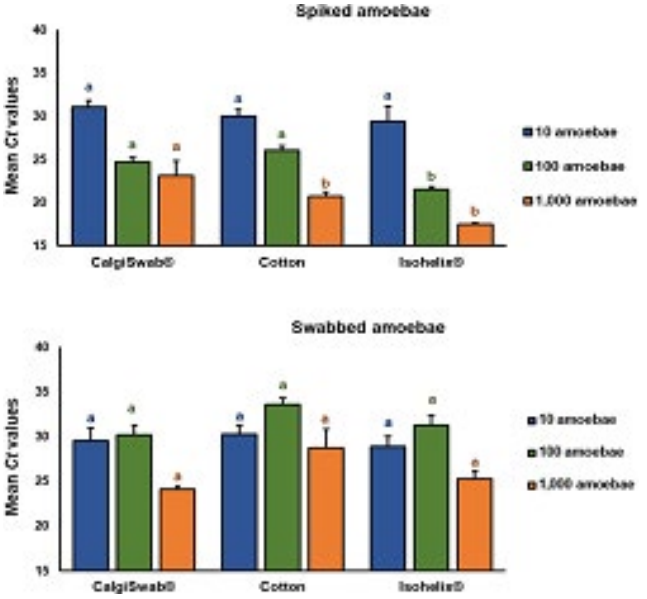

FIGURE 2 Spiked amoebae: qPCR *Ct* values of *N. perurans* for three clinical swab types spiked with different concentrations of amoebae (10, 100 and 1000/swab). Swabbed amoebae: qPCR *Ct* values of *N. perurans* for three clinical swab types used to detect amoebae from MYA plates seeded with different concentrations of amoebae (10, 100 and 1,000/plate). Bars represent the mean *Ct* values ($n = 10$ per concentration) (± s.e.m). Different letters represent statistically significant differences between swab types ($p < .05$) [Colour figure can be viewed at wileyonlinelibrary.com]

A two-way ANOVA was conducted to examine the effect of swab type and gill arch on detected DNA quantity during both trials (December 2018 and April 2019). For the December trial, there were statistically significant differences between swab types and between gill arches ($p = <0.001$). During the trial that was performed in April 2019, the results indicated similar statistical differences when the two-way ANOVA was conducted.

For the first trial in December 2018, a *post hoc* Tukey HSD test was performed to examine the differences between groups. Lower mean *Ct* values were observed for Calgiswab® swabs. However, there were no statistical differences between this swab type and the Isohelix® DNA buccal swabs ($p = .917$). The only statistical differences were found when comparing both these swab types to cotton ($p = .001$) (Figure 3). Significant differences were observed when looking at the results for different gill arches and swab types, but there was no significant interaction between the two factors.

TABLE 1 Percentages of positive and negative results during both trials: (A) first trial in December. (B) Second trial in April. (C) Mean results for both trials. Isohelix® and Calgiswab® present higher percentages of positive results throughout the experiment

	December trial		
	Calgiswab®	Isohelix®	Cotton
(A)			
Positives	60%	96.67%	56.67%
Negatives	40%	3.33%	43.33%
	April trial		
	Calgiswab®	**Isohelix®**	**Cotton**
(B)			
Positives	100%	96.67%	96.67%
Negatives	0%	3.33%	3.33%
	Mean		
	Calgiswab®	**Isohelix®**	**Cotton**
(C)			
Positives	80%	96.67%	76.67%
Negatives	20%	3.33%	23.33%

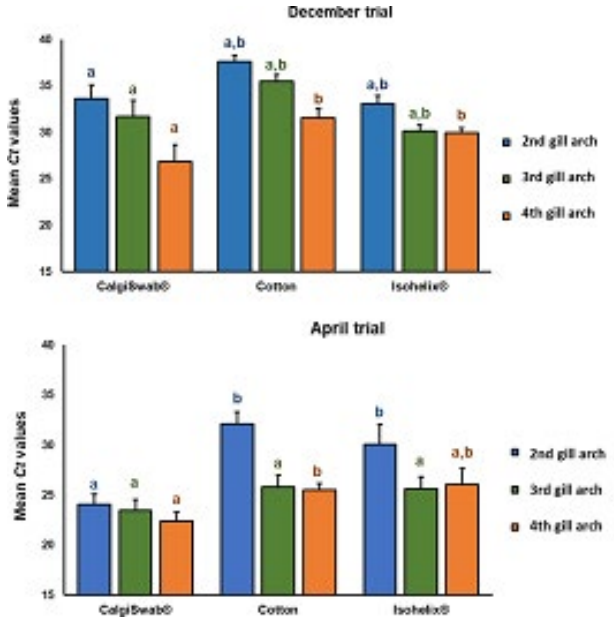

FIGURE 3 Results of the qPCR detection of *N. perurans* 18S rRNA sequences. Three clinical swab types were tested on AGD-infected fish ($n = 30$) during an AGD challenge trial in December 2018 and in April 2019 across the different gill arches. Bars represent the mean *Ct* values ($n = 10$ per swab type) (± s.e.m). Different letters represent statistically significant differences ($p < .05$), and same letters mean no statistical differences ($p > .05$) [Colour figure can be viewed at wileyonlinelibrary.com]

The same statistical approach was performed for the latter experiment in April 2019, presenting again lower mean *Ct* values within the Calgiswab® swabs (Figure 3), and statistical differences were found against the other two swab types (cotton: $p = .002$; Isohelix: $p = .003$); however, no statistically significant differences were seen between Isohelix® DNA buccal swabs and cotton swabs ($p = .991$) (Figure 3).

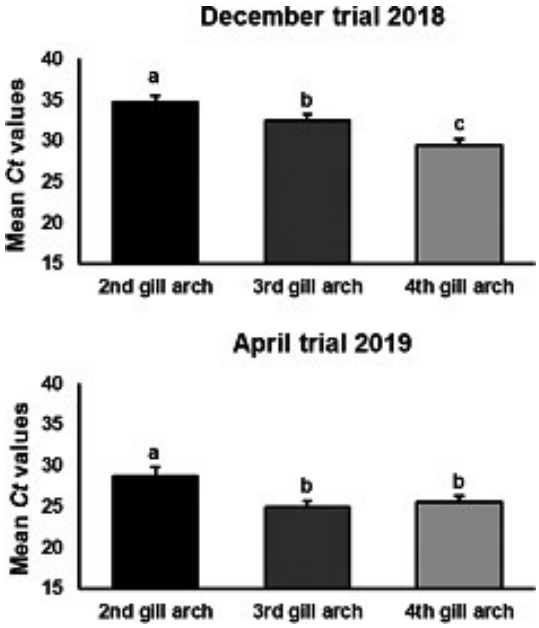

FIGURE 4 Results of the qPCR detection of *N. perurans* 18S rRNA sequences when only gill arches were compared, regardless of the swab type during both trials (December 2018 and April 2019). Bars represent the mean *Ct* values ($n = 30$) (± s.e.m). Different letters represent statistically significant differences (*post hoc* Tukey HSD test; $p < .05$), and same letters mean no statistical differences ($p > .05$)

Although both trials presented different results in terms of statistical differences, the tendency across both experiments was for Calgiswab® mean *Ct* values to be lower than the other two swab types.

With respect to the gill arches sampled, results from both trials showed a tendency for lower mean *Ct* values for swabbing of the 3rd and 4th gill arches relative to the 2nd. During the first trial in December 2018, it was demonstrated that the 4th gill arch presented the lowest mean *Ct* values and statistical differences were found (*post hoc* Tukey HSD test; 2nd gill arch versus 4th gill arch: $p < .001$; 3rd gill arch versus 4th gill arch: $p = .004$; 2nd gill arch versus 3rd gill arch: $p = .047$). Although the later trial in April 2019 showed no statistical differences between the 3rd and 4th gill arches (post hoc Tukey HSD test; $p = .905$), there were statistical differences when these were compared to the 2nd gill arch (post hoc Tukey HSD test; 2nd gill arch versus 4th gill arch: $p = .028$; 2nd gill arch versus 3rd gill arch: $p = .009$). Lowest mean *Ct* values were observed in swabbing the 3rd gill arches (Figure 4).

Estimates of the statistical correlation between gill score and *Ct* value for different swab types were examined for both trials by performing a Pearson correlation test. However, no statistical correlations were found between gill score and mean *Ct* value for any of the swab types when plotted against the recorded gill scores.

4 | DISCUSSION

During this study, the different properties of two swab types used globally in aquaculture (Isohelix® DNA buccal and classic cotton swabs) were compared against Calgiswab®, a calcium alginate swab used for specialist medical diagnostics. The potential utility of the latter lies in the fact that calcium alginate is wholly soluble in a solution of sodium citrate. The aims of this study were to assess the efficiency of the different swabs for the detection of the amoebic pathogen, *N. perurans*, through in vitro testing (spiking and plate swabbing) and in vivo testing of AGD-infected Atlantic salmon, and also to determine which of the tested gill arches could potentially demonstrate a higher amoebic load during testing. Whilst previous studies have focused on comparing different PCR techniques for the development of better pathogen quantification using non-lethal sampling methods (Bergmann & Kempter, 2011; Downes et al., 2017; Monaghan, Thompson, Adams, & Bergmann, 2015), this study focused on the type of swabbing material and location of the gill swabbing. The results showed that the swab material might not be critical for timely diagnosis of AGD, although Calgiswab® swabs presented lower *Ct* values during the in vivo trials, which suggests that higher quantities of DNA were retrieved using this approach and that it could therefore offer greater sensitivity. However, depending on the specific gill arch that was swabbed during the sampling of amoebae, differential gill loads were detected following analysis. When choosing which gill arch is swabbed, results suggest a strong tendency for higher amoebic load from the 3rd and 4th gill arches in comparison with the 2nd gill arch. Although the sampling of the gills was not assessed over time, higher numbers of amoebae in 3rd and 4th gill arches may suggest that these arches might provide an enhanced detection and timely treatment.

Following method development, prior in vitro testing proved that sodium citrate solution did not degrade amoebic DNA or affect the PCR. When different amoebae concentrations were spiked onto the different swabs, it was observed that both Calgiswab® and cotton swabs had an instant water-absorbing capacity. This capacity has been studied before in cotton swabs (Thomas, Mujawar, Upreti, & Sekhar, 2013) in which the recovery efficiency of *Bacillus* spores was suggested to be higher among the cotton swabs due to their major hydrophilicity index. In addition, other studies have investigated the use of calcium alginate dressings on blood coagulation, showing an improvement in the absorbance of blood and other fluids (Kneafsey et al., 1996; Segal, Hunt, & Gilding, 1998). In the present study, Isohelix® swabs were observed to absorb spiked drops containing amoebae more slowly than other swabs. Therefore, Isohelix® swabs could perhaps possess a less absorbent surface, suggesting that the sample might be more promptly released into the ethanol, resulting in a higher recovery of the amoebae. In contrast, the hydrophilic material of the Calgiswab® and cotton swabs may have led to a fuller absorption of the low-concentration amoeba sample, causing the sample to saturate the swab interior resulting in poorer recovery during agitation as observed in past experiments involving bacteria (Turner et al., 2010). However, further investigation on the properties of these materials and the interaction with parasitic species should be conducted in the future to validate this hypothesis. The greatest differences were observed when higher numbers of amoebae were spiked. As expected, higher numbers of spiked amoebae led to lower *Ct* values. This indicated that the detection of amoebae in vitro at lower concentrations from spiked samples was not improved with any of the tested swabs.

The subsequent in vitro experiment, in which swabs were used on agar plates containing different amoebic loads, enables clarification of the capacity of the different swabs to successfully collect the sample from agar plates. This experiment showed significant differences between the swabs. When the agar plates containing higher numbers of amoebae were swabbed, lower *Ct* values were obtained, as expected. Even though there were no significant differences between the Calgiswab® and Isohelix® swabs, both swabs presented lower *Ct* values than the cotton swabs, suggesting an enhanced collection of the amoebae from the substrate. During method development, first trials involved the use of larger volumes of sodium citrate (20 ml) which led to the creation of a mesh of the swab's material and captured cell fragments, leading to a poorer DNA quantification. Therefore, swabs were introduced into a smaller volume of sodium citrate and manually agitated for a standardized time for all samples. Using this approach, the swab was not fully dissolved, only the outer layer, to which the amoebae were nominally attached, was dissolved. In future experiments, a wider range of amoeba concentrations would provide a better understanding of the potentially enhanced sensitivity of the alginate swabs in comparison with the other tested swabs.

Whilst helpful in refining methodology, these in vitro models were not, however, realistic. During field sampling, biological fluids are commonly found within clinical samples. In the case of *N. perurans*, due to the high mucus secretion following an AGD infection (Roberts & Powell, 2003; Valdenegro-Vega, Crosbie, Cook, Vincent, & Nowak, 2014; Vincent, Morrison, & Nowak, 2006), these complex matrices can interact with the physical or chemical properties of the swab materials. Specifically, mucins have been considered to reduce non-specific binding of protein and can deter negatively charged molecules, like DNA (Hollingsworth & Swanson, 2004). During this study, a tendency for lower *Ct* values was found with the Calgiswab® swabs, but it was not always significant. However, the swabbing of different gill arches showed an interesting trend. The second gill arch presented higher *Ct* values in both experiments and with the use of the different swabs, meaning that a lower number of amoebae are presumably present in the second gill arch. In contrast, the third and fourth gill arches offered a better detection of amoebae, presumably due to the higher numbers of parasites in these gill arches.

The common practice of examining the 2nd gill arch when sampling for pathogens follows from the frequent observation that this arch is the preferred site for many gill-inhabiting parasites, for example the copepod *Ergasilus sarsi* (Kilian & Avenant-Oldewage, 2013). One of the principal determining factors for this is the water current over the gill surfaces which influences the available attachment surface, level of oxygenation and potential for dispersion of disseminules (Crafford, Luus-Powell, & Avenant-Oldewage, 2014;

Hanek & Fernando, 1978; Kilian & Avenant-Oldewage, 2013; Suydam, 1971; Turgut, Shinn, & Wootten, 2006). Hence, many gill pathogens tend to colonize the areas of the gill where there is more water flow (*e.g.* first and second gill arches) (Llewellyn, 1956; Davey, 1980; Dzika, 1999; Chapman, Lanciani, & Chapman, 2000; Matejusová, Simková, Sasal, & Gelnar, 2003). These factors may not, however, hold true for *N. perurans*, which, in addition to gaining protection within the host mucus layer, has been suggested to have wide environmental tolerances (Crosbie, Macleod, Forbes, & Nowak, 2005), including conditions found in marine sediments, that may allow it to thrive under conditions of lower oxygen and flow.

In the context of the aquaculture industry, the findings of the presented research can potentially improve methods employed for routine sampling. Whilst visiting fish farms for this experimental study, the general sampling regime consisted of the gill swabbing of all the gill arches. However, results from this study suggest that reduced fish stress and improved sensitivity could be achieved by sampling only third or fourth gill arches, with lower relative *Ct* values within this area translating to higher diagnostic sensitivity.

When looking at the correlation between gill score and *Ct* values, although correlations were not strong, trend lines suggested that higher gill scores lead to lower *Ct* values detected through qPCR. The fact that these correlations are not higher, however, provides a wider caveat, supporting previous suggestions that the number of amoebae present does not directly reflect the visible pathology (Adams & Nowak, 2001). In part, this may result from the fact that pathology may reflect historical events, for example tissue scarring, not the current location/activity of amoebae. Some studies have even reported the presence of *N. perurans* where gross pathology was not detected (Adams & Nowak, 2004a; Dyková & Novoa, 2001; Zilberg & Munday, 2000) and have also demonstrated less amoebae in sampled areas with higher visible pathology. The weak correlation between higher gill scores and lower *Ct* scores observed in this study warrants further investigation, as they clearly have a bearing on diagnostic outcomes, sensitivity and interpretation.

In conclusion, although this piece of work did not show consistently significant differences between different swab materials, there is a trend showing a higher level of amoeba detection with the use of Calgiswab® and Isohelix®, implicating an effect of the swab material in the recovery of amoebae. Cotton consistently proved the least effective swab material for the detection of amoebae across all experiments. The use of calcium alginate swabs presented an advantage compared to the Isohelix® as the swabs are kept in sodium citrate instead of ethanol, which is flammable for transport and storage. As sodium citrate does not interfere with DNA quantification, no drying step is needed in comparison with the use of ethanol which needs to be dried out from the sample, meaning a slightly faster DNA extraction method. However, further work needs to be performed in order to study this material in depth. Regarding the gill arch swabbing, it can be concluded that the gill or gill area that is chosen for swabbing during sampling has an impact on the success of retrieving parasites. This makes the third and fourth gill arches more appropriate tissue regions for detecting *N. perurans*, and therefore, swabbing of this region could translate to more timely diagnosis of AGD and could potentially lead to more successful treatment outcomes. Additionally, restricting the number of gills sampled during non-lethal sampling could reduce stress and minimize exposure of fish to additional infection by *N. perurans* or other pathogens.

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CONFLICT OF INTEREST

No potential conflict of interest was reported by the authors.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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