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Development of diagnostic assays for differentiation of atypical *Aeromonas salmonicida vapA* type V and type VI in ballan wrasse (*Labrus bergylta*, Ascanius)

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Abstract

Aeromonas salmonicida (*As*) is a highly heterogeneous bacterial species, and strains' host specificity has been reported. Ballan wrasse (*Labrus bergylta* Ascanius, 1767) is susceptible to atypical *As* (a*As*) *vapA* type V and type VI in Scotland and Norway. Identification of the bacterium is achieved by culture and molecular techniques; however, the available methods used to distinguish the *As* types are costly and time-consuming. This paper describes the development of a PCR and a restriction enzyme assay for the detection of a*As vapA* type V and type VI in ballan wrasse, respectively. Type V-specific primers were designed on conserved regions of the *vapA* gene, and the restriction enzyme assay was performed on the PCR products of the hypervariable region of *vapA* gene for the detection of type VI isolates. Amplification product was produced for type V (254 bp) and restriction bands (368 and 254 bp) for type VI isolates only. In addition, the assays detected type V and type VI isolates in spiked water samples and type V in diagnostic tissue samples. The assays are fast, specific and cost-effective and can be used as specific diagnostic tools for cleaner fish, to detect infectious divergence strains, and to manage and mitigate a*As* disease outbreaks through vaccine development.

KEYWORDS
atypical *Aeromonas salmonicida*, ballan wrasse, cleaner fish, diagnosis, polymerase chain reaction

1 | INTRODUCTION

Ballan wrasse (*Labrus bergylta*, Ascanius) is one of the main cleaner fish species used as a biological control in the salmon industry, to control sea lice (*Lepeophtheirus salmonis* Krøyer) infections of Atlantic salmon (*Salmo salar* Linnaeus). Atypical *Aeromonas salmonicida* (a*As*) is a highly heterogeneous bacterial species, and different strains infect fish in a host-specific manner (Gulla et al., 2019). The *vapA* type V and type VI strains are major bacterial pathogens of wild-caught and farmed ballan wrasse, and early detection of these strains could assist vaccine development for disease mitigation. The significance of the bacterium in wrasse disease outbreaks has been reported in Scotland and Ireland since the 1990s, where members of the family (e.g., *Ctenolabrus rupestris* and *C. exoletus*, L.) were used as cleaner fish for the first time (Frerchs et al., 1992; Treasurer & Cox, 1991; Treasurer & Laidler, 1994). In Norway, 32 cases of

atypical furunculosis were related to a*As* infection in ballan wrasse in 2015, although reports of outbreaks have declined by nearly half between 2016 and 2018 (Hjeltnes et al., 2019). However, a previous study reported a*As* as the most prevalent bacterial species isolated from ballan wrasse (farmed and wild) in Scotland (Hewson, 2019). The transmission of a*As* from ballan wrasse to farmed Atlantic salmon (*S. salar*) has not been reported, presumably resulting from immunization of Atlantic salmon prior to transfer to the sea or that the species is not susceptible to the two cleaner fish-associated a*As* types (V and VI) (Brooker et al., 2018; Evensen, 2016; Sommerset et al., 2005). Authorized antimicrobial treatments are being used to treat clinical outbreaks in cleaner fish when necessary (Brooker et al., 2018); however, concerns regarding welfare (e.g., stress related to handling) and antibiotic resistance (Watts et al., 2017) mean that the development of a vaccine for cleaner fish is of particular importance (Brooker et al., 2018).

Aeromonas salmonicida was first reported as *Bacillus salmonicida* in Germany (Emmerich & Weibel, 1894) as the causative agent of a disease affecting brown trout (*Salmo trutta*, L.), prior to reclassification into the genus *Aeromonas* (Griffin et al., 1953). Five subspecies have been described for *As* including *salmonicida* (Lehmann & Neumann, 1896; Schubert, 1967), *achromogenes* (Schubert, 1967; Smith, 1963), *masoucida* (Kimura, 1969a, 1969b), *smithia* (Austin et al., 1989) and *pectinolytica* (Austin & Austin, 2016; Holt et al., 1994; Pavan et al., 2000). In early years, the misconception that salmonids are predominantly susceptible to the bacterium led to the establishment of the term "typical" for a homogenous group of isolates (*A. salmonicida* subsp. *salmonicida*) causing furunculosis disease to salmonids and atypical for heterogeneous isolates, which infected predominantly nonsalmonid species and/or were phenotypically or biochemically different from subsp. *salmonicida* (McCarthy & Roberts, 1980). Although the classification of the atypical isolates was achieved, to some extent, with partial sequencing techniques in the past years (Beaz-Hidalgo et al., 2008; Kwon et al., 1997; Livesley et al., 1999; Lund et al., 2002; O'HIci et al., 2000), contrasting results were often found, and therefore, a more reliable diagnostic method was needed.

A virulence factor, the paracrystalline surface array protein (known as A-layer protein), has successfully been used for the differentiation of typical and atypical *As* strains (Gulla, Lund, et al., 2016; Gulla et al., 2019). This additional outer membrane protein plays an important role in host infection and providing protection for the bacterium by resisting host immune responses (Daly et al., 1996; Kay & Trust, 1991; Munn et al., 1982; Udey & Fryer, 1978). Lund and Mikkelsen (2004) reported defined groups for atypical isolates by sequencing the *vapA* gene, which encodes the A-layer protein. Subsequently, sequencing of the hypervariable region of the *vapA* gene enabled the allocation of 23 *vapA* types and most of them were host-specific (Gulla et al., 2019; Gulla, Lund, et al., 2016). Ballan wrasse is susceptible to *As* type V, predominant in Scotland, and type VI mainly in Norway (Gulla, Duodu, et al., 2016; Papadopoulou et al., 2020).

Several techniques have been developed for the identification of the bacterium during a disease outbreak including culture (Austin & Austin, 2016; Cipriano & Austin, 2011), serological (e.g., ELISA, IFAT) (Adams et al., 1995; Austin et al., 1989; Kawahara & Kusuda, 1987) and molecular assays (e.g. Gulla, Duodu, et al., 2016; Hiney et al., 1992; Livesley et al., 1999). Traditionally, isolation of *As* was achieved by culture, frequently on tryptone soya agar (TSA), blood agar (BA) or brain heart infusion agar (BHIA), where typical isolates produce the characteristic brown pigment (TSA only) (Cipriano & Austin, 2011). Difficulties in isolating the bacterium from carrier fish, the relatively slow growth of the bacterium on agar plates which can result in false-negative outcomes and the need for a quick screening tool led to the development of a number of conventional or quantitative polymerase chain reaction (PCR or qPCR) assays for *As* detection (e.g. Balcazar et al., 2007; Beaz-Hidalgo et al., 2008; Byers, Cipriano, et al., 2002; Byers, Gudkovs, et al., 2002; Gulla, Duodu, et al., 2016; Gustafson et al., 1992; Hiney et al., 1992; Keeling et al., 2013; Miyata et al., 1996; O'Brien et al., 1994; Rattanachaikunsopon & Phumkhachorn, 2012). Although very sensitive, the above methods cannot distinguish *As* at the strain type level, which is important for disease control during an outbreak.

The current paper describes the development of two novel, fast, specific and low-cost assays for the detection of a*As* type V and type VI in ballan wrasse. The assays can be used as a screening tool during disease outbreaks, to detect infectious strains and trace strain divergence, thus providing health certification and isolate identification for vaccine development.

TABLE 1 List of bacterial species used for specificity testing on newly designed *As vapA* type V primers and restriction enzyme assay for the detection of type VI in ballan wrasse samples

Species	Strain ID
Aeromonas salmonicida subsp. *salmonicida*	NCIMB 1102[b]
atypical Aeromonas salmonicida[a]	ORN6
atypical Aeromonas salmonicida (type VI)[a]	SAIC-CF-026
atypical Aeromonas salmonicida (type V)[a]	SAIC-CF-019
atypical Aeromonas salmonicida (type VI)[a]	SAIC-CF-035
Aeromonas salmonicida subsp. *achromogenes*	NCIMB 1110[b]
Vibrio (Listonella) anguillarum	NCIMB 6[b]
Vibrio ordalii	NCIMB 2167[b]
Vibrio splendidus	ATCC® 33125[b]
Pasteurella skyensis[a]	LN-45 XG
Pseudomonas anguilliseptica	NCIMB 2185

Abbreviations: ATCC, American Type Culture Collection; NCIMB, National Collection of Industrial Food and Marine Bacteria; SAIC-CF, Cleaner fish bacterial culture collection in Aquatic Vaccine Unit.
[a]Institute of Aquaculture bacterial culture collection.
[b]Type strain.

2 | MATERIALS AND METHODS

2.1 | Bacterial cultivation

Representative *As* strains, unrelated bacteria and commonly isolated bacteria from ballan wrasse were used for the assessment of the

newly developed assays. A list of the bacteria used in the study is provided in Table 1. *Aeromonas salmonicida* and *Pseudomonas anguilliseptica* isolates were plated on tryptone soya agar TSA (Oxoid), while *Vibrionaceae* isolates were cultured on seawater agar (SWA; Oxoid) and incubated at 22°C for 48 and 24 hr, respectively. *Pasteurella skyensis* was cultured on BA (TSA + 5% sheep blood, Thermo Fisher) at 22°C for 48 hr. The bacteria were then inoculated onto tryptone soya broth (TSB; Oxoid, UK; *As* isolates) and TSB + 2% NaCl (Oxoid, *Vibrionaceae* isolates) and incubated at 22°C for 48 hr and 24 hr, respectively, with continuous shaking at 150 rpm. All bacteria were centrifuged at 2,000 *g* for 10 min, and bacterial pellets were then washed with phosphate-buffered saline (PBS) and resuspended in PBS to an optical density (OD) of OD_{600} ~ 1.0. The bacterial colony-forming units (CFUs) per ml were counted by the 10-fold dilution method.

2.2 | DNA extraction

Bacterial DNA was extracted using a modified salt precipitation protocol (Aljanabi & Martinez, 1997) as described in Taslima et al. (2016). The samples were resuspended in 5 mM Tris, and sample concentration was measured and standardized at 50 ng/µl using NanoDrop 1000 (NanoDrop Technologies). The samples were stored at −20°C until use.

2.3 | a*As vapA* type V assay design

An atypical *As* type V PCR was developed and used in order to determine the presence or absence of type V isolates in ballan wrasse samples. Gulla, Lund, et al. (2016) sequenced the hypervariable region of the additional outer membrane of *As*—the paracrystalline surface protein (A-layer protein) gene (*vapA*)—and identified 14 different types. In total, 71 *vapA* gene sequences were downloaded representing the 14 different types identified from the GenBank database (Table S1). The sequences were aligned using ClustalW in BioEdit (Hall, 1999), and conserved regions for *As* type V within the *vapA* gene were identified as potential primer sites with *Aeromonas salmonicida* type V primers specific to these locations being designed using Primer-BLAST suit (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). The primers were screened for primer specificity (species-specific), in an in silico analysis using local nucleotide alignment tool "BLAST" (https://blast.ncbi.nlm.nih.gov/Blast.cgi) (Ye et al., 2012). The primers selected, Vspec—F: 5′-CCAACGGTTTCTGGAGTAATAACTTT-3′ and Vspec—R: 5′-TGCATCAGCAAC AGCGGTAGT-3′, were tested using routine PCRs consisting of 25 ng bacterial DNA (0.5 µl) from type strain *As* (ATCC 1102), 10 µM (0.25 µl) of each primer, 1 × Q5® Hot Start High-Fidelity Master Mix (2×) (NEB Biolabs), 10 mM dNTPs (2.5 µl), 1 × Q5 High GC Enhancer (1 µl) and Milli-Q water to reach 5 µl total volume. Following optimization, the following thermal cycling conditions were used: 1 cycle at 98°C for 1 min, 30 cycles at 95°C for 15 s, 57°C for 15 s and 72°C for 15 s, followed by 1 cycle at 72°C for 2 min and cooling at 4°C. PCR assay products were visualized using gel electrophoresis to confirm the presence of a*As* type V in the sample; single product at 254 base pairs (bp) indicates the presence of a*As* type V.

2.4 | Design of restriction enzyme assay for a*As vapA* type VI

Initially, the *vapA* gene was amplified using a previous protocol (Gulla, Lund, et al., 2016) and the obtained products (1 µl out of 5 µl) were digested with the restriction enzyme *ScaI* (cut site 5′-AGT▾ACT-3′, New England Biolabs®) at a final volume of 5 µl for 1 hr at 37°C according to conditions specified by the manufacturer. Restriction products (5 µl of enzyme digest) were visualized by gel electrophoresis to determine whether no product was present (no a*As* present in sample), a single band of 625 bp was present (a*As* other than type VI present in sample) or two bands of 368 bp and 254 bp were present (a*As* type VI present in sample).

2.5 | Assay analytical specificity and relative sensitivity

The specificity of a*As vapA* type V PCR and restriction enzyme assay (type VI) was tested against DNA of target and non-target bacterial species at 50 ng/µl (from active cultures of approx. 10^7 and 10^8 CFU/ml, for *As* and *Vibrionaceae* or *Pasteurella skyensis*) (Table 1). Initially, all bacterial DNAs were screened with a broad-range 16S rRNA PCR for the detection of bacterial DNA as described previously (Herlemann et al., 2011), and with *vapA* PCR (Gulla, Lund, et al., 2016) during which only *As* samples were expected to be amplified. Finally, all samples were screened with the newly developed a*As* type V and type VI assays described above. PCR and restriction enzyme products were sequenced (GATC Eurofins) following enzymatic cleanup where 5 µl of PCR product was mixed with 1 µl of exonuclease (New England Biolabs®) and 1 µl of shrimp alkaline phosphatase (New England Biolabs®) and digested in accordance with manufacturer's guidelines (Exo-CIP Rapid PCR Cleanup Kit, New England Biolabs®).

The relative sensitivity of the assays was assessed on water (sterile and tap water; 25 and 15 ml) spiked with a*As vapA* type V and type VI at an OD_{600} 1.0 (10^7 CFU/ml) and two liver samples from naturally infected individuals that had previously been confirmed to be positive for a*As vapA* type V by sequencing. The water samples were centrifuged at 5,100 *g* for 10 min, and the supernatant was removed prior to DNA extraction. The DNA was extracted from water and tissues samples with the method described earlier. The total a*As* concentration in the undiluted extracted DNA (600 ng/µl) from liver samples was approximately 10^4 CFU/ml (crossing point; Cp 28.58) and 10^7 CFU/ml (Cp 22.53) using a previously published qPCR to quantify (Gulla, Duodu, et al., 2016). Various concentrations were prepared for the two liver DNA samples (10, 25, 50, 100 and 600 ng/µl) in order to determine the appropriate screening concentration.

2.6 | Diagnostic validation of assays

Whole ballan wrasse larvae and tissue samples (liver-L and head kidney-HK) were collected randomly from healthy-looking individuals from a commercial ballan wrasse hatchery in Scotland between February and September in the same production year. The hatchery had a history of a*As* outbreaks in the previous years. A total of 140 whole larvae samples (various sizes from 2 to approx. 120 days post-hatch; dph, 20 samples/month; 10 newly hatch/live prey feeding larvae and 10 weaned larvae) and 120 tissue samples (60 liver and 60 head kidney various sizes ≥ 120 dph to 15 months old/pre-deployment) were gathered and fixed in 100% ethanol and stored at 4°C for later use. DNA was extracted from samples of 1–2 mm long and 1 mm thick using the protocol described previously. From those, 70 whole fish and 120 liver and head kidney (60 each) were screened with the assays developed above using 50 ng of total extracted DNA. The above samples were also screened with a qPCR protocol for *As* bacterial loads designed on *vapA* gene (Gulla, Duodu, et al., 2016) for cross-validation.

3 | RESULTS

All bacterial strains used for the validation of the newly design assays were screened with a generic *16S* PCR (Figure 1). Samples were then screened with *vapA* PCR (Gulla, Lund, et al., 2016), and only *As* samples were amplified (Figure 2). The newly designed primers for a*As vapA* type V PCR and restriction enzyme assay (type VI) yielded the expected products of 254 bp, and 368 and 254 bp, respectively (Figures 3 and 4). The amplification products were sequenced and the sequences confirmed to have 100% identity to *As vapA* gene by BLAST and type V and type VI following sequence alignment using ClustalW. PCR products were reported only for a*As vapA* type V and type VI isolates, as is shown in Figures 3 and 4, resulting in 100% specificity for the bacteria tested herein. The assays were able to detect a*As vapA* type V and type VI in water samples (sterile and tap water) spiked with a*As vapA* type V and type VI isolates (Figures 5 and 6).

Liver tissues naturally infected with a*As vapA* type V tested positive with the newly developed PCR assay for the majority of the concentrations tested (25, 50, 100 and 600 ng/μl) apart from the 10 ng/μl (Figures 7 and 8). However, based on the electrophoresis results, a concentration of 100 ng/μl would be appropriate for the screening of diagnostic samples to avoid false-positive results.

A total of 16 diagnostic samples (4/70 larvae, 8/60 L and 4/60 HK) randomly collected from apparently healthy individuals from commercial ballan wrasse tested positive with type V-specific PCR. Matching positive results were observed for the liver and kidney samples for two individuals. None of the diagnostic samples were positive for a*As vapA* type VI, apart from the positive control a*As vapA* type VI sample, which was amplified successfully. In addition, the same samples screened with a previously developed qPCR for *As* and 17 positive samples were identified (4/70 larvae, 8/60 L and 5/60 HK) 16 of which were in corroboration with the diagnostic PCR assay for a*As vapA* type V. These samples had medium (8/17)-to-low (9/17) bacterial loads corresponding to Cp value ≥25–30 and ≥31–35, respectively.

4 | DISCUSSION

Atypical *Aeromonas salmonicida* are commonly isolated bacteria from morbid ballan wrasse during bacterial disease outbreaks in the Northern Hemisphere (Hewson, 2019; Hjeltnes et al., 2019; Laidler et al., 1999). This fish species is susceptible to two a*As vapA* types (V and VI) (Gulla, Lund, et al., 2016). Type-specific diagnosis of a*As* can help in controlling the disease spread in ballan wrasse hatcheries and Atlantic salmon cage sites following deployment as cleaner fish. In this paper, we describe the development of two rapid, specific and low-cost assays for the detection of a*As vapA* type V and type VI in ballan wrasse.

A number of molecular assays (PCR and qPCR) are available for the detection of *As*. In particular, qPCR assays are highly sensitive and specific (Beaz-Hidalgo et al., 2008; Byers, Cipriano, et al., 2002; Gulla, Duodu, et al., 2016; Gustafson et al., 1992; Rattanachaikunsopon & Phumkhachorn, 2012). However, they require custom-designed probes, that is self-quenched primers and molecular beacons, which are costly (e.g., $200/500 samples). Furthermore, existing molecular assays are only able to detect *As* DNA at the broader species

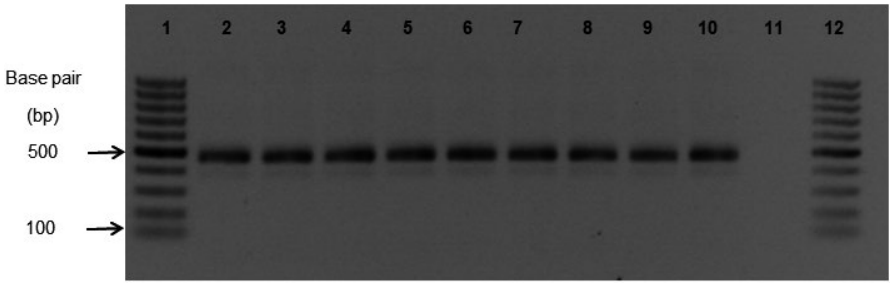

FIGURE 1 Agarose gel (1%) analysis for bacterial presence with *16S* PCR on DNA extracted from a variety of bacterial species at 50 ng/μl. From left to right 100-bp ladder (GeneRuler, Thermo Fisher Scientific; lanes 1 and 12), *Vibrio anguillarum* (lane 2), *Vibrio ordalii* (lane 3), *Vibrio splendidus* (lane 4), *Aeromonas salmonicida* subsp. *salmonicida* (lane 5), *Aeromonas salmonicida* subsp. *achromogens* (lane 6), atypical *Aeromonas salmonicida* (a*As*) (lane 7), a*As* type IV (lane 8), *Pasteurella skyensis* (lane 9), a*As* type V (lane 10) and no template control (lane 11). PCR product is of the expected size of 485 bp. PCRs were standardized for DNA samples at 25 ng

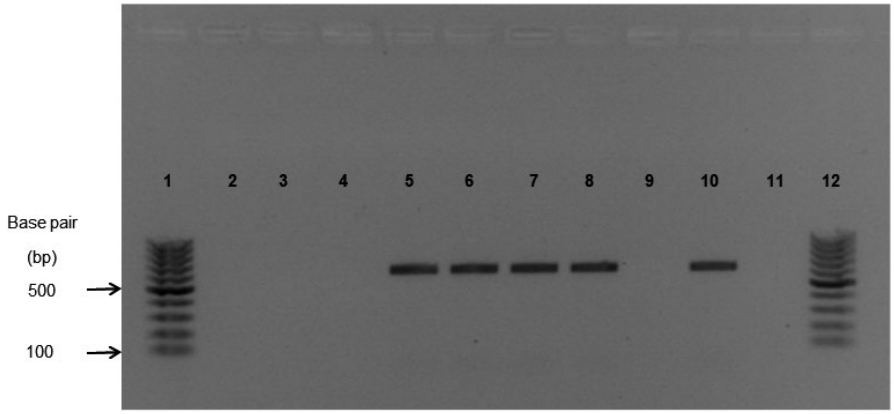

FIGURE 2 Agarose gel (1%) analysis for atypical *Aeromonas salmonicida* (a*As*) presence with *vapA* PCR (Gulla, Lund, et al., 2016) on DNA extracted from a variety of bacterial species at 50 ng/μl. From left to right 100-bp ladder (GeneRuler, Thermo Fisher Scientific; lanes 1 and 12), *Vibrio anguillarum* (lane 2), *Vibrio ordalii* (lane 3), *Vibrio splendidus* (lane 4), *Aeromonas salmonicida subsp. salmonicida* (lane 5), *Aeromonas salmonicida subsp. achromogens* (lane 6), atypical *Aeromonas salmonicicda* (a*As*) (lane 7), a*As* type IV (lane 8), *Pasteurella skyensis* (lane 9), positive control a*As* type V (lane 10) and no template control (lane 11). PCR product is of the expected size of 625 bp. PCRs were standardized for DNA samples at 25 ng

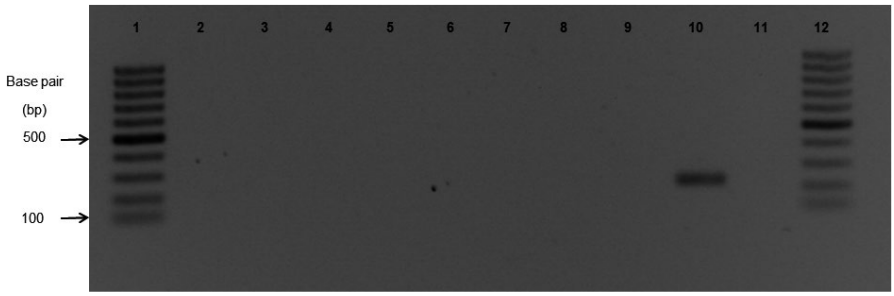

FIGURE 3 Specificity test for atypical *Aeromonas salmonicida* (a*As*) *vapA* type V newly designed primers on DNA samples extracted from a variety of bacterial species at 50 ng/μl. Samples run on an agarose gel (1%). From left to right 100-bp ladder (GeneRuler, Thermo Fisher Scientific; lanes 1 and 12), *Vibrio anguillarum* (lane 2), *Vibrio ordalii* (lane 3), *Vibrio splendidus* (lane 4), *Aeromonas salmonicida subsp. salmonicida* (lane 5), *Aeromonas salmonicida subsp. achromogenes* (lane 6), atypical *Aeromonas salmonicida* (a*As*) (lane 7), a*As* type IV (lane 8), *Pasteurella skyensis* (lane 9), a*As vapA* type V (lane 10) and no template control (lane 11). PCR product is of the expected size of 254 bp. PCRs were standardized for DNA samples at 25 ng

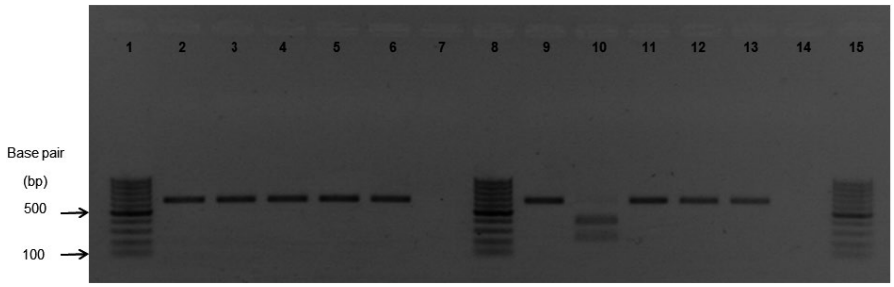

FIGURE 4 Specificity test for restriction enzyme assay on DNA samples extracted from a variety of bacterial species at 50 ng/μl. Isolated bacteria were screened with a broad-spectrum PCR for atypical *Aeromonas salmonicida* (a*As*) based on the *vapA* region; samples in lanes 2–7 (Gulla, Lund, et al., 2016) and PCR products restricted with *ScaI* (New England Biolabs®) in lanes 9–14. Samples run on agarose gel (1%). From left to right 100-bp ladder (GeneRuler, Thermo Fisher Scientific; 1, 8 and 15), *Aeromonas salmonicida sub. salmonicida* (lanes 2 and 9), a*As vapA* type VI (lanes 3 and 10), a*As vapA* type V (lanes 4 and 11), a*As vapA* (lanes 5 and 12), a*As vapA* type V (lanes 6 and 13) and no template control (lanes 7 and 14). PCR products are of the expected size of 625 bp and two bands for restriction enzyme at 368 and 254 bp. PCRs were standardized for DNA samples at 25 ng

level but cannot differentiate type strains. Such information previously depended on isolate sequencing techniques (Gulla, Lund, et al., 2016), which are time-consuming, expensive and require specialized technical understanding, in contrast with the conventional PCR and restriction enzyme assay described in this paper (e.g., $60/500 samples).

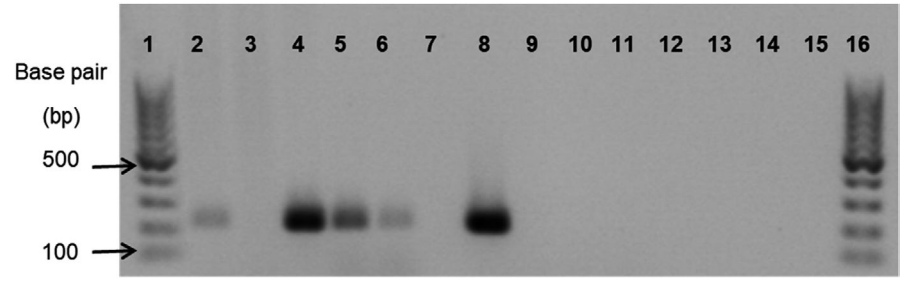

FIGURE 5 Agarose gel (1%) analysis for atypical *Aeromonas* salmonicida (a*As*) *vapA* type V assay. Sensitivity and specificity on bacterial DNA isolated from sterile or tap water spiked with a*As* type V or type VI (1 ml bacteria at 10^7 CFU/ml) after centrifugation. From left to right 100-bp ladder (GeneRuler, Thermo Fisher Scientific; lanes 1 and 16), tap water spiked with a*As vapA* type V (lane 2), tap water spiked with a*As vapA* type VI at 10^7 CFU/ml (lanes 3 and 7), sterile water (25 ml) spiked with a*As vapA* type V at 10^7 CFU/ml (lane 4) and DNA diluted 1/10 (lane 5) and 1/100 (lane 6), positive control a*As vapA* type V (lane 8) and no template control (lane 9). Positions 10 to 15 empty. PCR product is of the expected size of 254 bp. PCRs were standardized for DNA samples at 25 ng

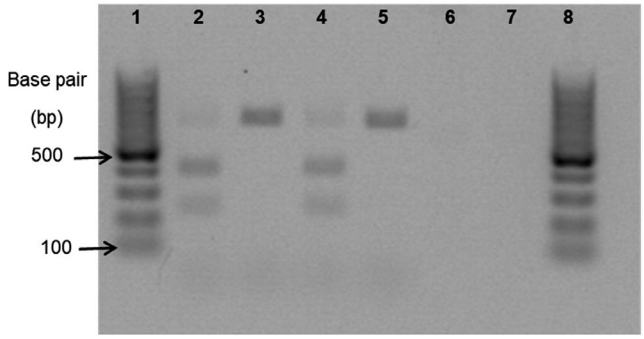

FIGURE 6 Agarose gel analysis for atypical *Aeromonas salmonicida* (a*As*) *vapA* type VI assay. Sensitivity and specificity on bacterial DNA isolated from 15-ml tap water spiked with a*As* type V or type VI (1 ml bacteria at 10^7 CFU/ml) after centrifugation. From left to right 100-bp ladder (lanes 1 and 8), tap water spiked with a*As vapA* type VI (lane 2), tap water spiked with a*As vapA* type V (lanes 3 and 5), positive control a*As vapA* type VI (lane 4) and no template control (lane 6). Position 7 empty. PCR product is of the expected size of 368 and 254 bp. PCRs were standardized for DNA samples at 25 ng

Several genes have been targeted for the diagnosis of *As* over the years including the *vapA* gene (Austin & Austin, 2016). This gene is one of the most preferable genes for molecular assay development as it encodes the outer membrane A-layer protein of the bacterium and has a significant role in its virulence (Daly et al., 1996; Udey & Fryer, 1978). In this study, both type-specific (V and VI) assays were designed based on the *vapA* gene. The assays have 100% specificity and did not cross-react with other *Aeromonas* subspecies, for example *achromogens* or atypical isolates and other bacteria that were tested (e.g., *Vibrio anguillarum*, *Vibrio splendidus*, *Pasteurella skyensis*), which are commonly isolated in cleaner fish (Hewson, 2019; Hjeltnes et al., 2019). Two closely related bacteria *As* subsp. *pectinolytica* and *A. hydrophila* were not included in the specificity test because the former has not been recovered in fish (Merino et al., 2015; Pavan et al., 2000), while the latter is mainly isolated in freshwater species (Austin & Austin, 2016). Nevertheless, the amplification products for the type V and type VI assays developed herein were sequenced and screened in silico (BLAST) against *A. hydrophila* and all the other four *As* subspecies (*achromogenes*, *masoucida*, *smithia* and *pectinolytica*)

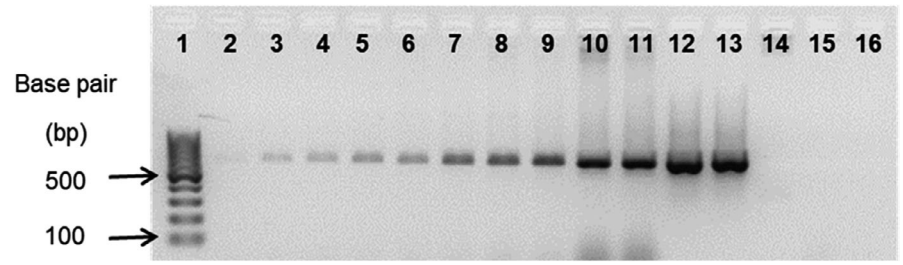

FIGURE 7 Ballan wrasse liver samples ($n = 2$) naturally infected with atypical *Aeromonas salmonicida* (a*As*) *vapA* type V at various concentrations (in duplicate 10, 25, 50, 100 and undiluted DNA; 600 ng/μl). Samples screened with the *Aeromonas salmonicida vapA* region PCR (Gulla, Lund, et al., 2016) and run on 1% agarose gel. Samples from left to right; 100-bp ladder (lane 1), DNA extracted from two liver samples of a concentration at 10 ng/μl (lanes 2 and 3), 25 ng/μl (lanes 4 and 5), 50 ng/μl (lanes 6 and 7), 100 ng/μl (lanes 8 and 9) and 600 ng/μl (lanes 10 and 11). Positive control: a*As vapA* type V and type VI (lanes 12 and 13, respectively), negative control: *Pseudomonas sp.* (lane 14), ballan wrasse genomic DNA (lane 15) and no template control (lane 16). PCRs were standardized for DNA samples at 25 ng

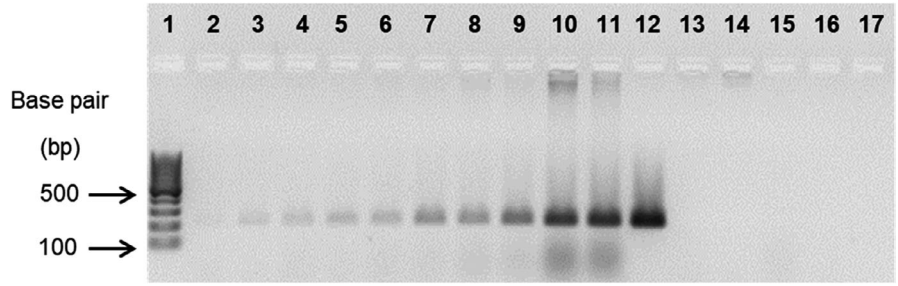

FIGURE 8 Assay sensitivity test on atypical *Aeromonas salmonicida* (a*As*) *vapA* type V primer set for two liver samples of ballan wrasse naturally infected with a*As vapA* type V. Total extracted DNA samples adjusted and tested at various concentrations (in duplicate 10, 25, 50, 100 and neat; 600 ng/μl). Samples run on 1% agarose gel from left to right; 100-bp ladder (GeneRuler, Thermo Fisher Scientific; lane 1), DNA extracted from 2 liver sample concentration at 10 ng/μl (lanes 2 and 3), 25 ng/μl (lanes 4 and 5), 50 ng/μl (lanes 6 and 7), 100 ng/μl (lanes 8 and 9) and 600 ng/μl (lanes 10 and 11). Positive control: a*As vapA* type V and type VI (lanes 12 and 13, respectively), negative control: *Pseudomonas sp.* (lane 14), ballan wrasse genomic DNA (lane 15) and no template control (lane 16). PCR product is of the size of 254 bp, as expected. Notice the negative PCR amplification (no band on gel) for a*As vapA* type VI samples. PCRs were standardized for DNA samples at 25 ng

with there being no identity to registered sequences. In addition, all the other available *As vapA* types were also aligned in silico for assay specificity assessment. The primer sites for type V and restriction site for type VI are in conserved regions of the *vapA* gene and are not cross-reactive with the other types. The newly designed primers successfully identified the a*As vapA* type V in diagnostic samples (liver and kidney samples), while type VI bacterial DNA was detected only within the positive control sample and not in the diagnostic samples. In addition, the same samples were positive when tested with the previously published qPCR assay (Gulla, Duodu, et al., 2016), with the exception of one additional positive kidney samples. This sample had a very high Cp value (34) and subsequently very low bacterial load. The qPCR used for cross-validation of the newly developed assays is a molecular beacon probe assay (Gulla, Duodu, et al., 2016). Molecular beacons are highly specific and more sensitive (i.e. lower detection limits) than conventional PCRs (Gulla, Duodu, et al., 2016; Manganelli et al., 2001), which explains the detection of these samples with low bacterial load with the qPCR and not the newly developed assays. The absence of positive a*As* vapA type VI is in corroboration with the previous report of rare isolation of this type in ballan wrasse in Scotland (Papadopoulou et al., 2020). The samples were screened at total DNA concentrations of 100 ng/μl to eliminate false-positive results; however, the relative sensitivity test demonstrated that samples could be screened at lower concentrations of 25 ng/μl. The sensitivity of the assays can be further optimized in order to increase the sensitivity (e.g., PCR cycles, sample concentration), if required. The diagnostic tools described in this study may also be used for lumpsucker (*Cyclopterus lumpus*, L.) tissue screening, as they are susceptible to a*As* (Hjeltnes et al., 2019), although validation will be required.

The positive results on type V and type VI spiked water samples support the potential use of the molecular assays for monitoring purposes in hatchery sites. In general, *Aeromonas salmonicida* isolation from environmental samples has been attempted on solid medium cultivation methods in the past with not encouraging results (Du et al., 2017; Teska & Cipriano, 1993). The bacterium is difficult to recover from these samples where mixed bacterial communities co-exist due to the lack of selective media and overgrowth of concomitant bacteria. Furthermore, diagnostics can be difficult where a change in physiological status (i.e., dormancy) of *As* occurs outside of the host—that is where it is still viable but not culturable (Popoff, 1984; Stevenson, 1977). All the above are strongly supporting that the use of molecular techniques like the ones developed herein is important for pathogen detection to control a disease outbreak in a commercial site.

In conclusion, we described the development of two novel type-specific assays for a*As* (type V and type VI) detection in ballan wrasse. The assays are fast, reliable, cost-effective and easy to perform. They can be applied as screening tools in disease outbreaks and health certification (e.g., transportation from hatchery to cage sites) for cleaner fish. They can also be used to facilitate autogenous vaccine development and vaccine trials for isolate confirmation at the type level (Papadopoulou et al., 2020). Future studies are required to optimize the assays for environmental samples (i.e., water samples) and screening of other cleaner fish species (i.e., lumpsucker samples). Furthermore, modification of the newly developed assays described in this paper for the development of qPCR assays could provide information on bacterial load at the type level in cleaner fish samples.

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CONFLICT OF INTEREST

The authors have no conflicts of interest to disclose.

DATA AVAILABILITY STATEMENT

Supplementary data file data are derived from public domain resources and upon approval of Dr. Snorre Gulla—https://doi.org/10.1111/jfd.12367 supplementary file Table S1.

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REFERENCES

Adams, A., Thompson, K. D., Morris, D., Farias, C., & Chen, S. C. (1995). Development and use of monoclonal antibody probes for immunohistochemistry, ELISA and IFAT to detect bacterial and parasitic fish pathogens. *Fish & Shellfish Immunology*, *5*, 537–547. https://doi.org/10.1016/S1050-4648(95)80040-9

Aljanabi, S. M., & Martinez, I. (1997). Universal and rapid salt-extraction of high quality genomic DNA for PCR-based techniques. *Nucleic Acids Research*, *25*, 4692–4693. https://doi.org/10.1093/nar/25.22.4692

Austin, B., & Austin, D. A. (2016). Aeromonadaceae representative (*Aeromonas salmonicida*). *Bacterial fish pathogens* (pp. 215–321). Springer.

Austin, D., McIntosh, D., & Austin, B. (1989). Taxonomy of fish associated *Aeromonas* spp., with the description of *Aeromonas salmonicida* subsp. *smithia* subsp. nov. *Systematic and Applied Microbiology*, *11*, 277–290. https://doi.org/10.1016/S0723-2020(89)80026-8

Balcazar, J. L., Vendrell, D., de Blas, I., Ruiz-Zarzuela, I., Girones, O., & Muzquiz, J. L. (2007). Quantitative detection of *Aeromonas salmonicida* in fish tissue by real-time PCR using self-quenched, fluorogenic primers. *Journal of Medical Microbiology*, *56*, 323–328. https://doi.org/10.1099/jmm.0.46647-0

Beaz-Hidalgo, R., Magi, G. E., Balboa, S., Barja, J. L., & Romalde, J. L. (2008). Development of a PCR protocol for the detection of *Aeromonas salmonicida* in fish by amplification of the *fstA* (ferric siderophore receptor) gene. *Veterinary Microbiology*, *128*, 386–394. https://doi.org/10.1016/j.vetmic.2007.10.004

Brooker, A. J., Papadopoulou, A., Gutierrez, C., Rey, S., Davie, A., & Migaud, H. (2018). Sustainable production and use of cleaner fish for the biological control of sea lice: Recent advances and current challenges. *Veterinary Record*, *183*, 383. https://doi.org/10.1136/vr.104966

Byers, H. K., Cipriano, R. C., Gudkovs, N., & Crane, M. S. J. (2002). PCR-based assays for the fish pathogen *Aeromonas salmonicida*. II. Further evaluation and validation of three PCR primer sets with infected fish. *Diseases of Aquatic Organisms*, *49*, 139–144. https://doi.org/10.3354/dao049139

Byers, H. K., Gudkovs, N., & Crane, M. S. J. (2002). PCR-based assays for the fish pathogen *Aeromonas salmonicida*. I. Evaluation of three PCR primer sets for detection and identification. *Diseases of Aquatic Organisms*, *49*, 129–138. https://doi.org/10.3354/dao049129

Cipriano, R. C., & Austin, B. (2011). Furunculosis and other Aeromonad diseases. In P. T. Woo, & D. W. Bruno (Eds.), *Fish diseases and disorders, vol. 3 Viral, bacterial and fungal infections* (2nd ed., pp. 424–483). CABI.

Daly, J. G., Kew, A. K., Moore, A. R., & Olivier, G. (1996). The cell surface of *Aeromonas salmonicida* determines in vitro survival in cultured brook trout (*Salvelinus fontinalis*) peritoneal macrophages. *Microbial Pathogenesis*, *21*, 447–461. https://doi.org/10.1006/mpat.1996.0075

Du, Y., Liu, Y., Xiao, P., Meng, L., & Liu, P. (2017). Development and application of a quantitative real-time polymerase chain reaction assay for the detection of *Aeromonas salmonicida*. *Journal of the World Aquaculture Society*, *48*, 574–582.

Emmerich, R., & Weibel, E. (1894). Über eine durch Bacterien erzeugte Seuche unter den Forellen.

Evensen, Ø. (2016). Development of fish vaccines: Focusing on methods. In A. Adams (Ed.), *Fish vaccines* (pp. 53–74). Springer.

Frerchs, G., Millar, S., & McManus, C. (1992). Atypical *Aeromonas salmonicida* isolated from healthy wrasse (*Ctenolabrus rupestris*). *Bulletin of the European Association of Fish Pathologists*, *12*, 48–49.

Griffin, P. J., Snieszko, S., & Friddle, S. (1953). A more comprehensive description of *Bacterium salmonicida*. *Transactions of the American Fisheries Society*, *82*, 129–138. https://doi.org/10.1577/1548-8659(1952)82[129:AMCDOB]2.0.CO;2

Gulla, S., Bayliss, S., Björnsdóttir, B., Dalsgaard, I., Haenen, O., Jansson, E., McCarthy, U., Scholz, F., Vercauteren, M., Verner-Jeffreys, D., Welch, T., Wiklund, T., & Colquhoun, D. J. (2019). Biogeography of the fish pathogen *Aeromonas salmonicida* inferred by *vapA* genotyping. *FEMS Microbiology Letters*, *366*, fnz074. https://doi.org/10.1093/femsle/fnz074

Gulla, S., Duodu, S., Nilsen, A., Fossen, I., & Colquhoun, D. J. (2016). *Aeromonas salmonicida* infection levels in pre- and post-stocked cleaner fish assessed by culture and an amended qPCR assay. *Journal of Fish Diseases*, *39*, 867–877. https://doi.org/10.1111/jfd.12420

Gulla, S., Lund, V., Kristoffersen, A. B., Sorum, H., & Colquhoun, D. J. (2016). *vapA* (A-layer) typing differentiates *Aeromonas salmonicida* subspecies and identifies a number of previously undescribed subtypes. *Journal of Fish Diseases*, *39*, 329–342. https://doi.org/10.1111/jfd.12367

Gustafson, C., Thomas, C., & Trust, T. (1992). Detection of *Aeromonas salmonicida* from fish by using polymerase chain reaction amplification of the virulence surface array protein gene. *Applied and Environmental Microbiology*, *58*, 3816–3825. https://doi.org/10.1128/AEM.58.12.3816-3825.1992

Hall, T. A. (1999). *BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT*. Paper presented at the Nucleic Acids Symposium Series.

Herlemann, D. P., Labrenz, M., Jürgens, K., Bertilsson, S., Waniek, J. J., & Andersson, A. F. (2011). Transitions in bacterial communities along the 2000 km salinity gradient of the Baltic Sea. *The ISME Journal*, *5*, 1571–1579. https://doi.org/10.1038/ismej.2011.41

Hewson, I. (2019). Technical pitfalls that bias comparative microbial community analyses of aquatic disease Ian Hewson. *Diseases of Aquatic Organisms*, *137*, 109–124. https://doi.org/10.3354/dao03432

Hiney, M., Dawson, M., Heery, D., Smith, P., Gannon, F., & Powell, R. (1992). DNA probe for *Aeromonas salmonicida*. *Applied and Environmental Microbiology*, *58*, 1039–1042. https://doi.org/10.1128/AEM.58.3.1039-1042.1992

Hjeltnes, B., Bang-Jensen, B., Bornø, G., Haukaas, A., & Walde, C. S. (2019). *The health situation in Norwegian aquaculture 2018*.

Holt, J. G., Krieg, N. R., Sneath, P. H., Staley, J. T., & Williams, S. T. (1994). *Bergey's manual of determinative bacteriology* (9th ed.). William & Wilkins.

Kawahara, E., & Kusuda, R. (1987). Direct fluorescent antibody technique for diagnosis of bacterial disease in eel. *Bulletin of the Japanese Society of Scientific Fisheries*, *53*, 395–399. https://doi.org/10.2331/suisan.53.395

Kay, W., & Trust, T. (1991). Form and functions of the regular surface array (S-layer) of *Aeromonas salmonicida*. *Cellular and Molecular Life Sciences*, *47*, 412–414.

Keeling, S., Brosnahan, C., Johnston, C., Wallis, R., Gudkovs, N., & McDonald, W. (2013). Development and validation of a real-time PCR assay for the detection of *Aeromonas salmonicida*. *Journal of Fish Diseases*, *36*, 495–503.

Kimura, T. (1969a). A new subspecies of *Aeromonas salmonicida* as an etiological agent of furunculosis on "Sakuramasu" (*Oncorhynchus masou*) and pink salmon (*O. gorbuscha*) rearing for maturity. Part 1. On the morphological and physiological properties. *Fish Pathology*, *3*, 34–44. https://doi.org/10.3147/jsfp.3.2_34

Kimura, T. (1969b). A new subspecies of *Aeromonas salmonicida* as an etiological agent of furunculosis on "Sakuramasu" (*Oncorhynchus masou*) and pink salmon (*O. gorbuscha*) rearing for maturity. Part 2.

on the serological properties. *Fish Pathology*, *3*, 45–52. https://doi.org/10.3147/jsfp.3.2_45

Kwon, M. G., Lee, J.-Y., Park, S., Iida, T., Hirono, I., & Aoki, T. (1997). RAPD analysis of atypical *Aeromonas salmonicida* isolated in Japan. *Fish Pathology*, *32*, 109–115. https://doi.org/10.3147/jsfp.32.109

Laidler, L. A., Treasurer, J. W., Grant, A. N., & Cox, D. I. (1999). Atypical *Aeromonas salmonicida* infection in wrasse (Labridae) used as cleaner fish of farmed Atlantic salmon, *Salmo salar* L., in Scotland. *Journal of Fish Diseases*, *22*, 209–213. https://doi.org/10.1046/j.1365-2761.1999.00148.x

Lehmann, K. B., & Neumann, R. O. (1896). *Atlas und grundriss der bakteriologie und lehrbuch der speziellen bakteriologischen diagnostik* (vol. *1*). Lehmann.

Livesley, M., Smith, S., Armstrong, R., & Barker, G. (1999). Characterisation of *Aeromonas* strains and species by pulsed field gel electrophoresis and principal components analysis. *Journal of Fish Diseases*, *22*, 369–375. https://doi.org/10.1046/j.1365-2761.1999.00186.x

Lund, V., Jenssen, L. M., & Wesmajervi, M. S. (2002). Assessment of genetic variability and relatedness among atypical *Aeromonas salmonicida* from marine fishes, using AFLP-fingerprinting. *Diseases of Aquatic Organisms*, *50*, 119–126. https://doi.org/10.3354/dao050119

Lund, V., & Mikkelsen, H. (2004). Genetic diversity among A-proteins of atypical strains of *Aeromonas salmonicida*. *Diseases of Aquatic Organisms*, *61*, 257–262. https://doi.org/10.3354/dao061257

Manganelli, R., Tyagi, S., & Smith, I. (2001). Real time PCR using molecular beacons. In T. Parish, & N. G. Stoker (Eds.), *Mycobacterium tuberculosis protocols* (pp. 295–310). Humana Press.

McCarthy, D., & Roberts, R. (1980) Furunculosis of fish-the present state of our knowledge. *Advances in Aquatic Microbiology*, *2*, 293–341.

Merino, S., de Mendoza, E., Canals, R., & Tomás, J. M. (2015). Functional Genomics of the *Aeromonas salmonicida* lipopolysaccharide O-Antigen and A-Layer from Typical and Atypical Strains. *Marine Drugs*, *13*, 3791–3808. https://doi.org/10.3390/md13063791

Miyata, A., Inglis, V., & Aoki, T. (1996). Rapid identification of *Aeromonas salmonicida* subspecies salmonicida by the polymerase chain reaction. *Aquaculture*, *141*, 13–24. https://doi.org/10.1016/0044-8486(95)01221-4

Munn, C. B., Ishiguro, E. E., Kay, W. W., & Trust, T. J. (1982). Role of surface components in serum resistance of virulent *Aeromonas salmonicida*. *Infection and Immunity*, *36*, 1069–1075. https://doi.org/10.1128/IAI.36.3.1069-1075.1982

O'Brien, D., Mooney, J., Ryan, D., Powell, E., Hiney, M., Smith, P. R., & Powell, R. (1994). Detection of *Aeromonas salmonicida*, causal agent of furunculosis in salmonid fish, from the tank effluent of hatchery-reared Atlantic salmon smolts. *Applied and Environmental Microbiology*, *60*, 3874–3877. https://doi.org/10.1128/AEM.60.10.3874-3877.1994

O'hIci, B., Olivier, G., & Powell, R. (2000). Genetic diversity of the fish pathogen *Aeromonas salmonicida* demonstrated by random amplified polymorphic DNA and pulsed-field gel electrophoresis analyses. *Diseases of Aquatic Organisms*, *39*, 109–119. https://doi.org/10.3354/dao039109

Papadopoulou, A., Garvey, K., Hill, T., Ramirez-Paredes, J. G., Monaghan, S. J., Baily, J. L., Davie, A., Katsiadaki, I., Verner-Jeffreys, D., Wallis, T., Migaud, H., & Adams, A. (2020). Novel atypical *Aeromonas salmonicida* bath challenge model for juvenile ballan wrasse (*Labrus bergylta*, Ascanius). *Journal of Fish Diseases*, https://doi.org/10.1111/jfd.13312

Pavan, M., Abbott, S., Zorzopulos, J., & Janda, J. (2000). *Aeromonas salmonicida* subsp. *pectinolytica* subsp. nov., a new pectinase-positive subspecies isolated from a heavily polluted river. *International Journal of Systematic and Evolutionary Microbiology*, *50*, 1119–1124. https://doi.org/10.1099/00207713-50-3-1119

Popoff, M. (1984). Bergey's manual of systematic bacteriology. In N. Krieg, & J. G. Holt (Eds.), *Genus III: Aeromonas Kluyver and van Niel 1936* (vol. *1*, pp. 545–548). Williams and Wilkins Co.

Rattanachaikunsopon, P., & Phumkhachorn, P. (2012). Detection of *Aeromonas salmonicida* by reverse transcription-multiplex polymerase chain reaction. *Bioscience, Biotechnology, and Biochemistry*, *76*, 665–670.

Schubert, R. H. (1967). The taxonomy and nomenclature of the genus *Aeromonas* Kluyver and Van Niel 1936. Part II. Suggestions on the taxonomy and nomenclature of the anaerogenic aeromonads. *International Journal of Systematic Bacteriology*, *17*, 273–279.

Smith, I. W. (1963). The classification of '*Bacterium salmonicida*'. *Microbiology*, *33*, 263–274.

Sommerset, I., Krossøy, B., Biering, E., & Frost, P. (2005). Vaccines for fish in aquaculture. *Expert Review of Vaccines*, *4*, 89–101. https://doi.org/10.1586/14760584.4.1.89

Stevenson, L. H. (1977). A case for bacterial dormancy in aquatic systems. *Microbial Ecology*, *4*, 127–133. https://doi.org/10.1007/BF02014283

Taslima, K., Davie, A., McAndrew, B. J., & Penman, D. J. (2016). DNA sampling from mucus in the Nile tilapia, *Oreochromis niloticus*: Minimally invasive sampling for aquaculture-related genetics research. *Aquaculture Research*, *47*, 4032–4037. https://doi.org/10.1111/are.12809

Teska, R. C., & Cipriano, J. D. (1993). Nonselective nature of Coomassie Brilliant Blue agar for the presumptive identification of *Aeromonas salmonicida* in clinical specimens. *Diseases of Aquatic Organisms*, *16*, 239–242. https://doi.org/10.3354/dao016239

Treasurer, J., & Cox, D. (1991). The occurrence of *Aeromonas salmonicida* in wrasse (*Labridae*) and implications for Atlantic salmon farming. *Bulletin of the European Association of Fish Pathologists*, *11*, 208–210.

Treasurer, J. W., & Laidler, L. A. (1994). *Aeromonas salmonicida* infection in wrasse (Labridae), used as cleaner fish, on an Atlantic salmon, *Salmo salar* L., farm. *Journal of Fish Diseases*, *17*, 155–161. https://doi.org/10.1111/j.1365-2761.1994.tb00208.x

Udey, L., & Fryer, J. (1978). Immunization of fish with bacterins of *Aeromonas salmonicida*. *Marine Fisheries Review*, *40*, 12–17.

Watts, J. E., Schreier, H. J., Lanska, L., & Hale, M. S. (2017). The rising tide of antimicrobial resistance in aquaculture: Sources, sinks and solutions. *Marine Drugs*, *15*, 158. https://doi.org/10.3390/md15060158

Ye, J., Coulouris, G., Zaretskaya, I., Cutcutache, I., Rozen, S., & Madden, T. L. (2012). Primer-BLAST: A tool to design target-specific primers for polymerase chain reaction. *BMC Bioinformatics*, *13*, 134. https://doi.org/10.1186/1471-2105-13-134

SUPPORTING INFORMATION

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