

Comparing CRISPR-Cas and qPCR eDNA assays for the detection of Atlantic salmon (*Salmo salar* L.)

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Abstract

Molecular techniques offer sensitive, specific, noninvasive monitoring of target species from a variety of environmental samples. We recently developed a CRISPR-Cas-based eDNA assay for rapid single-species detection as a route to a simple, cost-effective biosensor device. CRISPR-Cas-based diagnostic assays use isothermal conditions in combination with a highly specific sequence recognition system. This CRISPR-Cas assay was designed to target *Salmo salar*, and we previously demonstrated its utility in eDNA samples from sites in Ireland. The aim of this study was to validate our assay in two larger sample sets from Canada ($n = 16/n = 63$) in comparison with an independent *S. salar* qPCR assay. We demonstrate that overall, the CRISPR-Cas assay performs similarly to qPCR for assessing the presence or absence of *S. salar* from eDNA and provides a viable alternative approach where qPCR assay design and application have proven to be challenging.

KEYWORDS

CRISPR-Cas, eDNA, fresh water, qPCR, *Salmo salar*

1 | INTRODUCTION

The coupling of environmental DNA (eDNA) with quantitative PCR (qPCR) has become one of the most widely applied contemporary methods for single-species detection in fresh water (Thomsen & Willerslev, 2015). This approach has underpinned numerous publications, reporting the successful design and application of qPCR assays for the detection of a wide range of species (Rees et al., 2014; Tsuji et al., 2019). Recently, we reported the development and application of CRISPR-Cas to eDNA as an alternative to qPCR for the detection of *Salmo salar* in Europe (i.e., Burrishoole Catchment, Ireland)

(Williams et al., 2019). This new approach was originally developed for clinical diagnostic applications (Chen et al., 2018; Gootenberg et al., 2018; Myhrvold et al., 2018; Tsou et al., 2019) and utilizes recombinase polymerase amplification (RPA) coupled to CRISPR-Cas technology. Following pre-amplification using RPA, the CRISPR-Cas system consists of two main elements: a guide RNA specific to the target and an effector Cas12a nuclease (Chen et al., 2018). The potential advantages of using CRISPR-Cas as opposed to qPCR include a higher likelihood of distinguishing sympatric taxa, reduced sensitivity to inhibitors, and its greater adaptability for rapid on-site detection (Williams et al., 2019). More resolute detection of

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rare/invasive/threatened species from catchment to regional scale, using this high-resolution method, could overcome some of the issues associated with sympatric taxa detection using qPCR (Wilcox et al., 2013).

Our *S. salar* CRISPR-Cas detection assay (Williams et al., 2019) required further assessment in other unrelated eDNA study sets to confirm its validity. We compared the performance of our CRISPR-Cas-based system to a qPCR assay that was designed to specifically detect the presence of *S. salar* in eDNA samples extracted from freshwater habitats. Our study sites were two watersheds in eastern Canada, both of which have seen a decline in their *S. salar* populations in recent years. These samples included the Miramichi and Jacques-Cartier rivers (DFO, 2018; Veinott, 2018). The Miramichi River is located in New Brunswick and was previously recognized as a world-renowned *S. salar* fishery (Cunjak et al., 1990) and highly valued by the local economy (Gardner Pinfold, 2011). *S. salar* numbers in the Miramichi are in steep decline, with causes including overfishing, habitat degradation, high water temperatures, and low discharge (DFO, 2018). An additional stressor to the Miramichi *S. salar* population will undoubtedly be the presence of Smallmouth Bass (*Micropterus dolomieu*), an apex predator whose presence was confirmed in the main Southwest Miramichi during 2017 (O'Sullivan et al., 2020). Human activities, such as pollution but dams, in particular, have had a significant impact on *S. salar* as exemplified by the Jacques-Cartier River (CBJC, 2020). Thus, *S. salar* was reintroduced in this river during the 1970s after a dam was built at the mouth of the river in 1913, preventing migration to spawning sites. While the numbers partially recovered up to the 1990s, the population is still supported by stocking and fishing is forbidden since 2009 (MFFP, 2020). Habitat quality degradation continues to be an important stressor for this population. The significant threat to *S. salar* populations at both of these rivers necessitates close monitoring and provides new sampling sites for our validation of our CRISPR-Cas assay.

Monitoring of *S. salar* and other fish species has traditionally relied on the sighting and often capture of organisms. These methods

are expensive, labor-intensive, potentially harmful to the species of interest (Snyder, 2003), and are known to miss rare taxa, such as endangered or invasive species, due to their low capture probabilities (Magnuson et al., 1994). Although contemporary qPCR based eDNA methodologies have overcome many of the problems associated with traditional monitoring, they still have limitations. Previous studies have shown that, depending on the location of mismatches, measurable qPCR signals can be produced with up to five base pair mismatches (Whiley & Sloots, 2005). The need for robust assay design to prevent cross-amplification of nontarget species has been highlighted in the literature (Wilcox et al., 2013); however, some species may still be indistinguishable using the qPCR approach. With the global biome under threat and current biodiversity loss, there is a greater need for high-resolution detection methods. Using isothermal CRISPR-Cas technology has the potential to fill this gap.

In this paper, we present the results of this validation study; comparing qPCR with CRISPR-Cas data, for the detection of *S. salar* from 79 sampling sites across two rivers in eastern Canada. We propose strict criteria for using CRISPR-Cas technology as a presence/absence detection system and highlight the benefits of adapting this methodology for single-species detection in other areas of research.

2 | MATERIALS AND METHODS

2.1 | Study sites

The Miramichi Watershed is located in New Brunswick, Canada (Figure 1), and spans $\approx 14,000$ km² of connected waterways (Cunjak & Newbury, 2005). A total of 63 sites were sampled throughout the watershed in November 2018 (Table S1). The Jacques-Cartier Watershed is located in Quebec, Canada (Figure 1). A total of 16 sites were sampled along the Jacques-Cartier River and its tributaries in September 2017 (Table S2).

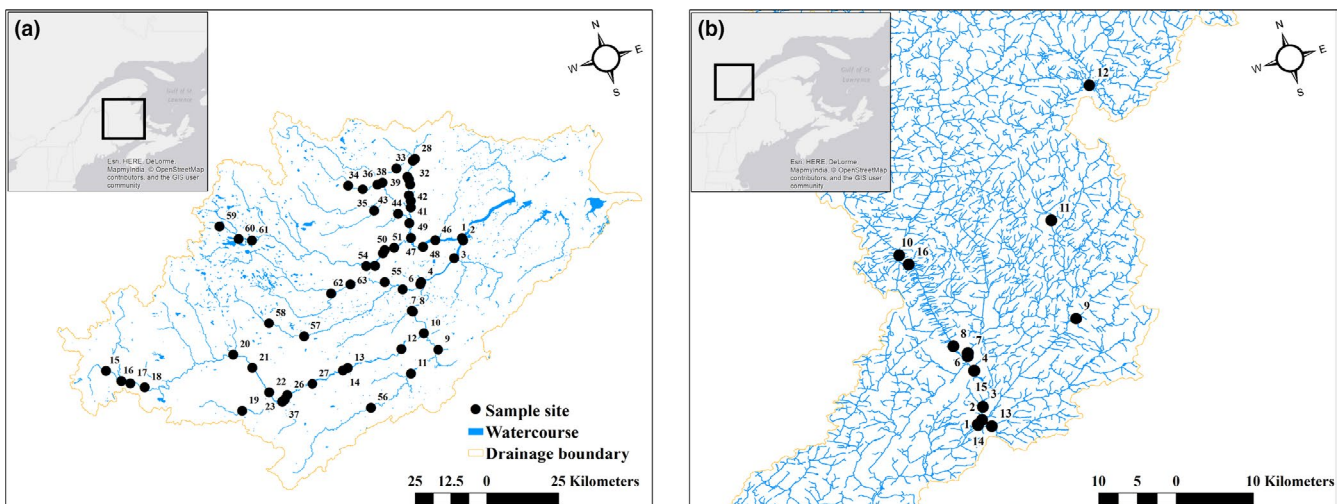


FIGURE 1 Location of sampling sites within eastern Canada. Sampling locations within (a) Miramichi Watershed, New Brunswick, and (b) Jacques-Cartier Watershed, Quebec

2.2 | Field sampling

Water samples were collected from the Miramichi Watershed following a protocol established by Carim et al. (2016). As previously stated in Carim et al. (2016), the protocol employed has been used to collect >5,000 samples and no field contamination has been reported (see also McKelvey et al., 2016; O'Sullivan et al., 2020; Wilcox et al., 2016). At each site, 3.5 L of water was pumped through a pre-packaged filter (cellulose nitrate membrane filters: Whatman—47 mm diameter/0.45 μ l), housed within a Nalgene 145-2020 Analytical Test Filter Funnel—250 ml capacity. During pumping (via a Geopump 2 Peristaltic Pump), a new pair of sterilized disposable gloves were worn at each site to mitigate against potential contamination. Filters were removed from the funnel using sterile tweezers, a new pair at each site, and transported in a cooler with ice. Filtered samples were then stored at -20°C at the University of New Brunswick, until they were shipped to University Laval for qPCR analysis.

Collection of water samples throughout the Jacques-Cartier National Park was performed by the team from the Ministry of Forests, Wildlife and Parks (MFFP) and filtered using syringes. A 250-ml water sample was taken at each sampling station and was filtered in the field using a 0.7- μ m glass microfiber filter (Whatman GF/F, 25 mm) and syringes (BD 60 ml; Franklin Lakes, NJ, USA). As with the Miramichi Sampling Campaign, a new pair of sterilized disposable gloves were worn and a new pair of sterile tweezers were used to remove filters from the funnel at each site. All filters were preserved in 2-ml microtubes containing 700 μ l of Longmire's lysis/preservation buffer and then frozen at -20°C until extraction (detailed in Leduc et al., 2019).

No negative field controls were performed during sampling. To account for this, several "negative stations" were selected with known absence of *S. salar* (i.e., sites located upstream of a waterfall and *S. salar* absence confirmed by electrofishing). As expected, we did not detect *S. salar* DNA at any of these sites (9, 11, 13, and 16 for Jacques-Cartier Watershed and 59, 60, and 61 for Miramichi Watershed), using either detection method. Any contamination during the field sampling stage would be detected using both qPCR and RPA-CRISPR-Cas detection methods. Separate negative controls were included during eDNA extraction and both detection protocols to account for any contamination at these stages.

2.3 | eDNA extraction

DNA from both watersheds was extracted following the protocol developed by Goldberg et al., (2011). DNA was extracted in multiple batches with a negative control included in each batch and used for qPCR, to account for potential contamination during extraction. (All extraction negative controls were blank using qPCR and therefore not used for RPA-CRISPR-Cas detection). The extracted DNA was

subsequently stored at -20°C prior to use for qPCR amplification at IBIS (Institut de Biologie Intégrative et des Systèmes), University of Laval, Quebec. Following qPCR analysis, the remainder of the samples (between 15 and 20 μ l) were shipped on dry ice to Dublin City University, Ireland, and stored at -20°C prior to RPA-CRISPR-Cas detection.

2.4 | qPCR assay

In order to specifically identify *S. salar*, a segment of the mitochondrial DNA cytochrome oxidase subunit 1 (COI) was used. COI sequences of *S. salar* and closely related species were obtained from the BOLD database (Barcode of Life Database <http://www.boldsystems.org/index.php/>). Primers and probes used are detailed in Hernandez et al. (2020). In brief, these were designed to maximize the number of mismatches between target and closely related species. The specific sequences were as follows: forward primer 5'-CCCCGGAATGAATAACATAAGTTTT-3', reverse primer 5'-AATGGCCCCCAGAATTGAA-3', and probe 5'-CTAGCAGGTAATCTTGC-3'. The amplicon length was 205 bp.

The presence of *S. salar* was tested in six technical replicates for each eDNA and extraction control sample. When testing eDNA samples, "SPUD" was incorporated into each reaction and a standard curve was added to each plate analyzed. "SPUD" acts as an internal positive control to identify the presence of inhibitors in the sample (Nolan et al., 2006). The "SPUD" assay consists of a known concentration of the SPUD amplicon, specific primers, and a TaqMan probe, producing reproducible C_T values between 23 and 24. An increased C_T value indicates qPCR inhibition (Nolan et al., 2006). The amplification was carried out in a final volume of 20 μ l including: 0.9 μ M of each primer, 0.25 μ M probe, 10 μ l TaqMan Environmental Master Mix 2.0 (Life Technologies), and 3.9 μ l SPUD and 2 μ l DNA. PCR conditions were as follows: 2 min at 50°C , 10 min at 95°C , 50 cycles of 15 s at 95°C , and 60 s at 60°C on a PCR 7500 Fast Real-Time instrument (Life Technologies).

The standard curve was generated using a synthetic 500 bp DNA template (gBlocks, IDT) designed from the COI sequence (see Supplementary Information). To determine the sensitivity of the qPCR assay, the synthetic DNA was serially diluted until the fluorescence signal corresponding to a single molecule was reached (Foorootan et al., 2017). For the primers and probes used in this study, one molecule was amplified at a C_T of 38.9 ± 0.63 (the PCR cycle where the fluorescence detection threshold is reached). Consequently, this value was used as a cutoff threshold, with any signal exceeding 39 C_T considered a PCR artifact and eliminated from the analysis (see Hernandez et al., (2020) for limit of detection, amplification efficiency, intercept, and r^2 values).

To confirm assay specificity, a selection of positive samples was sent for Sanger sequencing. All sequences matched back to *S. salar* when run through BLAST (NCBI).

2.5 | RPA-CRISPR-Cas assay

CRISPR-Cas assays were carried out as in Williams et al. (2019). Recombinase polymerase amplification products were generated using the TwistAmp Basic Kit (TwistDx), following manufacturer's instructions. Species-specific primers, targeting *S. salar*, were designed (forward primer: 5'-GTTATTAATCCCATCAAACGAC TAGCTTGAGG-3' and reverse primer: 5'-GGCTAATTTAATGGGA GGGGTATGGTTATGATAG-3') to target the mitochondrial DNA NADH dehydrogenase subunit five region. For Cas12a detection, 2.52 μ M Alt-R Acidaminococcus sp. BV3L6 (A.s) Cas12a nuclease (IDT) was preassembled with 3.2 μ M of a *S. salar*-specific crRNA (5'-UACCCUCCAAAACCCCUAUC-3') in PBS at room temperature for 20 min. The Cas12a-crRNA complex was diluted to a final concentration of 50 nM Cas12a: 62.5 nM crRNA in a solution containing 1x Binding Buffer (20 mM Tris-HCl, pH 7.5, 100 mM KCl, 5 mM MgCl₂, 1 mM DTT, 5% glycerol, 50 μ g/ml heparin), 50 nM ssDNA-FQ reporter (5'-/56-FAM/TTATT/3IABkFQ/-3'), and 2 μ l of RPA product. Reactions (20 μ l, 96-well plate format) were incubated in a LightCycler 480 Instrument (Roche) for 120 min at 37°C with fluorescence measurements taken every 30 s ($\lambda_{\text{exc}} = 485$ nm, $\lambda_{\text{em}} = 535$ nm).

To prevent contamination, all RPA and CRISPR-Cas reactions were set up in a designated DNA clean room. In order to identify potential contamination, an RPA negative and CRISPR-Cas negative (in triplicate) was carried out for each analysis series.

Background-corrected fluorescence values were calculated by subtracting the mean fluorescence value obtained from reactions carried out in the absence of target DNA ($n = 3$). To account for interplate variation, fluorescence values were normalized against the mean fluorescence of the positive control, consisting of 0.04 ng/ μ l of *S. salar* tissue DNA. Assay sensitivity was determined by serially diluting DNA extracted from *S. salar* until no fluorescence detection was seen. This occurred below 0.04 pg/ μ l (as reported in Williams et al. (2019)). DNA was extracted from tissue samples from farmed *S. salar* using the DNeasy Blood and Tissue Kit (Qiagen) following manufacturer's instructions. To deem a replicate as positive, a threshold value of ten times above the background standard deviation was used.

2.6 | Positive/negative criteria

We used the following criteria when applying the CRISPR-Cas assay to determine whether a given sample is either positive or negative for *S. salar* eDNA. The CRISPR-Cas assay used three amplification replicates per site with three detection replicates per amplification replicate. During the detection stage of the assay, only standard deviations < 0.5 were deemed acceptable, and if > 0.5 , then the whole run was discarded. Samples were considered "definitely" positive for *S. salar* if at least two out of three replicates were positive and "probably" positive if one out of three replicates were positive. The criteria used for the qPCR assay were that a given sample was

considered positive for *S. salar* if one out of six replicates detected amplification. A selection of samples was sent for Sanger sequencing to confirm assay specificity. In order to avoid bias when determining positive/negative status of a sample, the CRISPR-Cas assay analysis was blinded to the qPCR data and only compared once all samples were processed. A chi-square test was used to determine whether the CRISPR-Cas detection was statistically different from the qPCR detection. For this test, the "probably" positive results using the CRISPR-Cas method were deemed positive to allow binary analysis of the data.

3 | RESULTS

In general, both detection methodologies showed specific detection of *S. salar*. No *S. salar* was detected at "negative stations" using either methodology. All extraction negative controls showed no positive amplification indicating the absence of contamination during DNA extraction. For RPA-CRISPR-Cas detection, all negative controls showed no positive amplification demonstrating the absence of contamination during detection. Therefore, we can assume that positive detection of *S. salar* comes from the sampled water and not contamination during any stage of the experimental procedure.

3.1 | Miramichi watershed

In the Miramichi Watershed, 63 sites were sampled with *S. salar* positively detected at 42 sites using qPCR and 35 sites using CRISPR-Cas (Figure 2). All of the positive sites using CRISPR-Cas were also positive using qPCR. C_T values for positive detections ranged from 24.2 ± 0.08 to 38.3 ± 0.5 , with 39 samples having six out of six positive qPCR replicates. Site 54 had four out of six positive detections, while sites 44 and 53 detected *S. salar* in three out of six replicates. Amplicons from eight of the 42 positive sites were analyzed, and all confirmed as *S. salar* using Sanger Sequencing (Table S5). Samples 56 and 57 were deemed negative using qPCR as the single positive replicate at each site had a C_T value that exceeded 40 (Table S1). When using CRISPR-Cas detection, 31 of the 35 sites that showed positive detection of *S. salar* were positive in at least two out of three replicates and considered "definitely" positive. The other four sites were positive in one out of three replicates and considered "probably" positive (Table S3). Direct comparison of the results shows 11.1% of the sites were incongruous. At these seven sites, qPCR detected *S. salar* presence, while CRISPR-Cas did not.

3.2 | Jacques-Cartier National Park

In the Jacques-Cartier National Park 16 sites were sampled, of which *S. salar* was positively detected in nine sites using qPCR and eleven sites using CRISPR-Cas (Figure 3). C_T values for positive amplification ranged from 35.3 ± 0.8 to 37.9. Of the nine samples that tested positive

FIGURE 2 *Salmo salar* detection in the Miramichi Watershed. Dots represent the sampling sites. Magenta shading is negative detection, blue shading is positive qPCR detection, green shading is “definitely” positive detection with CRISPR whereby at least two out of three replicates were positive, and orange shading is “probably” positive detection using CRISPR whereby one out of three replicates was positive

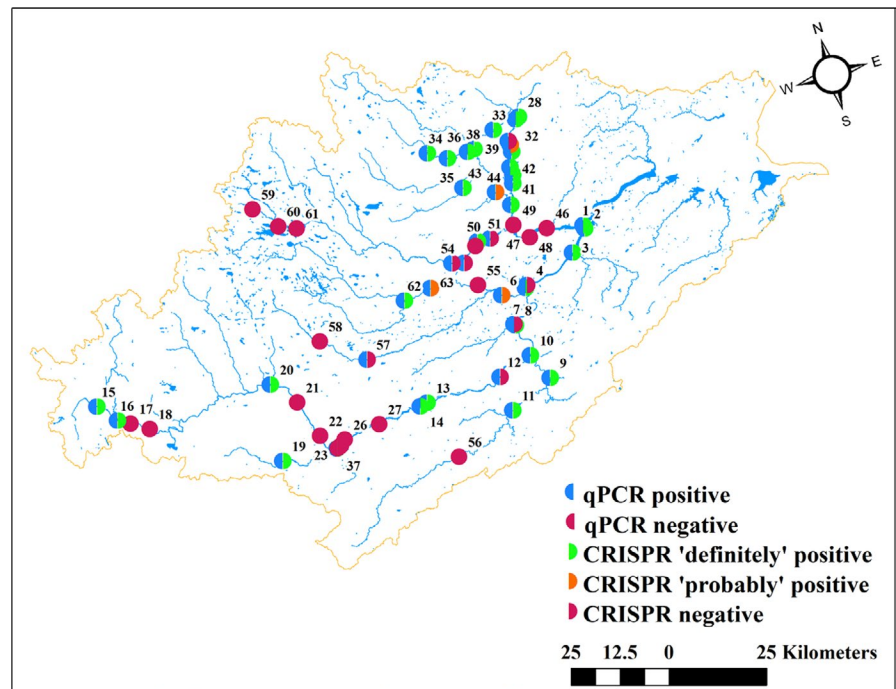
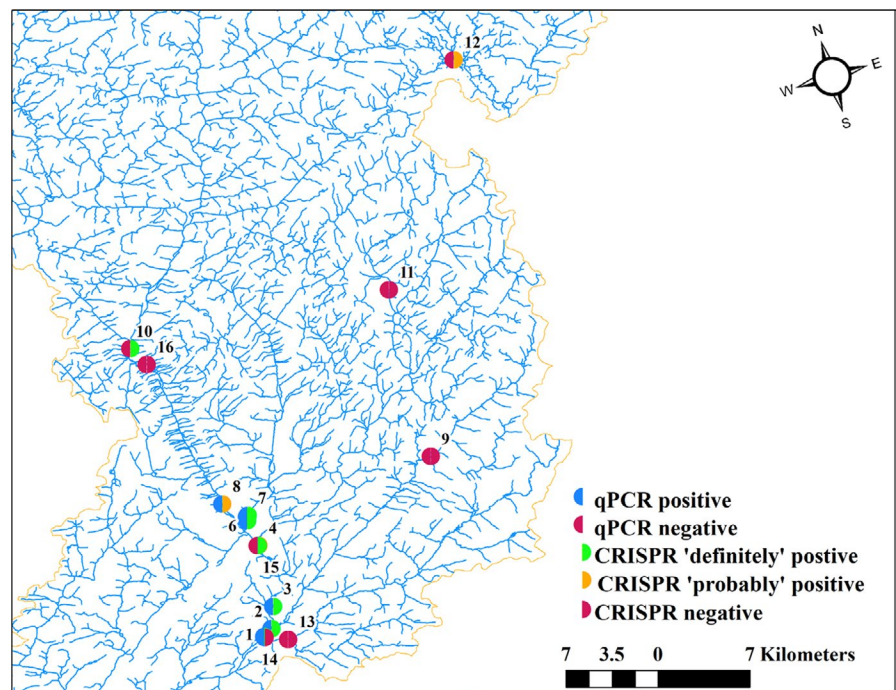


FIGURE 3 *Salmo salar* detection in the Jacques-Cartier Watershed. Dots represent the sampling sites. Magenta shading is negative detection, blue shading is positive qPCR detection, green shading is “definitely” positive detection with CRISPR whereby at least two out of three replicates were positive, and orange shading is “probably” positive detection using CRISPR whereby one out of three replicates were positive



using qPCR, five sites showed positive amplification in six out of six replicates. Site 4 had one out of six positive detection, sites 1 and 14 detected three out of six replicates, and site 2 positively detected *S. salar* in five out of six replicates (Table S2). Amplicons from the nine positive sites were analyzed, and all of them confirmed as *S. salar* using Sanger Sequencing (Table S6). When using CRISPR-Cas detection, eight of the 11 sites were deemed “definitely” positive with at least two out of three replicates identifying *S. salar*. The other three sampling sites were “probably” positive as only one out of three replicates showed positive

detection (Table S4). In the Jacques-Cartier study, 25% of samples were discordant between qPCR and CRISPR-Cas detection.

A chi-square test of independence was performed to determine whether there was a statistically significant difference between the CRISPR-Cas method and qPCR detection at each of the sites. For this, the “probably” positive results from the CRISPR-Cas method were deemed positive to allow for a binomial comparison: Miramichi Watershed: $\chi^2 (1, N = 63) = 1.636, p = 0.2008$; Jacques-Cartier Watershed: $\chi^2 (1, N = 16) = 0.533, p = 0.4652$. Given $p > 0.05$ at

both sites, we can report the number of *S. salar*-positive detections did not differ statistically by detection method.

4 | DISCUSSION

Our results indicate that using CRISPR-Cas for single-species detection is comparable to qPCR. However, given the potential of false negatives (Furlan et al., 2016), when validating new qualitative assays for eDNA detection, it is vital we set out strict criteria to ensure that everyone using the methodology is following the same criteria. Therefore, we must take direction from other fields that have been using diagnostic assays of low copy number DNA for many years. When initial concentrations of DNA are very low (<100 copies), discrepancies between qPCR replicates are expected (Ellison et al., 2006). In clinical diagnostics, it is suggested that one positive qPCR replicate is sufficient to determine whether cancer cells are still present in a patient after remission (van der Velden et al., 2007); however, others suggest there needs to be consistency between replicates to accept as positive (Bustin & Mueller, 2005). In the eDNA space, there seems to be a lack of consensus regarding how many positive qPCR replicates are required to deem the species present in the sampling site (Ficetola et al., 2015; Giguet-Covex et al., 2014; Willerslev et al., 2014). Due to the risk of taxa being present at low densities, some accept a single positive qPCR replicate (O'Sullivan et al., 2020), although Goldberg et al. (2016) suggest that results of this variety, which cannot be replicated, should be interpreted with caution.

In this validation study, the qPCR assays were performed with six amplification replicates per sampling location with one out of six positive indicating presence of *S. salar* at the sampling site. This criterion assumes that no positive detection would be seen if the species were absent from the site, with Sanger sequencing used to confirm sequence identity. A single replicate was deemed positive if the C_T value was less than the $C_T > 39$ cutoff. This cutoff is based on the C_T value of one molecule as measured during the LOD calculation of the assay, which used serially diluted synthetic DNA until the fluorescence signal corresponding to a single molecule was reached. In clinical diagnostics, a clear cycle cutoff value is routinely used for qPCR-based assays (Caraguel et al., 2011; Elfving et al., 2014; Tom & Mina, 2020) and is accepted as best practice based on established criteria known as the MIQE guidelines. The fluorescence signal generated on real-time instruments allows one to calculate the efficiency of a qPCR and relate the C_T value to the number of DNA molecules in a starting sample using a standard curve. Absolute quantification is challenging for qPCR assays and, in reality, is a relative quantitative method especially when compared to the more recent quantitative method of digital droplet PCR (Hindson et al., 2013). Quantitation in relation to RPA-CRISPR-Cas assays is in its infancy, and its mode of detection is different to qPCR. It is currently a qualitative method of detection and can only be used for yes/no diagnostics. The quantitative aspect of CRISPR-Cas assays will develop over time particularly when the enzyme kinetics of the various Cas nucleases are more

fully characterized (Cofsky et al., 2020). Currently, it is effectively an endpoint detection assay whereby the target is fully amplified using RPA and then detected using CRISPR-Cas with a fluorescent reporter to increase the specificity of the assay. This process means fluorescence is not detected during sigmoidal amplification like in qPCR, and therefore, different criteria are required for data handling. To account for background noise, blank samples (no DNA template) are run on each plate enabling background-subtracted fluorescence of samples. As with qPCR, we can set a threshold and any signal above that threshold is deemed positive. We suggest that a value 10× greater than the standard deviation of the background should be used to this effect. In order to compare sensitivities of each method, we can calculate LODs using DNA samples such as gBlocks, plasmid DNA constructs, or tissue extracts.

Based on the selected criteria, 86.1% of the qPCR and CRISPR-Cas data reported the same result and the number of positives did not differ statistically between methods. There were, however, several locations with discrepancies. Furlan et al. (2016) discussed the importance of considering eDNA distribution within the sample when designing an eDNA study. The discordant results seen in this validation study could be because of detection failure caused by replicates containing no target molecules due to variation in subsampling of eDNA extracts for the different analyses (Furlan et al., 2016). As discussed in Jerde (2019), increasing the number of samples and number of replicates would increase the strength of eDNA evidence. However, in this study, insufficient quantities of eDNA did not allow the CRISPR-Cas assay to be repeated as many times as the qPCR assay; likely, another contributing factor to the discordant results for 13.9% of the samples.

Due to the different chemistry involved in DNA amplification, the CRISPR-Cas assay might work where qPCR does not (Kersting et al., 2014). Kersting et al. (2014) focused on detection from blood but many of the inhibitors that are common with eDNA extraction procedures such as ethanol contamination were considered. RPA (the isothermal amplification step in the CRISPR-Cas assay) was shown to outperform PCR in the presence of such inhibitors. Although this is beyond the scope of this validation study, it is possible that where water contaminants are high and inhibit qPCR they may not inhibit RPA-CRISPR-Cas-based assays. It could also be used where qPCR assays are unable to be designed for systems where two closely related species are found in sympatry. The high level of specificity that comes with an RPA-CRISPR-Cas assay due to the four sequence-dependent components (Williams et al., 2019) might enable previously indistinguishable species to be separated and therefore detected. This could be of huge importance in cases where rare endemic and closely related invasive species coexist (Fukumoto et al., 2015) and cannot be distinguished using qPCR.

Overall, we have shown that RPA-CRISPR-Cas methodology is comparable to qPCR for *S. salar* detection. Although qPCR is the more established method for single-species detection from fresh water, CRISPR-Cas adds to the eDNA toolbox, providing an alternative method which has the potential to detect single-species where a qPCR approach is unsuitable. This promising method requires

further exploration to assess its ability to detect closely related species or subspecies and to resist environmental inhibition.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTION

MAW, FR, LB, and APMCD designed the research; MAW performed the CRISPR-Cas experiments and analyzed the CRISPR-Cas data; AMOS designed the field study for the Miramichi Watershed and provided samples; JA designed the field study for the Jacques-Cartier Watershed and provided samples; CH developed the primers and probe, performed the experiments, and analyzed the qPCR data; LB coordinated the collaborative team effort; MAW and APMCD interpreted comparative data and wrote the paper; and all authors contributed to and approved the final draft.

DATA AVAILABILITY STATEMENT

All data pertaining to this work are contained in the main text and Supplementary Information file.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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