Detection of *Aeromonas salmonicida* by Reverse Transcription-Multiplex Polymerase Chain Reaction

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*Aeromonas salmonicida* is one of the major fish pathogens causing economically devastating losses in aquaculture. *A. salmonicida* subsp. *salmonicida* is a typical *A. salmonicida* causing furunculosis, while the other subspecies are atypical strains causing ulcer diseases. PCR-based methods of detecting *A. salmonicida* suffer from the drawback that they do not distinguish living (pathogenic) from dead cells. In this study, a method of detecting *A. salmonicida* was developed based on reverse transcription-multiplex PCR (RT-MPCR) using two sets of primers, SV1/SV2 and SF1/SF2, specific to the *vapA* gene and the *fstB* gene of *A. salmonicida* respectively. This method was found to detect *A. salmonicida* specifically with detection limits of 10 CFU in pure culture and 30 CFU in the presence of tissue debris. It was also found distinguish not only between viable and nonviable cells but also between typical and atypical strains of *A. salmonicida*. Using RT-MPCR, two DNA fragments, of 542 and 1,258 bp, were amplified from RNA of typical *A. salmonicida*, whereas only one DNA fragment, of 542 bp, was amplified from the RNA of the atypical ones. The proposed assay was also used successfully to detect *A. salmonicida* in artificially infected rainbow trout (*Oncorhyncus mykiss*).

**Key words:** *Aeromonas salmonicida*; multiplex PCR; reverse transcription-multiplex PCR

The genus *Aeromonas* are Gram negative, non spore forming, oxidase positive, rod shaped, facultatively anaerobic bacteria that occur ubiquitously and autochthonously in aquatic environments. They have been found in brackish, fresh, estuarine, marine, chlorinated, and unchlorinated water supplies worldwide.¹ Some of them have been implicated as human pathogens causing gastroenteritis, soft-tissue and wound infections, pneumonia, and bacteremia²-³ while others cause a broad range of infections in cold- and warm-blooded animals.¹-⁶ The ubiquity of *Aeromonas* species in aquatic environments provides ample opportunity for animals, particularly fish, to come into contact with and to ingest the organisms. Such contact may lead to infection which, depending on the species and the virulence of the strains encountered, may have life-threatening consequences.

The taxonomy of the genus *Aeromonas* has undergone continual change due to the addition of newly described species and the reclassification of existing taxa. Historically, *Aeromonas* has been placed in the family Vibrionaceae, but later it was proposed that it be placed in its own family, the Aeromonadaceae.⁷ Before the year 2000, the genus consists of 15 species, including *A. hydrophila*, *A. caviae*, *A. veronii* biotype *sobria*, *A. veronii* biotype *veronii*, *A. jandaei*, *A. schubertii*, *A. salmonicida*, *A. sobria*, *A. media*, *A. eucrenophila*, *A. trota*, *A. allosaccharophila*, *A. encheleia*, *A. bestiarum*, and *A. popoffii*.⁸ Recently, three new species were described: *A. culicicola*,⁹ *A. simiae*,¹⁰ and *A. molluscorum*.¹¹

*A. salmonicida* is a major fish pathogen that causes economically devastating losses in aquaculture. *A. salmonicida* subsp. *salmonicida* comprises a homogeneous group and is referred to as a typical *A. salmonicida*. This subspecies is the causative agent of furunculosis, a disease restricted mostly to salmonid fish. It involves hemorrhagic septicemia, including the presence of furancles (boils) on the flanks. Other subspecies of *A. salmonicida*, *achromogenes*, *masoucida*, *smithia*, and *pectinolytica*,¹²-¹⁵ along with an increasing number of isolates reported for various fish species and geographical areas that are not included in any of the described subspecies of *A. salmonicida*, are referred to as atypical strains. These are heterogeneous in terms of molecular and phenotypic characteristics. They cause various ulcer diseases and atypical furunculosis in various fish species, including goldfish, eel, marine flat fish, and salmonids.¹⁻⁶

The traditional methods for the detection of *A. salmonicida*, which include culture-based techniques and phenotypic characterization based on morphological and metabolic characteristics, have many disadvantages. They are time-consuming and laborious due to the lack of an effective selective media for *A. salmonicida* and its slow growth characteristics. *A. salmonicida* generally take more than 48 h to appear on trypticase soy agar or brain heart infusion (BHI) agar. The traditional methods are considered to be insensitive due to difficulty in the recovery of *A. salmonicida* from fish that do not show clinical signs of disease, and from the aquatic environment. Although pre-incubation of pathogenic material for 48 h in trypticase soy broth can improve recovery of the pathogen,¹⁰ the process is prolonged. Moreover, the existence of a viable but not culturable state and the high incidence of covert infection by *A. salmonicida* makes these methods incapable of detecting the pathogen. To overcome the drawbacks of the traditional methods,

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Abbreviations: MPCR, multiplex polymerase chain reaction; RT-PCR, reverse transcription-polymerase chain reaction; RT-MPCR, reverse transcription-multiplex polymerase chain reaction; BHI, brain heart infusion
culture-independent molecular methods have been developed. Many DNA probes and PCR primers have been designed for rapid and specific detection of A. salmonicida in pure culture and in fish tissue. 17-19 Most of these methods are based on the use of 16S rDNA and the vapA gene, a gene encoding a subunit protein of the A-layer, 20 as target genes but these molecular methods suffer from the disadvantage that they do not distinguish living and hence pathogenic cells from dead cells. Therefore, a molecular method with discriminative power as between living and dead cells remains to be described.

Here, we propose a molecular method of detecting of A. salmonicida based on reverse transcription-multiplex PCR (RT-MPCR) using primers specific to the vapA and fstB genes. It is capable of distinguishing not only between living and dead cells but also between typical and atypical strains of A. salmonicida. In this study, the method was validated on A. salmonicida pure cultures and on the cultures present in tissue debris. It was also used to detect the bacteria in artificially infected fish.

Material and Methods

Bacterial strains and culture conditions. The bacterial strains and growth conditions used in this study are listed in Table 1. They include both Gram positive and Gram negative bacteria. All bacterial stock cultures were stored as frozen cultures in appropriate liquid media containing 20% glycerol (vol/vol) at −80 °C.

Bacterial cultures for total RNA and genomic DNA extraction were prepared as follows. A single colony of each bacterial strain was inoculated into 5 mL of an appropriate liquid medium and grown under the growth conditions used in this study. They include

Reverse transcription-PCR (RT-PCR), reverse transcription-multplex PCR (RT-MPCR), and multiplex PCR (MPCR) conditions. RT-PCR and RT-MPCR amplifications of total RNA were carried out in a Thermocycler 480 (Perkin-Elmer, Waltham, MA) using the SuperScript™ III One-Step RT-PCR System with Platinum® Taq DNA polymerase (Invitrogen, Carlsbad, CA). RT-PCR and RT-MPCR were performed in 50μL volumes containing 5μL of RNA, 25μL of 2x reaction mix (a buffer containing 0.4 mM of each dNTP, and 3.2 mM MgSO4), 0.5μM of each primer, and 2 μL of SuperScript™ III RT/Platinum Taq™ Mix. The thermal cycle was programmed so that reverse transcription was followed immediately by PCR or MPCR amplification. Reverse transcription experiments were done at 60 °C for 35 min. PCR and MPCR experiments were done under the following conditions: denaturation at 94 °C for 2 min, followed by 40 cycles at 94 °C for 30s, 60 °C for 30s, and 72 °C for 1 min, and a final cycle at 72 °C for 5 min.

Table 1. Bacteria and Growth Conditions Used in This Study

<table>
<thead>
<tr>
<th>Bacterial strains</th>
<th>Growth conditions</th>
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<tbody>
<tr>
<td>Aeromonas allosaccharophila ATCC 51208</td>
<td>BHI agar and broth, 26 °C</td>
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<tr>
<td>Aeromonas bestiarum ATCC 51108</td>
<td>BHI agar and broth, 30 °C</td>
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<tr>
<td>Aeromonas caviae ATCC 15468</td>
<td>BHI agar and broth, 30 °C</td>
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<tr>
<td>Aeromonas encheleia ATCC 51929</td>
<td>BHI agar and broth, 26 °C</td>
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<td>Aeromonas enteropelogenes ATCC 49803</td>
<td>BHI agar and broth, 30 °C</td>
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<td>Aeromonas eucnophila ATCC 23309</td>
<td>BHI agar and broth, 30 °C</td>
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<tr>
<td>Aeromonas hydrophila ATCC 7966</td>
<td>BHI agar and broth, 30 °C</td>
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<tr>
<td>Aeromonas ichthioamia ATCC 49904</td>
<td>BHI agar and broth, 30 °C</td>
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<tr>
<td>Aeromonas media ATCC 33907</td>
<td>BHI agar and broth, 22 °C</td>
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<tr>
<td>Aeromonas popoffii ATCC BAA-243</td>
<td>BHI agar and broth, 30 °C</td>
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<tr>
<td>Aeromonas salmonicida subsp. achromogenes ATCC 10801</td>
<td>BHI agar and broth, 26 °C</td>
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<tr>
<td>Aeromonas salmonicida subsp. masoucida ATCC 27013</td>
<td>BHI agar and broth, 26 °C</td>
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<td>Aeromonas salmonicida subsp. salmonicida ATCC 14174</td>
<td>BHI agar and broth, 26 °C</td>
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<tr>
<td>Aeromonas salmonicida subsp. smithia ATCC 49395</td>
<td>BHI agar and broth, 26 °C</td>
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<tr>
<td>Aeromonas schuberti ATCC 43700</td>
<td>BHI agar and broth, 30 °C</td>
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<td>Aeromonas sobria ATCC 43979</td>
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<td>Aeromonas trota ATCC 49657</td>
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<td>Lactococcus garvieae ATCC 49156</td>
<td>BHI agar and broth, 37 °C</td>
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<td>Listonella anguillarum ATCC 43310</td>
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<td>Photobacterium damselae subsp. piscicida ATCC 17911</td>
<td>Marine agar and broth, 26 °C</td>
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<td>Pseudomonas anguilliseptica ATCC 33660</td>
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<tr>
<td>Streptococcus iniae ATCC 29177</td>
<td>BHI agar and broth, 37 °C</td>
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<tr>
<td>Streptococcus phoceae ATCC 51973</td>
<td>BHI agar and broth, 37 °C</td>
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<tr>
<td>Tenacibaculum maritimum ATCC 43397</td>
<td>Flexibacter medium, 26 °C</td>
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<tr>
<td>Vibrio azidovu ATCC 33909</td>
<td>Marine agar and broth, 26 °C</td>
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<tr>
<td>Vibrio salmonicida ATCC 43839</td>
<td>Marine agar and broth, 15 °C</td>
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For MPCR amplification, MPCR reaction mixtures of 50μL contained 25μL of 2× PCR Master Mix (Promega, San Luis Obispo, CA), 5μL of DNA, and 0.5μL of each primer. MPCR amplifications were carried out in a Thermocycler 480 under conditions of 1 cycle of 94°C for 4 min, followed by 30 cycles of 94°C for 45 s, 60°C for 1 min, and 72°C for 3 min, and a cycle of 72°C for 10 min.

The RT-PCR, RT-MPCR, and MPCR products were electrophoresed through 1.5% agarose gels in Tris acetate EDTA buffer, and the gels were then stained with 0.5μg/mL of ethidium bromide solution. The molecular sizes of the products were estimated by comparison with pgEM the DNA marker (Promega, San Luis Obispo, CA).

Sensitivity of RT-MPCR. The sensitivity of RT-MPCR was evaluated using pure cultures of A. salmonicida subsp. aachromogenes ATCC 10801, A. salmonicida subsp. masoucida ATCC 27013, A. salmonicida subsp. salmonicida ATCC 49385, and A. salmonicida subsp. smithia ATCC 49395. Twenty mL of appropriate liquid media were inoculated with 2mL of overnight bacterial cultures and incubated at 26°C until the cultures reached log phase. Each of the cultures was diluted in the liquid medium to obtain samples with cell numbers of 1, 10, 20, 30, and 40 CFU/mL. The RNA extracted from the various samples was used as template in RT-MPCR amplification.

The sensitivity of RT-MPCR was also evaluated in the presence of tissue debris. Kidneys were removed aseptically from rainbow trout (Oncorhyncus mykiss), homogenized with Tris EDTA buffer (1mT Triss-HCl, 0.5mM EDTA, pH 8.0) (10% wt/vol), autoclaved, and stored at −20°C. One hundred-μL aliquots of the kidney homogenates were seeded with serial dilutions of each of the various pure bacterial cultures to obtain samples with cell numbers of 100, 200, 300, 400, and 500CFU/mL. The RNA extracted was used as template in RT-MPCR amplification. The experiments were performed in triplicate for each bacterial strain to confirm the results.

Ability of RT-MPCR to discriminate between viable and nonviable cells. RT-MPCR was evaluated for its ability to discriminate between viable and nonviable cells of A. salmonicida subsp. aachromogenes ATCC 10801, A. salmonicida subsp. masoucida ATCC 27013, A. salmonicida subsp. salmonicida ATCC 49385, and A. salmonicida subsp. smithia ATCC 49395. Twenty mL of BHI broth was inoculated with 2mL of overnight bacterial cultures, and this was incubated at 26°C until the cultures reached log phase. Two-μL aliquots of each bacterial strain were subjected to RT-MPCR and MPCR, respectively.

To confirm the results, the experiments were performed in triplicate for each bacterial strain.

Fish preparation. Rainbow trout (Oncorhyncus mykiss) were purchased from a farm in Ubon Ratchathani Province, Thailand. They were maintained in plastic tanks (150 L) for 2 weeks to acclimate them to laboratory conditions prior to the experiments. Throughout the acclimation period and subsequent experiments, the fish were held at 15°C under a 12-h light/12-h dark photoperiod and fed 5% body weight twice a day with a commercial fish diet (S.W.T. Co., Bangkok, Thailand). Before the experiments, the fish were determined to be free of external parasites by procedures suggested by the American Fisheries Society Fish Health Section.22 All experiments were conducted in 50-L aquaria. Fish weighing 10±2 g were stocked in the aquaria (10 fish per aquarium) 24 h prior to the experiments.

Determination of median lethal dose (LD₅₀). Groups of 10 fish at an average weight of 10g were used to determine the LD₅₀ of A. salmonicida for rainbow trout. Each group of fish was kept in a 50-L aquarium at 15°C throughout the experiments. Ten-fold serial dilutions were prepared from a bacterial culture at a concentration of 10⁷ CFU/mL. One hundred μL of each dilution was injected intra-peritoneally into each fish. As control, the same volume of physiological saline was used instead of the bacterial suspension. Each dilution trial was performed in five replicates. Mortality was recorded daily for 2 weeks. Dead fish were removed from the aquaria daily. Their livers and kidneys were aseptically streaked on appropriate agar plates. After incubation at 26°C for 24 h, the colonies grown on the agar were confirmed to be A. salmonicida with an API 20NE Rapid Identification System Test Kit.

The median lethal dose (LD₅₀) was calculated by the method of Reed-Muench as follows:23

\[
\log LD_{50} = a + \log b + c
\]

where \(a\) = (mortality higher than 50% − 50%)/(mortality higher than 50% − mortality lower than 50%); \(b\) = dilution rate in this experiment; \(c\) = the log of the minimum dilution rate, when the mortality higher than 50%.

Identification of A. salmonicida in artificially infected fish. In this experiment, A. salmonicida subsp. aachromogenes ATCC 10801, A. salmonicida subsp. masoucida ATCC 27013, A. salmonicida subsp. salmonicida ATCC 49385, and A. salmonicida subsp. smithia ATCC 49395 were used to infect fish separately. For each bacterial strain, three groups of 10 rainbow trout each were maintained in 50-L aquaria at a temperature of 15°C. Each trout was intraperitoneally injected with 100μL of bacterial suspension at a dose causing 50% mortality (LD₅₀). As control, fish were inoculated with 0.1μL of physiological saline instead of the bacterial suspension. Dead fish were removed from the experimental aquaria, and the kidneys were removed aseptically and homogenized with Tris EDTA buffer (1mT Triss-HCl, 0.5mM EDTA, pH 8.0) (10% wt/vol). Samples were subjected to total RNA extraction and subsequently to RT-MPCR. In parallel, a loopful of the fish kidney was streaked onto BHI agar and incubated at 26°C. The fish used as controls were maintained and processed in the same way as the infected ones.

Results

Evaluation of specificity and functionality of primers

Each primer pair was initially evaluated separately for specificity and functionality by RT-PCR using RNA extracted from all of the bacteria listed in Table 1. The primer pairs used in the experiments were SV1/SV2 and SF1/SF2. Using primer pair SV1/SV2, RT-PCR products of 542 bp were obtained from A. salmonicida subsp. aachromogenes ATCC 10801, A. salmonicida subsp. masoucida ATCC 27013, A. salmonicida subsp. salmonicida ATCC 49385, and A. salmonicida subsp. smithia ATCC 49395 (Fig. 1a). No RT-PCR amplification was detected in the other bacteria. The identity of the RT-PCR products was confirmed by nucleotide sequencing, which gave the expected results (data not shown). Using primer pair SF1/SF2, only RT-PCR amplification of the RNA extracted from A. salmonicida subsp. salmonicida ATCC 49385 was positive. It gave a product of 1,258 bp (Fig. 1b). Sequencing of the RT-PCR product confirmed that the product was amplified

Fig. 1. Agarose Gel Electrophoresis of Products from RT-PCR Using Primer Pairs SV1/SV2 (a) and SF1/SF2 (b).

Lane 1, marker; lane 2, A. salmonicida subsp. aachromogenes; lane 3, A. salmonicida subsp. masoucida; lane 4, A. salmonicida subsp. salmonicida; lane 5, A. salmonicida subsp. smithia; lane 6, L. garvicae.
from the gene of interest (data not shown). No RT-PCR product was obtained from the other bacteria.

To confirm that the negative results of the RT-PCR amplifications were not due to the quality of the RNA, RNA extracted from the bacteria that gave no RT-PCR product was subjected to RT-PCR using universal primers specific to 16S rDNA (primer FD1: 5'-AGAGTTTGATCCTGGCTCAG-3', and primer RP2: 5'-ACG GCC TACCTTGTTACGACTT-3'). Each of the tested strains gave an RT-PCR product of expected size and sequence (data not shown).

To develop an RT-MPCR assay, all primers were further evaluated for functionality and specificity. For each RNA template, RT-MPCR was performed using all four primers, SV1, SV2, SF1, and SF2. The results of the agarose gel electrophoresis analysis of the RT-MPCR products were in agreement with those obtained from the experiments using the primer pairs separately. RT-MPCR of the RNA extracted from *A. salmonicida* subsp. *salmonicida* ATCC 49385 produced two DNA fragments, of 1,258 bp and 542 bp, while that of the RNA extracted from *A. salmonicida* subsp. *achromogenes* ATCC 10801, *A. salmonicida* subsp. *masoucida* ATCC 27013, and *A. salmonicida* subsp. *smithia* ATCC 49395 produced a DNA product of 542 bp (Fig. 2). No RT-MPCR product was found for the other bacteria tested. These results indicate that all the primers maintain their functionality and specificity in the RT-MPCR and that they can be used to detect of *A. salmonicida*.

**Sensitivity of RT-MPCR**

The sensitivity of RT-MPCR was evaluated using *A. salmonicida* subsp. *achromogenes* ATCC 10801, *A. salmonicida* subsp. *masoucida* ATCC 27013, *A. salmonicida* subsp. *salmonicida* ATCC 49385, and *A. salmonicida* subsp. *smithia* ATCC 49395, both in pure culture and in the presence of tissue debris. In the experiments on pure cultures, RT-MPCR was performed using RNA isolated from pure cultures of the tested bacteria at various cell concentrations. In all the tested bacteria, the RNA isolated from cultures at cell concentrations of 10 CFU/mL or more gave the expected results on RT-MPCR (Fig. 5a) and MPCR (Fig. 5b) respectively, the products were observed as expected. For *A. salmonicida* subsp. *salmonicida* ATCC 49385, two DNA bands were observed, of 524 bp and 1,258 bp, while for *A. salmonicida* subsp. *achromogenes* ATCC 10801, *A. salmonicida* subsp. *masoucida* ATCC 27013, and *A. salmonicida* subsp. *smithia* ATCC 49395, only one DNA band was observed, of about 542 bp. Furthermore, the same results were reached by MPCR following heat treatment of the cells (Fig. 5d). Enumeration of the culture on BHI agar plates revealed that heating at 100°C for 15 min rendered the cell culture nonviable, indicating that the bacterial DNA was stable and capable of being amplified following loss of viability. In contrast, the RNA from the heat-killed cells could not be amplified by RT-MPCR due to rapid degradation of the RNA following the loss of cell viability (Fig. 5c). Thus the detection of RNA by RT-MPCR should provide a more sensitive indicator of cell viability than detection of gene sequences by MPCR.

**Ability of RT-MPCR to discriminate between viable and nonviable cells**

The ability of RT-MPCR to distinguish between viable and nonviable *A. salmonicida* cells was examined by performing RT-MPCR as well as MPCR with RNA and DNA isolated from both living and heat-killed cells. When the RNA and DNA of *A. salmonicida* were extracted from live cells and amplified by RT-MPCR (Fig. 5a) and MPCR (Fig. 5b) respectively, the products were observed as expected. For *A. salmonicida* subsp. *salmonicida* ATCC 49385, two DNA bands were observed, of 524 bp and 1,258 bp, while for *A. salmonicida* subsp. *achromogenes* ATCC 10801, *A. salmonicida* subsp. *masoucida* ATCC 27013, and *A. salmonicida* subsp. *smithia* ATCC 49395, only one DNA band was observed, of about 542 bp. Furthermore, the same results were reached by MPCR following heat treatment of the cells (Fig. 5d). Enumeration of the culture on BHI agar plates revealed that heating at 100°C for 15 min rendered the cell culture nonviable, indicating that the bacterial DNA was stable and capable of being amplified following loss of viability. In contrast, the RNA from the heat-killed cells could not be amplified by RT-MPCR due to rapid degradation of the RNA following the loss of cell viability (Fig. 5c). Thus the detection of RNA by RT-MPCR should provide a more sensitive indicator of cell viability than detection of gene sequences by MPCR.
Detection of Aeromonas salmonicida

Several DNA-based PCR methods have been developed to detect *A. salmonicida*. The primers used in these methods are specific to the *vapA* gene,\(^{17}\) a cryptic 423-bp DNA fragment,\(^{18}\) and 16S rDNA.\(^{24}\) Although they are rapid, sensitive and specific, they have several drawbacks. They do not distinguish between viable and nonviable cells. Hence they tend to produce false positive results through the amplification of target DNA from nonviable bacteria. Furthermore, they do not distinguish between typical and atypical strains of *A. salmonicida*. To overcome these drawbacks, an RT-MPCR assay was developed to detect viable cells of *A. salmonicida* and to discriminate between typical and atypical strains. It is based on the detection of mRNA transcribed from the *vapA* gene and the *fstB* gene of *A. salmonicida*. Since the products of the *vapA* gene and the *fstB* gene are considered to be virulent factors for *A. salmonicida* produced only in virulent cells,\(^{17,21}\) it provides information on the potential virulence of the bacteria.

**Discussion**

Bacterial mRNA has a very short half life, usually measured in minutes,\(^{25}\) and detection of it decreases comparatively quickly with loss of bacterial cell viability.\(^{26}\) Processes that render cells nonviable and thus disrupt cellular transcription result in rapid losses of cellular mRNA. Therefore, an assay system based on the detection of mRNA should provide a sensitive indicator of cell viability as compared with methods that rely on the amplification of DNA and rRNA, which is extremely stable.\(^{27}\) Recent investigations have described the potential use of mRNA detection by RT-PCR methods to identify viable bacterial cells of human pathogens such as *Listeria monocytogenes*\(^{28}\) and *Vibrio cholerae*\(^{29}\) in complex environments such as food. RT-PCR was also used to detect viable cells of a fish pathogen, *Renibacterium salmoninarum*, in Atlantic salmon (*Salmo salar* L.).\(^{30}\) Several further reports on *Legionella pneumo-
With its specificity and sensitivity, our method detected also between typical and atypical strains of bacteria. This primed the amplification of a 542-bp DNA fragment of the \textit{vapA} gene from both typical and atypical strains of \textit{A. salmonicida}. The other set of primers, consisting of SF1 and SF2, was specific to the \textit{fisB} gene of the \textit{A. salmonicida} subspecies \textit{salmonicida}. It codes for ferric siderophore receptor B, a protein involved in the regulation of iron uptake. Using these primers, a 1,258-bp DNA fragment of the \textit{fisB} gene from the \textit{A. salmonicida} subspecies \textit{salmonicida} alone was amplified. Thus these are subspecies \textit{salmonicida}-specific primers. \textit{A. salmonicida} can grow under conditions of iron-restriction, as evidenced in \textit{vitro} by multiplication in the presence of iron chelators, but typical and atypical strains of \textit{A. salmonicida} regulate iron uptake by different mechanisms. Among the typical strains of \textit{A. salmonicida}, the mechanism is siderophore-dependent. Hence the presence of siderophore receptors and major iron-regulated outer membrane proteins is consistent and homogeneous within \textit{A. salmonicida} subs. \textit{salmonicida}.\textsuperscript{32} On the other hand, among atypical strains of \textit{A. salmonicida}, the regulation of iron uptake is siderophore-independent. For this reason, siderophore receptor proteins were not found in this group of \textit{A. salmonicida}.

In using the RT-MPCR assay to detect \textit{A. salmonicida} in pure culture, a detection limit of 10 CFU was observed. This is comparable to the detection limit determined by Gustafson \textit{et al.}\textsuperscript{17} by PCR using primers specific to the \textit{vapA} gene to detect \textit{A. salmonicida}. However, the detection limit of 30 CFU was determined using the assay to detect \textit{A. salmonicida} in the presence of tissue debris. Although the sensitivity of the assay in the detection of \textit{A. salmonicida} in a complex substrate was lower, it was still used successfully to detect bacteria in artificially infected fish without an enrichment step. This accomplishment is not surprising because the number of the pathogenic bacteria in the infected fish was expected to be far beyond the detection limit of the assay according to the report of Rose \textit{et al.}\textsuperscript{33}

They found that Atlantic salmon kidney and intestine contained $1.2 \times 10^6 \pm 1.0 \times 10^5$ and $1.2 \times 10^4 \pm 1.7 \times 10^3$ CFU of \textit{A. salmonicida} per mL of antigen extraction buffer respectively, as determined by enzyme-linked immunosorbent assay (ELISA). Given the sensitivity of our RT-MPCR assay, we believe that it can be used to detect \textit{A. salmonicida} in carrier fish and in water taken from a tank containing infected fish. Experiments are ongoing in our laboratory to confirm this.

In conclusion, this is the first report of the successful use of RT-MPCR with two sets of primers, SV1/AV2 and SF1 and SF2, to detect \textit{A. salmonicida}. This method makes it possible to avoid the false positive results of PCR-based detection methods. It was found to distinguish not only between viable and nonviable cells but also between typical and atypical strains of bacteria. With its specificity and sensitivity, our method detected \textit{A. salmonicida} in pure cultures, in samples mixed with tissue debris, and in artificially infected fish. Experiments are underway on the use of this method to detect \textit{A. salmonicida} in carrier fish and in tank water surrounding infected fish.

References


Mycobacterium leprae,\textsuperscript{31} and \textit{Escherichia coli}\textsuperscript{32} can be found to support these statements.