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PCR-based assays for the fish pathogen *Aeromonas salmonicida*. II. Further evaluation and validation of three PCR primer sets with infected fish

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ABSTRACT: Two *Aeromonas salmonicida*-specific polymerase chain reaction (PCR) tests and 1 *A. salmonicida* subsp. *salmonicida*-specific PCR test were used to screen salmonid populations that were either overtly or covertly infected with *A. salmonicida* subsp. *salmonicida*. It was demonstrated that these PCR assays could be used to replace the biochemical testing currently employed to confirm the identity of *A. salmonicida* isolates cultured from infected fish. The AP and PAAS PCR assays were also capable of direct detection of *A. salmonicida* in overtly infected fish, with mucus, gill and kidney samples most likely to yield a positive result. Culture was a more reliable method for the direct detection of *A. salmonicida* in covertly infected salmonids than was the direct PCR testing of tissue samples, with the AP and PAAS PCRs having a lower detection limit (LDL) of approximately 4×10^5 colony-forming units (CFU) g^{-1} sample.

KEY WORDS: *Aeromonas salmonicida* · Polymerase chain reaction · Detection · Identification · Validation · Naturally infected fish · Covert infections

INTRODUCTION

The bacterial pathogen *Aeromonas salmonicida* is responsible for a range of clinical syndromes in a wide variety of fish species, including furunculosis of salmonids caused by *A. salmonicida* subsp. *salmonicida* (also referred to as typical *A. salmonicida*), which is exotic to Australia (DPIE 1996). The threat that this disease poses to the Australian salmonid industry is compounded by the fact that the bacterium can be present in a clinically inapparent or 'covert' state of infection that is very difficult to detect. Infections may remain covert or they may become overt, depending upon environmental conditions such as trauma and poor water quality that stress carrier hosts (Hiney et al. 1997). Thus, covertly infected fish play a significant role in the epizootiology of the disease. The extensive range of known carriers of *A. salmonicida* (Hiney & Olivier 1999) increases the opportunities for transmission of the microorganism to susceptible fish. Furthermore, because there are no restrictions on access to sea-cage sites, feral populations of fish are attracted by the abundance of food. Consequently, covertly infected populations used in mariculture could act as an additional source of contagion among feral fish.

The current standard method for detecting carriers is culture from kidney and spleen of fish subjected to the stress-induced furunculosis (SIF) assay (Bullock & Stuckey 1975, McCarthy 1977). This process is resource intensive, time consuming and expensive. Polymerase chain reaction (PCR) assays are being developed for the detection and identification of *Aeromonas salmonicida*, and this technology may offer an alternative means of detecting latent infections. Such an approach could address the most significant problems associated with either the detection of covert

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infections or identification of atypical isolates of *A. salmonicida* that are difficult to classify phenotypically. It is important to note that the application of non-culture-based, 'proxy' methods such as PCR, enzyme-linked immunosorbent assay (ELISA) and western blotting require extensive validation (Hiney 1997). The PCR assays used in this study have been evaluated previously (Byers et al. 2002 in this issue) at the *in vitro* level, the 'sterile seeded microcosm' level and the 'non-sterile incurred microcosm' stage in accordance with the framework proposed by Hiney & Smith (1998). The final stage of validation depends on field trials. In this study, salmonid populations in the USA, which were infected with *A. salmonicida* subsp. *salmonicida*, were examined. The PCR assays were used to screen these populations for the presence of *A. salmonicida*, and the performance of these assays was compared to that of culture and biochemical testing for the detection and identification of *A. salmonicida*.

MATERIALS AND METHODS

Overt infection in Arctic char by bath exposure. Experiments involving overt infection in Arctic char *Salvelinus alpinus* with *Aeromonas salmonicida* subsp. *salmonicida* were conducted at the National Fish Health Research Laboratory (NFHRL; Kearneysville, West Virginia, USA). Labrador and Nauyuk strains of Arctic char (n = 18) were placed in water containing 10^5 colony-forming units (CFU) of *A. salmonicida* ml^{-1} for 30 min. Fish were then transferred to 50 l aquaria, maintained at approximately 12.5°C and monitored daily for clinical signs of disease, including the development of lesions, lethargy, colour change and other abnormal behaviour. Fish that displayed any signs of disease were euthanised using benzocaine at 100 mg l^{-1}. Unexposed control fish (n = 10) were maintained in a separate tank and sampled at the end of the experiment.

Overt infection in rainbow trout and Atlantic salmon induced by stress. Experiments to induce overt infection in rainbow trout *Oncorhynchus mykiss* and Atlantic salmon *Salmo salar* with *Aeromonas salmonicida* subsp. *salmonicida* were also conducted at NFHRL. Some of the Atlantic salmon used had been vaccinated (against *A. salmonicida* subsp. *salmonicida*) on an annual basis (in May) with the Aquavet oil emulsion vaccine. Thus, the fish used in these experiments had received a vaccine dose approximately 1 yr prior to sampling. Rainbow trout (n = 20), unvaccinated Atlantic salmon (n = 20) and vaccinated Atlantic salmon (n = 24) suspected to be covertly infected with *A. salmonicida* were subjected to the SIF assay. This assay was conducted as described by McCarthy (1977) except that fish were injected intraperitoneally with 0.1 ml prednisolone 21-hemisuccinate (Sigma) rather than prednisolone 21-acetate, at a rate of 20 mg kg^{-1} fish. The fish were held at 18°C. Aquaria were checked daily and dead fish were removed for processing as described below. On Day 8 post-administration of the steroid, any remaining rainbow trout were euthanised, and on Day 9 post-administration, any remaining Atlantic salmon were euthanised and processed for detection of *A. salmonicida*.

Cultural identification and quantification of bacterial flora from fish. Bacteria were isolated from the infected fish tissues (gill, spleen, kidney and intestine) and mucus, and were identified according to the method of Cipriano et al. (1992).

Detection of *Aeromonas salmonicida* in tissue samples collected from covertly infected salmonids. *A. salmonicida* subsp. *salmonicida* had previously been isolated from Atlantic salmon that had survived epizootics of furunculosis in raceways at the Richard Cronin National Salmon Station (RCNSS; Sunderland, Massachusetts, USA) and, therefore, this population was believed to be covertly infected (Cipriano et al. 1996e). These fish were age 3+ reconditioned kelts and were held at the hatchery for the ongoing New England Salmon Restoration Program. Because these fish could not be sacrificed for sampling, detection of *A. salmonicida* was attempted only from mucus samples of individual salmon (n = 100).

Mucus, gill, spleen, kidney and intestine samples were also collected from the following:

(1) Experimental Atlantic salmon (n = 40) also maintained at the same facility. These Atlantic salmon were age 2+ excess domestic stock and were receiving run-off water from the covertly infected re-conditioned kelts, described previously. Ongoing mortality was reported in this population and *Aeromonas salmonicida* was isolated from dead fish.

(2) Age 1+ brown trout (n = 20) located at a Vermont Fish and Wildlife fish culture station (Bennington, Vermont, USA). Ongoing mortality had been reported in this population and *Aeromonas salmonicida* had previously been isolated from these fish.

(3) Rainbow trout (n = 20) and unvaccinated Atlantic salmon (n = 20) held at NFHRL. These fish were covertly infected with *Aeromonas salmonicida* as indicated by results of SIF assays that were conducted on a proportion of each population.

Whenever lethal samples were obtained, individual fish were euthanised in tricaine methane sulphonate (MS-222, Argent Chemical Laboratories). The tissue samples were divided into 2 equal aliquots: one portion was used for direct PCR tests, and the other portion was used for quantitative culture of *Aeromonas salmonicida*.

PCR identification of *Aeromonas salmonicida* from fish tissue. Mucus, gill, intestine, kidney and spleen samples were taken from the overtly infected (diseased) fish. DNA was extracted and amplified by PCR as described previously (Byers et al. 2002).

RESULTS

Generation of overt infection in salmonids

Overt infection was generated in Arctic char via a bath challenge and, in covertly infected rainbow trout and Atlantic salmon (both vaccinated and unvaccinated), via SIF assays.

Culture-based identification of *Aeromonas salmonicida* in overtly infected fish tissues

Both mixed bacterial cultures and pure cultures of *Aeromonas salmonicida* subsp. *salmonicida* (as identified by the biochemical tests employed by NFHRL) were isolated on Coomassie Brilliant Blue (CBB) agar from tissues of necropsied fish. Identification of suspected *A. salmonicida* isolates based on the AP and PAAS combined PCR tests, and of suspected *A. salmonicida* subsp. *salmonicida* isolates based on the MIY PCR test agreed with the biochemical test results in all cases. PCR of pure cultures was faster than phenotypic screening. Typically PCR tests yielded results in 1 to 2 d, compared to 4 d for classical biochemical testing.

Direct PCR detection of *Aeromonas salmonicida* in overtly infected fish tissues

All dead fish obtained prior to Day 8 post-administration of the SIF assay were PCR positive for all samples collected, without the need for an enrichment culture phase prior to PCR. Of the surviving fish that were euthanised after Day 8, 1 vaccinated Atlantic salmon and 3 rainbow trout were PCR negative for all tissue samples. Overall, at least 1 sample was PAAS and AP PCR positive for 95% of all fish tested (Table 1).

Mucus was the sample most likely, while intestine was least likely, to yield a PCR-positive result (Table 1). No sample that was positive for *Aeromonas salmonicida* by PCR screening was negative for *A. salmonicida* by culture on CBB agar. Presence of *A. salmonicida* subsp. *salmonicida* as determined by PCR correlated with positive results based on the biochemical testing of isolates of mucus and kidney samples taken from the rainbow trout.

Quantification of bacterial flora from covertly infected fish

A variety of bacteria, including *Aeromonas salmonicida*, were isolated from the 100 mucus samples taken from the age 3+ Atlantic salmon kelts sampled nonlethally at RCNSS. These bacteria were subsequently identified using biochemical tests (data not shown). *A. salmonicida* was not isolated among the experimental age 2+ domestic Atlantic salmon held at the RCNSS, nor from the brown trout located at Bennington. The pathogen was isolated from the rainbow trout and the unvaccinated Atlantic salmon located at NFHRL.

The levels of *Aeromonas salmonicida* determined to be present in the samples ranged from 8.3×10^2 to 2×10^6 CFU g^{-1} (Table 2). The number and type of *A. salmonicida* culture-positive samples found in the lethally sampled fish (n = 40) were 8 mucus, 9 gill, 3 kidney, 2 spleen and 2 intestine (Table 2). Six fish were culture positive by gill samples only, and 4 fish were culture positive by mucus samples only. No fish was culture positive based solely on kidney, spleen or intestine samples. As shown previously with overt infections, results from the AP and PAAS combined PCR tests and the MIY PCR test agreed with the biochemical tests currently employed by NFHRL to identify *A. salmonicida* and *A. salmonicida* subsp. *salmonicida* (Table 2).

The PCR assays were tested on a variety of the normal bacterial flora isolated from these samples, including *Pseudomonas diminuta*, *P. pseudoalcaligenes*, *P. fluorescens*, *Aeromonas hydrophila*, *Commomonas tarrigania*, *Shewanella putrefasciens*, *Acinetobacter* sp., *Staphylococcus* sp. and *Moraxella* sp. No false positive reactions occurred, further demonstrating the specificity of the PCR tests for *Aeromonas salmonicida*.

Table 1. Proportion of samples from overtly infected salmonids demonstrated, by direct PCR, to be infected with *Aeromonas salmonicida*

Tissue	Salmonid test group			
	Arctic char	Rainbow trout	Atlantic salmon (vaccinated)	Atlantic salmon (unvaccinated)
Mucus	18/18 (100%)	16/20 (80%)	18/24 (75%)	16/16 (100%)
Gill	6/18 (33%)	12/20 (60%)	17/24 (71%)	16/16 (100%)
Spleen	5/18 (28%)	7/20 (35%)	18/24 (75%)	16/16 (100%)
Kidney	8/18 (44%)	8/20 (40%)	19/24 (79%)	16/16 (100%)
Intestine	2/18 (11%)	7/20 (35%)	17/24 (71%)	16/16 (100%)

Table 2. Comparison of PCR testing of *Aeromonas salmonicida* isolates and samples of covertly infected fish. CFU: colony-forming units; NFHRL: National Fish Health Research Laboratory; RCNSS: Richard Cronin National Salmon Station. +: positive; –: negative; (+): extremely weak positive reaction

Salmonid species	Location	Fish no.	Sample	*A. salmonicida* CFU g^{-1}	PCR of isolates AP	PAAS	MIY	PCR of tissues AP	PAAS	MIY
Atlantic salmon 3+ kelts	RCNSS	18	Mucus	2.0E+03	+	+	+	–	–	–
		23	Mucus	7.7E+03	+	+	+	–	–	–
		56	Mucus	8.3E+02	+	+	+	–	–	–
		63	Mucus	5.0E+03	+	+	+	–	–	–
		66	Mucus	1.1E+03	+	+	+	–	–	–
		78	Mucus	1.0E+03	+	+	+	–	–	–
		97	Mucus	2.5E+03	+	+	+	–	–	–
		100	Mucus	1.4E+04	+	+	+	–	–	–
Rainbow trout	NFHRL	5	Mucus	1.3E+04	+	+	+	–	–	–
			Gill	6.7E+04	+	+	+	–	–	–
			Spleen	1.7E+05	+	+	+	–	–	–
			Kidney	4.0E+05	+	+	+	+	+	–
			Intestine	3.9E+04	+	+	+	–	–	–
		18	Spleen	2.0E+06	+	+	+	+	+	(+)
			Kidney	1.9E+05	+	+	+	–	–	–
			Intestine	1.8E+05	+	+	+	–	–	–
		19	Mucus	1.3E+05	+	+	+	+	+	(+)
			Gill	1.3E+04	+	+	+	–	–	–
		20	Mucus	1.9E+04	+	+	+	–	–	–
Atlantic salmon (unvaccinated)	NFHRL	1	Gill	2.0E+03	+	+	+	–	–	–
		2	Mucus	7.1E+04	+	+	+	–	–	–
		5	Mucus	5.0E+04	+	+	+	–	–	–
			Kidney	6.3E+03	+	+	+	–	–	–
		7	Mucus	2.0E+05	+	+	+	–	+	–
		9	Gill	2.9E+04	+	+	+	–	–	–
		11	Gill	5.0E+04	+	+	+	–	–	–
		15	Gill	2.5E+04	+	+	+	–	–	–
		17	Gill	1.0E+05	+	+	+	+	+	–
		18	Gill	1.0E+04	+	+	+	–	–	–
		19	Mucus	2.9E+05	+	+	+	+	+	–
		20	Mucus	2.3E+04	+	+	+	–	–	–
			Gill	6.0E+04	+	+	+	–	–	–

Direct PCR detection of *Aeromonas salmonicida* from covertly infected fish tissue

All samples taken from the age 2+ Atlantic salmon domestic stock at RCNSS and the brown trout from Bennington were negative for *Aeromonas salmonicida* by culture and by direct PCR screening.

Results from the direct PCR screening of samples taken from fish that were positive for *Aeromonas salmonicida* by culture are given in Table 2. No tissue sample that was positive for *A. salmonicida* by PCR screening was negative for *A. salmonicida* by culture. These results show that culture was more reliable than direct PCR for detection of *A. salmonicida* in covertly infected fish (Table 3).

Table 3. Comparison of cultural isolation of *Aeromonas salmonicida* vs direct PCR detection of *A. salmonicida* in tissues from covertly infected salmonids

Source of covertly infected tissue	Positive by culture (%)	Positive by direct PCR (%)
Atlantic salmon, 3+ kelts, RCNSS (n = 100)	8	0
Rainbow trout, NFHRL (n = 20)	20	15
Atlantic salmon (unvaccinated), NFHRL (n = 20)	55	15

DISCUSSION

The SIF assay was used to generate overt infection in rainbow trout and Atlantic salmon held at NFHRL, thereby confirming that these populations were covertly infected. In addition, bath challenge with *Aeromonas salmonicida* generated overt, albeit experimental, infection in Arctic char. Bacteria isolated from these overtly infected fish populations were successfully identified as *A. salmonicida* using the AP and PAAS PCRs, and as *A. salmonicida* subsp. *salmonicida* using the MIY PCR. All PCR results were in agreement with the biochemical tests currently employed by NFHRL to identify *A. salmonicida* and *A. salmonicida* subsp. *salmonicida*.

The AP and PAAS PCRs were also used to detect *Aeromonas salmonicida* in tissue and mucus samples taken from the overtly diseased fish. Cipriano et al. (1992, 1994b, 1996a,c,d) have previously reported levels of *A. salmonicida* in overtly infected fish, including Atlantic salmon, ranging from 10^3 to 10^7 CFU g^{-1}, and even as high as 10^9 CFU g^{-1}, in both kidney and mucus samples. Given that the detection limits of the PCRs cover at least part of this range (Byers et al. 2000), it was not surprising to find that direct PCR detection of overtly infected tissues was possible in many instances. The fact that mucus yielded PCR products more often than tissues, especially intestine, may indicate that the pathogen occurs in higher numbers in some locations than in others or that some types of infected tissues may inhibit the PCR more than others.

Aeromonas salmonicida was detected by culture in covertly infected salmonid populations, e.g. the Atlantic salmon kelts at RCNSS, and rainbow trout and Atlantic salmon populations held at NFHRL. The pathogen load ranged between 8.3×10^2 and 2×10^6 CFU g^{-1} sample. These levels are higher than those reported by Nomura et al. (1993) of 10^2 CFU g^{-1} in pink salmon *Oncorhynchus gorbuscha* and 10^1 CFU g^{-1} kidney in chum salmon *O. keta*, but are within the range reported by other researchers, as cited by Hiney et al. (1997).

Aeromonas salmonicida was cultured predominantly from the gill and mucus samples of covertly infected fish. Only 3 of the 15 culture-positive, lethally sampled salmonids (n = 40) had systemic infections, i.e. kidney, spleen or both were culture positive. However, 2 of these systemically infected fish were not culture positive using either gill or mucus samples, demonstrating the importance of sampling more than 1 host site when attempting to detect *A. salmonicida* from covertly infected fish (Cipriano et al. 1996b, Bernoth 1997). The predominance of the external surfaces of the fish as the site of pathogen carriage is also consistent with the findings of Cipriano et al. (1994a, 1996a,b). The PCR assays correctly determined the identity of the *A. salmonicida* isolates in a shorter time than that required by conventional biochemical testing, again demonstrating their usefulness with regard to confirming the identity of colonies presumptively identified as *A. salmonicida* by characteristic growth on CBB agar.

PCR tests detected *Aeromonas salmonicida* in the covertly infected rainbow trout and Atlantic salmon populations held at NFHRL, but culture methods were more reliable. The PCR assays completely failed to yield positive results for the age 2+ Atlantic salmon held at RCNSS, which was most likely due to pathogen concentrations below the detection limits of these tests. Our results indicated that, for covertly infected tissues, the level of detection for the AP and PAAS PCRs was approximately 4×10^5 CFU g^{-1} sample. Although there were instances where the PCRs detected lower numbers than this, detection at lower levels was inconsistent. In no instance was a sample PCR positive but culture negative, and thus false positive results with these PCR assays were not encountered during this survey. In addition, no particular tissue sample site was consistently PCR negative, suggesting that the main constraint on direct detection was the lower detection limit (LDL) of the respective PCR assays. It is interesting to note that the LDL of the PCRs does not equate to those levels determined previously by the seeded tissue studies (Byers et al. 2000). This observation highlights the danger of inferring the performance of an assay from laboratory studies alone.

The failure to detect *Aeromonas salmonicida* by either culture or PCR in the age 2+ experimental Atlantic salmon held at RCNSS or the brown trout from Bennington could be due to a number of factors. Firstly, as the LDL of culture is estimated to be around 10^2 CFU g^{-1} sample (Cipriano 1997), the occurrence of the target pathogen at levels lower than this would produce false negative results. Secondly, only single-point inspections were performed on these populations, and it has been noted that such inspections can produce erroneous results (Cipriano et al. 1994a), possibly as a result of the proposed transient nature of covert infections (Scallan et al. 1993). It has also been found that sampling at multiple points over time can provide greater accuracy in the cultural detection of *A. salmonicida* in covertly infected salmonids (Cipriano et al. 1997). Unfortunately, it was not possible to incorporate such a sampling regimen in this study. These issues may have been resolved by use of the SIF assay, which has been reported to be more reliable than culture and to provide the most definitive single-point assay for the detection of covert *A. salmonicida* infections (Cipriano et al. 1997). However, it was not possible to conduct the SIF assay on these salmonid populations because of the lack of appropriate facilities at RCNSS. Thirdly, the prevalence of the pathogen may have been at such low levels that a larger sample size was required.

Hiney & Smith (1998) in their discussion on the validation of proxy methods state that the 'major role [of validation] is in providing rational grounds for rejecting techniques whose application to environmentally derived matrices has been shown to be invalid.' Since the primary aim of this study was to use PCR to establish the presence or absence of *Aeromonas salmonicida*, as opposed to predicting the consequences of the presence of the pathogen, it was pertinent to use the principle of comparative validation (Hiney & Smith 1998), i.e. the performance of the PCRs was compared to previously validated, standard techniques.

This study demonstrated that PCR technology can be used to replace the biochemical testing currently used to confirm the identity of *Aeromonas salmonicida* isolates cultured from both overtly and covertly infected salmonids. The AP and PAAS PCR assays were also capable of direct detection of *A. salmonicida* in overtly infected fish, with mucus, gill and kidney samples most likely to yield a positive result. Culture, however, was a more reliable and sensitive method for the detection of *A. salmonicida* in covertly infected salmonids than direct PCR testing of tissue samples. It is highly recommended, therefore, that any target pathogen that may be present in the tissues of covertly infected fish be first concentrated by a culture step, as used in this study, prior to PCR. Still, it is important to note that culture alone did not detect *A. salmonicida* in all salmonid populations either, but the development of a medium selective for *A. salmonicida*, and the use of multiple-point sampling regimens, could possibly alleviate some of the problems associated with the detection of this pathogen among covertly infected fish.

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