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ISOTHERMAL RECOMBINANT POLYMERASE AMPLIFICATION AND CRIPSR(CAS12A) ASSAY DETECTION OF *RENIBACTERIUM SALMONINARUM* AS AN EXAMPLE FOR WILDLIFE PATHOGEN DETECTION IN ENVIRONMENTAL DNA SAMPLES

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ABSTRACT: Improving rapid detection methods for pathogens is important for research as we collectively aim to improve the health of ecosystems globally. In the northern hemisphere, the success of salmon (*Oncorhynchus* spp.) populations is vitally important to the larger marine, aquatic, and terrestrial ecosystems they inhabit. This has led to managers cultivating salmon in hatcheries and aquaculture to bolster their populations, but young salmon face many challenges, including diseases such as bacterial kidney disease (BKD). Early detection of the BKD causative agent, *Renibacterium salmoninarum*, is useful for managers to avoid outbreaks in hatcheries and aquaculture stocks to enable rapid treatment with targeted antibiotics. Isothermal amplification and CRIPSR-Cas12a systems may enable sensitive, relatively rapid, detection of target DNA molecules from environmental samples compared to quantitative PCR (qPCR) and culture methods. We used these technologies to develop a sensitive and specific rapid assay to detect *R. salmoninarum* from water samples using isothermal recombinase polymerase amplification (RPA) and an AsCas12a RNA-guided nuclease detection. The assay was specific to *R. salmoninarum* (0/10 co-occurring or closely related bacteria detected) and sensitive to 0.0128 pg/μL of DNA (approximately 20–40 copies/μL) within 10 min of Cas activity. This assay successfully detected *R. salmoninarum* environmental DNA in 14/20 water samples from hatcheries with known quantification for the pathogen via previous qPCR (70% of qPCR-positive samples). The RPA-CRISPR/AsCas12a assay had a limit of detection (LOD) of >10 copies/μL in the hatchery water samples and stochastic detection below 10 copies/μL, similar to but slightly higher than the qPCR assay. This LOD enables 37 C isothermal detection, potentially in the field, of biologically relevant levels of *R. salmoninarum* in water. Further research is needed to develop easy-to-use, cost-effective, sensitive RPA/CRISPR-AsCas12a assays for rapidly detecting low concentrations of wildlife pathogens in environmental samples.

Key words: Bacterial kidney disease, CRISPR, DETECTR, eDNA, environmental DNA, *Renibacterium salmoninarum*, Salmonid disease.

INTRODUCTION

Throughout their range, Pacific salmon (*Oncorhynchus* spp.) play a vital role in their ecosystem and in human industries (Gilberston 2003; Schindler et al. 2003). These species are prey for critically endangered predators, sources of nitrogen in forests where spawning of rivers occurs, culturally important species for indigenous people, and economically viable species for fisheries and aquaculture (Schindler et al. 2003; Connors et al. 2019; Atlas et al. 2021). Many populations are threatened or endangered nationally or regionally in the Pacific Northwest and Alaska in the US and in British Columbia, Canada; thus management and conservation of key salmonid species and stocks are a high priority (Scheuerell et al. 2005; Connors et al. 2019).

Habitat loss, fisheries, toxins, and disease have hindered salmonid recovery efforts (Weitkamp 2000; Naish et al. 2007; Lundin et al. 2019). Response by management includes cultivation of native salmonid species in hatchery and release programs. However, in hatcheries viral, fungal, parasitic, and bacterial

diseases may cause loss of stock through acute mortality, impeded reproduction, or decreased survival (Morrison et al. 1990; Sano 1995; Boerlage et al. 2020).

Renibacterium salmoninarum is a Gram-positive bacterium found world-wide infecting salmon species in both wild and captive environments (Bayliss et al. 2018). It causes bacterial kidney disease (BKD) in salmonids and can lead to mortality rates as high as 70–80% in *Oncorhynchus tshawytscha* (Banner et al. 1983; O'Farrell et al. 2000). In the Pacific Northwest of the US, salmon hatcheries are regularly at risk of BKD outbreaks, slowing recovery of salmon species in the region (Maule et al. 1996). Early detection of this pathogen in hatcheries, aquaculture, and wild environments might enable better management of disease outbreaks, reduce pathogen spread in the environment, and reduce injudicious use of antibiotics (Marti et al. 2014; Radhouani et al. 2014).

Current diagnostic methods for *R. salmoninarum* require a series of steps, including necropsy, tissue collection, swabbing, culturing, and PCR or quantitative PCR (qPCR) in laboratory settings, which are costly and time consuming (Delghandi et al. 2020; Jansson et al. 2022). These methods generally require a fully equipped microbiological or molecular laboratory and a skilled laboratory technician, which may lead to time lags from sample collection to pathogen detection of 5–10 d or longer, depending on technique (Evelyn et al. 1990; Faisal et al. 2010). Methods for qPCR that might be implemented in the field are available (Biomeme's Franklin platform, Biomeme, Philadelphia, Pennsylvania, USA) or in development but are limited by factors including complicated extraction methods and PCR inhibition (Voelker et al. 2022). For management agencies, delays and high costs may limit the scale and scope of testing, monitoring, and treatment.

Cas enzymes are endonucleases that when integrated with a specific RNA sequence will cleave a DNA strand at a specific location. In contrast to other Cas enzymes that specifically cleave only one nucleic acid strand, Cas12 and Cas13 will cleave all nucleic acids present in a sample, making them suitable as targeted DNA or RNA detection systems by cleaving reporter molecules when activated (Chen et al. 2018). Recent advances in isothermal amplification methods such as loop-mediated amplification (LAMP) or recombinase polymerase amplification (RPA; TwistDx, Berkshire, UK) can increase the amount of target DNA at a single temperature (isothermal). Paired with a reporting system such as CRISPR (clustered regularly interspaced short palindromic repeats) technology that uses Cas-enzyme activity to cleave nucleic acids, fully isothermal, sensitive, fast, and specific assays may be developed for a variety of targets (Chen et al. 2018; Knott and Doudna 2018; Fasching et al. 2022), enabling speedy detection with a fluorometer while eliminating the need for a thermocycler. This may be made accessible and cost effective for a variety of target organisms.

In wild marine and aquatic systems, these assays have been developed into fast, easy-to-use assays for detection of fish species and of a virus impacting fisheries and aquaculture (Chaijarasphong et al. 2019; Williams et al. 2019, 2021; Baerwald et al. 2020). The system, in principle, would enable detection of any species of interest in a highly specific and sensitive way. Our aim was to develop an isothermal RPA-CRISPR/AsCas12a assay for detection of *R. salmoninarum* from environmental water samples.

METHODS

Recombinase polymerase amplification primer design

We targeted the major soluble antigen 1 (MSA1) gene of *R. salmoninarum* for RPA primer design; this was also the target for the previously validated qPCR assay (Chase et al. 2006). It is an important virulence factor, unique to *R. salmoninarum*, found in variable copy numbers in all strains (Rhodes et al. 2004; Chase et al. 2006). The PrimedRPA Python program (Higgins et al. 2019) was used to identify potential forward and reverse RPA primers. Three complete gene sequences were obtained

from NCBI GenBank (accession nos. AY986794.1, AY986795.1, and AF123888.1) and aligned using the PrimedRPA alignment algorithm (Higgins et al. 2018). Using the consensus sequence produced, 100 potential RPA primer sets were identified (see Supplementary Material); we then selected 10 pairs having the following characteristics: a GC content between 37% and 57%, a maximum dimerization under 41%, the amplicon length between 120 bp and 275 bp, and covering variable regions of the gene (see the Supplementary Material parameter file). These 10 primer pairs were then checked for specificity using Nucleotide BLAST (NCBI 2021). Product regions contained within the potential RPA primers in silico were imported into an open-source software, CRISPOR (Concordet and Haeussler 2018), for identification of six potential crRNAs that did not overlap with their respective primer regions (Concordet and Haeussler 2018). The crRNAs were combined with the AsCas12a enzyme to operate as the signal for cleavage enzymatic activity. Thus, they were designed to be specific to the target region and for AsCas12a activity required a protospacer adjacent motif (PAM) identification site of the base pairs TTTN at the leading end of the guide sequence, so sites were identified based on these parameters. The crRNAs and RPA primer pairs were used for in vitro testing of multiple combinations in the laboratory, leading to eight potential assays (Supplementary Material Table S1). Both RPA primers and crRNAs were synthesized by Integrated DNA Technologies (IDT, Coralville, Iowa, USA).

DNA extraction from cultured bacteria

Bacterial isolates were extracted from stock cultured isolates stored in glycerol at −80 C at the U.S. Geological Survey (USGS), Western Fisheries Research Center (WFRC), US Geological Survey (USGS), Seattle, Washington, US. These bacterial culture isolates were extracted using the Qiagen DNeasy Blood and Tissue Kit (Qiagen, Valencia, California, USA) following the manufacturer's instructions for Gram-positive bacteria as described in Chase et al. (2006) and were originally cultured from hatchery salmon tissues in the case of *R. salmoninarum* or were obtained as standards and recultured at the WFRC laboratory. All extraction products were quantified via a Qubit fluorometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA).

RPA reaction methods

All RPA reactions were performed using the TwistAmp Basic RPA kit (TwistDx) and modified to increase the number of samples per reaction The RPA assay included a final concentration of 0.48 μM of each forward and reverse primer, 1× TwistAmp rehydration buffer and PCR DNA/RNAase-free sterile water, 1/5 of the RPA pellet, and 14 nM magnesium acetate (MgOAc) in a total volume of 10 μL per reaction including 1 μL of template. To achieve these single reactions, each RPA pellet was resuspended with 40 μL of master mix made of primers, TwistAmp rehydration buffer, and molecular-grade sterile water, mixed by pipetting, and then split into 8 μL aliquots for single reactions. For each single reaction, 1 μL of culture extract (ranging from 0.5 to 2 ng/μL) or environmental DNA extract (ranging from 0.05 to 20 ng/μL) was added. Finally, 1 uL of 140 mM MgOAc was added to achieve a final concentration of 14 mM to activate reaction. Reactions were incubated at 37 C for 20 min, with a manual mixing step at 4 min (see Supplementary Material Protocol 1). The RPA product was visualized using a 2100 Bioanalyzer (Agilent, Santa Clara, California, USA) to ensure the correct fragment length was amplified.

CRISPR-AsCas12a reactions and visualization

All Cas12a reactions were conducted using commercially available As Cas12a nuclease, custom crRNA molecules, and reporter molecules synthesized by IDT. The reaction mix included 2.52 μM As Cas12a (Cpf1; *Acidaminococcus sp. BV3L6*) nuclease, 3.2 μM crRNA, 0.05 μM reporter (5′-FAM-TTATT-IABK-FQ-3′), and 1× NEB Buffer 2.1 (New England Biosystems, Ipswich, Massachusetts, USA) and PCR DNA/RNAase-free sterile water to a final volume of 18 μL before template. Each reaction included 2 μL of RPA product for a final volume of 20 μL reactions. A One-Step RT-PCR light cycler (Applied Biosystems, Waltham, Massachusetts, USA) measured fluorescence every 30 s over a 120-min period while incubating the reactions at 37 C (see Supplementary Material Protocol 1).

Pilot study to select candidate primer-guide combination

For initial screening, each combination of RPA primers and crRNA was tested against the closest bacterial relatives and three isolates of *R. salmoninarum* (Supplementary Material Table S2). One assay was chosen based on its rapid detection of all three *R. salmoninarum* isolates, without detection of three nontarget bacterial species (*Arthrobacter globiformis*, *Vibrio ordalii*, and *Micrococcus luteus*).

Sensitivity and specificity testing

The specificity and sensitivity of the selected primer-guide combination (Supplementary Material Table S2) was assessed using serial dilutions of DNA extracted from cultures. For specificity testing, cultured isolates of seven *R. salmoninarum* stocks and 10 nontarget bacteria were used in triplicate: these were selected based on their use in validating the qPCR assay developed previously (Chase et al. 2006; Supplementary Material Table S3).

Sensitivity testing was performed using three of the cultured isolates, serially diluted from 1.16 to 1.28 ng/μL to approximately 1.16 to 1.28×10^{-7} ng/μL (~0.00001 pg/μL). Testing was done in triplicate using isolates extracted from glycerol (*R. salmoninarum* Willamette, *R. salmoninarum* CHLM92-026-2, and *R. salmoninarum* M05-51046-90v) and triplicate no-template controls (NTC). Additional standard dilutions to approximately 0.0002 ng/μL, 0.00002 ng/μL, and 0.000002 ng/μL were tested with *R. salmoninarum* M05-51046-90v. A synthetic gene fragment (gBlock) typically used to determine the exact limit of detection of an assay could not be synthesized because of the crRNA sequence being repeated in the amplicon region. Therefore *R. salmoninarum* M05-51046-90v dilutions used in the sensitivity testing were tested using the validated qPCR assay to directly compare RPA-CRISPR/AsCas12a results, performed in the University of Washington laboratory, to quantification via qPCR, performed at the USGS WFRC laboratory (Chase et al. 2006).

Environmental DNA (eDNA) sample testing

Water samples (1 L) were collected at Carson National Fish Hatchery (US Fish and Wildlife Service, Carson, Washington, USA) at monthly intervals from March to October 2021, stored at −20 C, then filtered through cellulose nitrate filter membranes with 5 μm pore size (Whatman, Fisher Scientific). The eDNA was extracted from the filters using the DNeasy Blood and Tissue Kit (Qiagen) with a modified protocol for pretreatment of Gram-positive bacteria, as follows. Filters were placed in PowerBead tubes (Qiagen) and homogenized for 2 min at 4.0 m/s with 360 μL enzymatic lysis buffer, then incubated for 30 min at 37 C. Next, 40 μL of Proteinase K and 400 μL lysis buffer (AL buffer) were added and thoroughly mixed with the samples, which were incubated for 30 min at 56 C. Then 400 μL of 95% ethanol was added and mixed. The bead tube was then centrifuged 10,000 × G for 1 min, and the supernatant was transferred to a spin column (Qiagen). All subsequent steps were performed according to the manufacturer's instructions. The DNA was eluted into 200 μL AE elution buffer and stored at −20 C until qPCR analysis. A negative extraction control (extraction buffers only) was included in the extraction procedure to identify any contamination of equipment and reagents during this procedure. The Carson hatchery water samples used were a subset of samples from a larger project that optimized detection of *R. salmoninarum* from aquatic eDNA using qPCR. The qPCR protocol included an internal positive control (IPC) qPCR using TaqMan Exogenous IPC Reagents (EXO-IPC, Thermo Fisher Scientific) to test for PCR inhibition. The IPC qPCR was run in triplicate on each DNA sample in 12 μL volume containing 6 μL TaqPath Master Mix (Thermo Fisher Scientific), 1 μL EXO-IPC mix, 0.2 μL EXO-IPC DNA, and 5 μL DNA template, or for the no template control (NTC) sterile water, using the default cycle conditions of 10 min initial heat activation at 95 C, followed by 40 cycles of denaturing at 95 C for 15 s and annealing and extension at 60 C for 1 min. Environmental DNA samples were considered inhibited if samples displayed >1 quantification cycle (Cq) shift relative to the NTC. Quantitative PCR was performed using the ViiA 7 real-time PCR System (Applied Biosystems) and analyzed with the *Vii*A 7 RUO 1.3 software associated with the system. A published *R. salmoninarum* qPCR assay (Chase et al. 2006) was used to test for *R. salmoninarum* DNA in each sample. This qPCR assay was run in triplicate in 12 μL volumes containing 1× TaqPath Master Mix (Thermo Fisher Scientific), 800 nM each forward primer and reverse primer, 200 nM probe, and 5 μL of DNA extract using the default cycle conditions described above. A five-point serial dilution (5×10^1 to 5×10^5

copies per reaction) of DNA standard (gBlock double-stranded DNA fragment; IDT) was included with each PCR run to estimate the mean DNA concentration in the field samples. Positive amplification controls, negative filtered water controls, negative DNA extraction controls, and NTCs were included in each qPCR run. We estimated the limit of detection, limit of quantification, and PCR efficiency as described by Klymus et al. (2019).

We analyzed the same eDNA samples using the RPA-CRISPR/AsCas12a assay to detect *R. salmoninarum* and compared the results to the qPCR results. Samples included qPCR-positive samples and qPCR-negative samples from locations in the hatchery with no fish or detectable *R. salmoninarum*. As all qPCR assays occurred using 5 µL of template DNA, and the RPA-CRISPR/AsCas12a reactions occurred using 1 µL of reaction, qPCR quantifications were calculated to copies/µL by dividing the quantifications by five for direct comparison with the RPA-CRISPR/AsCas12a results.

RPA-CRISPR/AsCas12a analysis

The threshold for detection was set at 0 fluorescent intensity (FAM), as there was no passive dye used to add background fluorescence nor any seen above 0. To determine whether this assay could be made semiquantitative based on time to detection or should be made presence-absence within a defined time, we determined the time to cross the threshold for all replicates of each dilution.

We compared the RPA-CRISPR/AsCas12a assay results from dilutions of a cultured isolate (*R. salmoninarum* M05-51046-90v) along with triplicate NTCs (performed at the University of Washington laboratory) to the quantification of the same dilutions using the qPCR assay with a gBlock standard curve (performed within 24 h at USGS after transport on ice). The quantity detected by qPCR was used to determine an approximate limit of detection (LOD, copies per volume) for the RPA-CRISPR/AsCas12a assay of the same concentrations, and limit of detection (LOD) was determined as the lowest concentration of *R. salmoninarum* DNA where 95% of replicates were positive upon detection (Klymus et al. 2019). We used these results to determine the probability of detection based on copy number/µL via logistic regression analysis and a predictive model using R version 4.2.1 (R Core Team 2022).

For the Carson Hatchery eDNA samples, the CRISPR/Cas12a fluorescence results were cross-validated with the qPCR. The time at which fluorescence surpassed the detection threshold and the assay sensitivity for eDNA samples were determined by comparing detections to the qPCR quantity via standard curve creation. Probability of detection was determined via logistic regression of detection via RPA-CRISPR/AsCas12a compared to the amount of DNA in the sample determined via qPCR.

RESULTS

Pilot study to select candidate primer-guide combination

The list of RPA primer and crRNA combinations tested in vitro and their resulting detections are included in Supplementary Material Tables S1 and S2. The primers and crRNA selected for further testing were Rs_RPA_F: 5′-GACGGCCAATGGGTACTGCAAAAGC CAAATGC-3′, Rs_RPA_R: 5′-AGTAGACCG TGTCACACCAATCGATGTTTTAC-3′, and Rs_crRNA: 5′-*TTTG* GTAATCCCGGTGA TACTCCTG-3′ (the PAM site is in italics).

Sensitivity and specificity testing

Using the selected primer-guide combination for RPA-CRISPR/AsCas12a identified in the pilot study results, all DNA extracts of isolated cultures (n=9, representing seven strains of *R. salmoninarum*) were detected, and no nontarget species isolates (n=10) were detected with fluorometer values of DNA concentration ranging from 1 to 4 ng/µL (Fig. 1 and Supplementary Material Table S3).

The LOD was approximately 1.16×10^{-5} ng/µL to 1.33×10^{-5} ng/µL; mean detection time was 6.22 min. Detections in 66.7% of replicates occurred as low as 2.7×10^{-6} ng/µL with a mean time to detection of 37 min. The lowest detection occurred for 1.6×10^{-6} ng/µL (detection time of 47 min; Fig. 2).

The time to cross the fluorescence threshold (FAM = 0) was calculated for each dilution in the sensitivity testing (Fig. 3). For total DNA concentrations greater than 0.0128 pg/µL, positive detections occurred in less than 10 min.

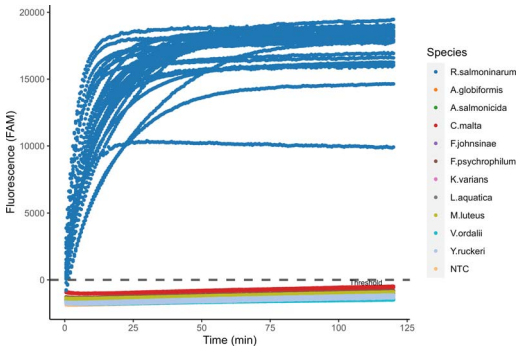

FIGURE 1. Results from the specificity testing for the RPA-CRISPR/AsCas12a assay using nontarget bacterial species (n=10) that are closely related or expected to be found in the same environment, and the DNA extracts from seven isolates of *Renibacterium salmoninarum*, each run in triplicate. The nontarget bacteria tested were *Arthrobacter globiformis*, *Aeromonas salmonicida*, *Carnobacterium maltaromanticum*, *Flavobacterium johnsinae*, *Flavobacterium psychrophilum*, *Kocuria varians*, *Listeria aquatica*, *Micrococcus luteus*, *Vibrio ordalii*, and *Yersina ruckeri*. All lines above threshold are from *R. salmoninarum*.

Concentrations lower than 0.0128 pg/µL were detected in fewer than three replicates up to 65 min after the start of reaction. One NTC replicate had a final FAM fluorescence of 6.9 at 107 min.

Testing the same dilutions of one isolate in triplicate both by RPA-CRISPR/AsCas12a and qPCR assays showed that the LOD for the RPA-CRISPR/AsCas12a assay was approximately 6 copies/µL within 5 min and approximately 1 copy/µL in 2 of 3 replicates within 65 min (Fig. 3). In the qPCR assay, below approximately 10 copies/µL only one or two of three replicates detected *R. salmoninarum* in the same dilutions. We estimated the limit of detection for the qPCR assay at 5.8 copy/qPCR, the limit of quantification at 42 copy/qPCR, and PCR efficiency was 95.14% with an $R^2 = 0.99$. Logistic regression analysis determined that the starting quantity of DNA was significantly related to detection via RPA-CRISPR/AsCas12a, where low concentrations (1–10 copies/µL) were less likely to be detected than were higher concentrations (P<0.0001; Fig. 4).

eDNA sample testing

Fourteen eDNA samples from Carson Hatchery had at least one replicate (36 replicates total) where both the RPA-CRISPR/AsCas12a assay and the qPCR detected the bacterium (Table 1); the lowest copy number detected by RPA-CRISPR/AsCas12a was 3.27 copies/µL. In six samples, the qPCR assay detected *R. salmoninarum* in at least one replicate, where the RPA-CRISPR/AsCas12a assay did not detect *R. salmoninarum* even after the 90-min cutoff. Environmental samples that were detected by qPCR but not detected by RPA-CRISPR/AsCas12a ranged in concentration from an average of 0.88 copies/qPCR to 8.33 copies/qPCR (Fig. 5). In samples with low concentrations (<10 copies/µL average), detection among technical replicates was stochastic for both assays. Neither assay detected *R. salmoninarum* in any of the nine control (*R. salmoninarum* not present) samples, nor in any of the NTC replicates for either assay. No instances of PCR inhibition were found in our environmental samples.

DISCUSSION

We developed a sensitive and specific assay using isothermal amplification with an RPA-CRISPR/AsCas12a system to detect *R. salmoninarum*. We found the assay to be specific for *R. salmoninarum* when tested against 10 closely related and co-occurring bacteria. The limit of detection, determined by comparing RPA-CRISPR/AsCas12a results to qPCR quantification of the same isolate, was found to be approximately 6 copies/µL of target DNA. When testing with eDNA samples collected at a Chinook salmon hatchery (Carson Hatchery), we found a similar LOD to the sensitivity through isolate DNA testing. Although it appears that detection in eDNA samples with quantifiable *R. salmoninarum* via qPCR was stochastic below approximately 10 copies/µL via RPA-CRISPR/AsCas12a. This trend is also seen with qPCR assays that also have high rates of false negatives with DNA concentrations <10 copies/µL (Forootan et al. 2017).

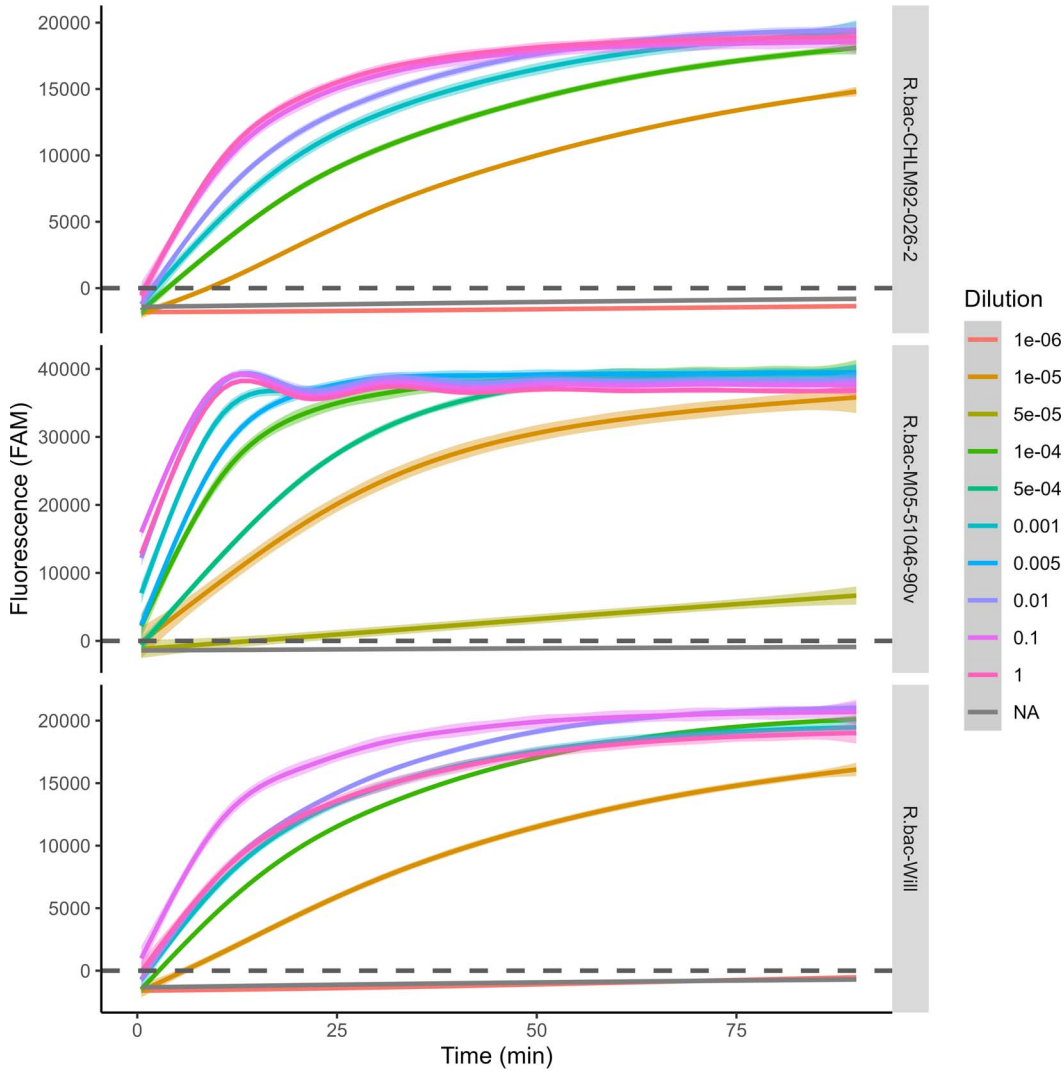

FIGURE 2. Results of the RPA-CRISPR/AsCas12a sensitivity trial using genomic DNA from *Renibacterium salmoninarum* isolates. Dilutions represented by darkness of the line start with 1 being the highest concentration from 1.16–1.33 ng/μL of total DNA to dilution 1×10^{-6}, which is approximately 1.16–1.33 $\times 10^{-6}$ ng/μL. Triplicates are collapsed at each time point, and the mean values for each time point are represented across time points to view the overall trend of fluorescence throughout the reaction time of up to 90 min for each isolate tested. The NAs are negative controls.

Based on our detection times with the RPA-CRISPR/AsCas12a assay (detection of *R. salmoninarum* above >10 copies/μL within approximately 10–30 min, results suggest that this assay should be run for no more than 90 min to avoid missing potential low quantity positives while avoiding false positives beyond that time. A limitation of the RPA-CRISPR/AsCas12a assay that may have led to the lower analytical sensitivity compared to the established qPCR assay could be the small starting volume of DNA template used in the RPA reaction (1 μL DNA in 10 μL reactions compared to 5 μL DNA in 12 μL qPCR reactions). Smaller volumes decrease cost per reaction for management scalability but involve a risk of nondetects if the target DNA is in low concentration in the sample because detections in replicates are more likely

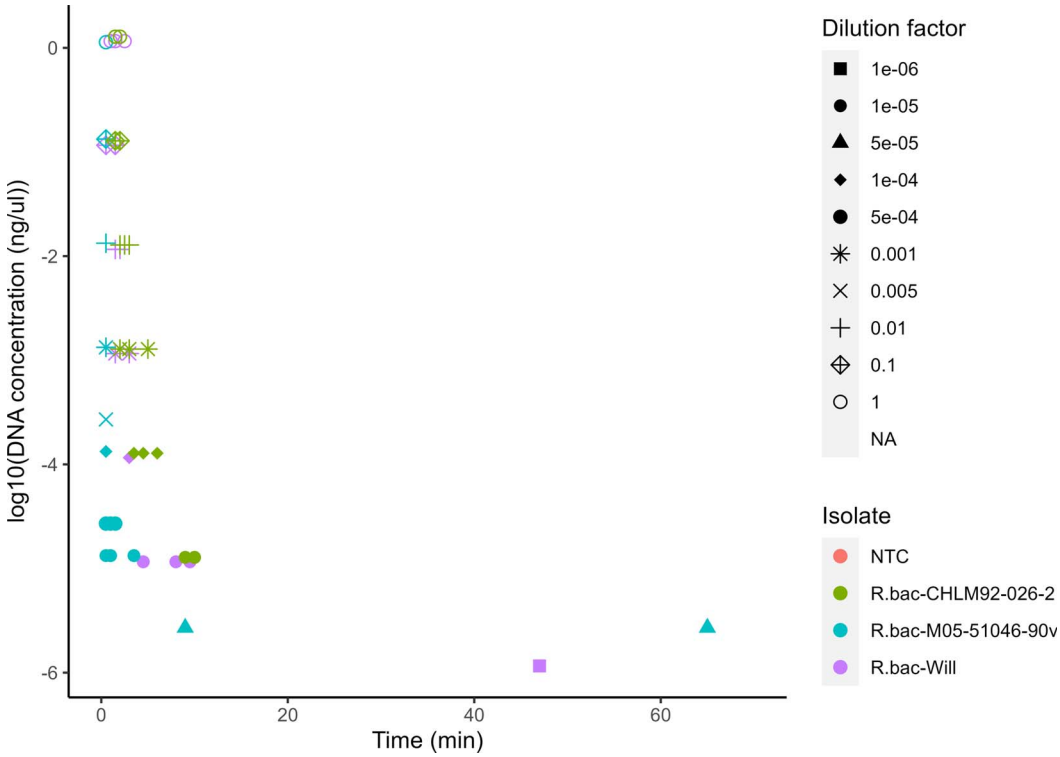

FIGURE 3. Time to detection for starting concentrations of all positive *Renibacterium salmoninarum* isolate replicates from the sensitivity testing of the RPA-CRISPR/AsCas12a assay. Total DNA concentration is shown as the log of concentration, with starting concentrations ranging from 1.133 ng/μL to 1.16×10^{-6} ng/μL. Dilutions represented by the different shapes and start with 1 being the highest concentration from 1.16–1.33 ng/μL of total DNA to dilution 1×10^{-6}, which is approximately 1.16–1.33 $\times$ 10^{-6} ng/μL. NAs represent no DNA or no template controls (NTCs).

since the probability of pipetting the low-concentration target approaches zero (Gold et al. 2023). Increasing the volume of the reaction may increase the sensitivity of this assay.

The eDNA sample detection time varied more than the pure isolates. This may be due to the presence of more nontarget DNA slowing the time to interaction between the Cas12a enzyme-crRNA complex and target DNA. Thus, it may be that enzymatic exhaustion could be a limiting factor for these assays as the more nontarget DNA with which the enzyme interacts, the less enzyme is left to bind to low concentration target DNA. Therefore, a higher concentration of Cas12a-enzyme/crRNA may lead to increased sensitivity, but this would involve the increased cost of using more enzyme per reaction.

The qPCR was more sensitive when detecting eDNA compared to RPA-CRISPR/AsCas12a. Detection of *R. salmoninarum* in water is probably related to highly localized infected fish stock actively shedding bacteria through feces because *R. salmoninarum* shows limited survival outside the host (Austin and Rayment 1985; Balfry et al. 1996). Therefore, in a case with potential for disease outbreak, one would expect a relatively high bacterial load being shed from multiple fish. Thus, the LOD for slightly lower sensitivity using RPA-CRISPR/AsCas12a compared to qPCR is not likely to be of practical importance in detecting biologically relevant quantities of *R. salmoninarum* in water samples. More research would be needed to understand the concentration that is likely to lead to a disease outbreak in

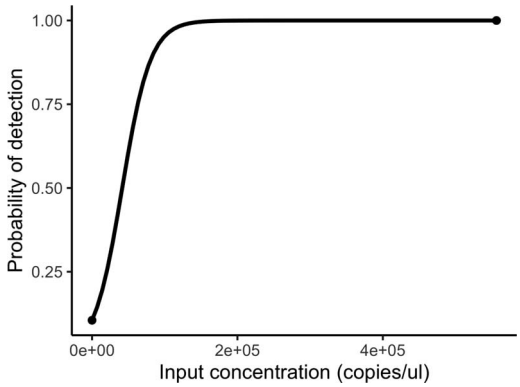

FIGURE 4. The probability of the RPA-CRISPR/AsCas12a detecting *Renibacteriuim salmoninarum* based on the copy numbers/μL determined by qPCR of the same sample dilutions. Probabilities were calculated from the logistic regression model fit to the detection results from the isolate (M05-51046-90v) dilutions tested by both qPCR and RPA-CRISPR/AsCas12a assays. Detections were those that crossed the fluorescence intensity (FAM)=0 threshold, and quantities were determined via the qPCR and standard curve.

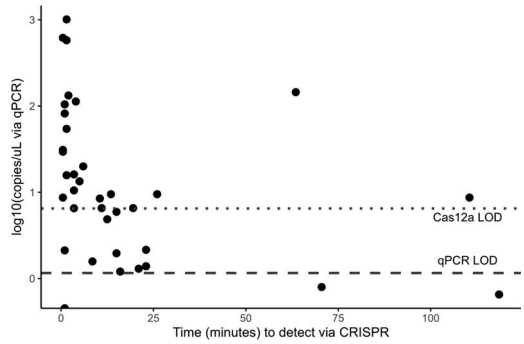

FIGURE 5. *Renibacterium salmoniarum* detections from eDNA samples taken throughout the Carson salmon hatchery quantified via qPCR and then found positive via RPA-CRISPR/AsCas12a assay. Limit of detection (LOD) determined through the isolate sensitivity testing of the RPA-CRIPSR/AsCas12a is represented at 6.49 copies/μL by the dotted line, and the qPCR assay is represented at 1.16 copies/μL by the dashed line for comparison.

salmon stocks. A screening tool for managers of hatcheries or aquaculture facilities that detects a pathogen load >10 copies/μL within 30 min is probably adequate for indicating an immediate need to implement antibiotic treatment and fish stock isolation, and to further test for quantity of pathogen present or detection of disease in fish.

Overall, this technology, not requiring a thermocycler, shows promise as a possible screening tool for managers. Further developments are still needed. Once faster field DNA extraction methods are developed and the enzymes are lyophilized, the time from collection to detection could be as little as 1 h, as the CRISPR reaction detected most pathogens within 30 minutes. Although this study determined fluorescence via a light cycler as would a qPCR assay, many other methods for detection of reporter could be used, such as spectrophotometry, UV light exposure and visualization, or lateral flow (Gootenberg et al. 2018; Shin et al. 2022). Another development would be single-tube reactions. Some progress has been made in this with Cas13a (Baerwald et al. 2020), but more work needs to be done to develop a true single-tube Cas12a reaction (Wang et al. 2020; Qiu et al. 2022). Baerwald et al. (2020) were able to translate a single-tube Cas13a assay into a lateral flow system after crude extractions from swabs to detect target DNA in a field setting; this is an important advancement for moving assays from the laboratory to field applications.

TABLE 1. Agreements and disagreements between detections of *Renibacterium salmoninarum* in the quantitative PCR (qPCR) replicates and the RPA-CRISPR/AsCas12a replicates for all environmental DNA samples, performed in triplicate with triplicate no template controls performed by each assay.

	+ qPCR	− qPCR
+ CRISPR	36 (n=14 samples)	0
− CRISPR	18 (n=6 samples)	33 (n=11 samples)

The use of CRISPR detection systems is still in the early stages, so protocols are not as streamlined and well established as those for qPCR. Other isothermal methods such as LAMP can also be used to amplify targets. However, LAMP methods require designing and using six primers rather than two as is used for RPA, despite comparable sensitivity between the two methods (Zou et al. 2020).

Although RPA is proprietary, making use limited by supply, the ease of use as demonstrated by previous researchers was preferred for our study, and its use in the SHERLOCK and DETECTR systems previously showed it increased sensitivity of the assay compared to other isothermal reactions (Mustafa and Makhawi 2021). This technology, although not yet fully developed, has the potential to detect pathogens without the need for a molecular diagnostic laboratory (Baerwald et al. 2020). This may assist in limiting the spread of disease within these hatchery or aquaculture fish stock populations and decrease the spread of this pathogen to wild fish populations, as well as by determining when bacteria are not present and reducing the unnecessary use of antibiotics that might lead to increases in multiple-drug-resistant bacteria (Radhouani et al. 2014).

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DATA ACCESSIBILITY

All data files, metadata, and scripts for processing the data are found on a publicly available GitHub repository: https://github.com/erdagnese/CRISPR-assaydev-analysis.

SUPPLEMENTARY MATERIAL

Supplementary material for this article is online at http://dx.doi.org/10.7589/JWD-D-22-00128.

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