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Development of sensitive, high-throughput one-tube RT-PCR-enzyme hybridisation assay to detect selected bacterial fish pathogens

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ABSTRACT: Bacterial monitoring and surveillance is critical for the early detection of pathogens to avoid the spread of disease. To facilitate this, an efficient, high-performance and high-throughput method to detect the presence of femotgram amounts of ribosomal RNA from 4 bacterial fish pathogens: *Aeromonas salmonicida; Tenacibaculum maritimum* (formerly *Flexibacter maritimus*); *Lactococcus garvieae;* and *Yersinia ruckeri* was developed. The system uses NucleoLink™ strips for liquid- and solid-phase PCR in 1 tube, to perform RT-PCR-enzyme hybridisation assays (RT-PCR-EHA) detecting 4 fg or less of rRNA from pure cultures and between 1 and 9 CFU per 200 µl sample volume from selective-enrichment culture media. The liquid-phase amplicons were visualised by gel electrophoresis and the solid-phase amplicons detected using internal probes and visualised using colorimetric detection and *p*-nitrophenylphosphate.

KEY WORDS: RT-PCR-EHA · RT-PCR-ELISA · NucleoLink · High-throughput · Hybridisation

INTRODUCTION

Asymptomatic carriage of bacterial pathogens poses a serious risk for the spread of disease in fish populations. With covert infection, fishes show no signs of disease although the bacterium is present within the host. It is only when the fishes are stressed that the disease becomes evident. Under aquaculture conditions the risk of stress is increased and a significant proportion of the stock may become infected. Consequently, the detection of asymptomatic carriers is an essential strategy for effective disease control (Bullock & Stuckey 1975).

Statistically relevant disease surveillance and monitoring requires testing large numbers of fish. A system that accommodates high numbers of samples needs to be streamlined, high-throughput and involve minimal handling of samples at every step. Therefore, to detect asymptomatic carriage of bacterial pathogens in fish, a cost-effective, high-throughput, sensitive and specific system is required for surveillance and monitoring purposes.

Reverse transcriptase polymerase chain reaction (RT-PCR) is a well-developed technique used to detect specific RNA sequences and determine levels of gene expression (Koo & Jaykus 2000). The sensitivity and specificity achieved in a well-designed RT-PCR make it an ideal tool for use in the surveillance and monitoring of covert infections. The high sensitivity of RT-PCR makes the detection of very low numbers of bacteria possible and, due to the short half-life of RNA compared with DNA, RT-PCR gives a more accurate approximation of live bacterial carriage than regular PCR. However, restrictions due to sample size, the presence of non-target nucleic acids, PCR inhibitors and the logistics of high-throughput sampling could limit its usefulness.

The restrictions posed by sample size and non-target nucleic acids can be addressed by coupling PCR with selective-enrichment culture (Thisted Lambertz et al.

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1996). Selective-enrichment increases the amount of target bacterium and helps reduce the number of competing microflora (Fitter et al. 1992, Swaminathan & Feng 1994, Witham et al. 1996). The impact of PCR inhibitors such as selective components and fish tissues can be minimised by using a high-throughput and high-quality nucleic acid extraction system (Wilson & Carson 2001). When performing RT-PCR, handling RNA pre-PCR can be minimised by using a 1-step RT-PCR protocol. This saves time, reduces costs and decreases the chance of false-positive reactions arising from excessive handling and pipetting (Limbach et al. 1999). Large numbers of samples are most easily processed in a 96-well system. While most thermal cyclers accommodate a 96-well format, visualisation of high-throughput RT-PCR amplicons by gel electrophoresis is cumbersome and laborious. Also, the diagnostic result produced from gel electrophoresis relies wholly on the specificity of the primers and the stringency of the PCR reaction conditions. For disease diagnosis, an increased level of confidence is desired and can be achieved with a specific internal probe that verifies the amplicon sequence.

The most convenient high-throughput DNA hybridisation technique uses 96-well micro-well ELISA-like technology. These methods can be significantly more sensitive than Southern blotting, and processing time is greatly decreased through shorter hybridisation times and easier high-throughput sample-processing. Current RT-PCR-ELISA methods typically suffer from excessive handling, as RT-PCR amplicons are manually transferred from the PCR to the ELISA tray (Barlic-Maganja & Grom 2001, Liolios et al. 2001, Rey et al. 2001), thereby increasing cost, time and, importantly, the risk of cross-contamination. A protocol for the amplification and verification of PCR amplicon in a 1-tube system would be highly desirable, particularly for diagnostic applications.

PCR-enzyme hybridisation has been developed in a 1-tube format for the amplification and detection of DNA (NucleoLink™, Nalge Nunc International) and optimised to suit specific bacterial pathogens (Grennan et al. 2001, Wilson et al. 2002). Using this system, a biphase PCR reaction occurs, with amplicon generated bound to the surface and free in liquid phase. The proprietary term for this process is 'Detection of Immobilised Amplified Product in a One-Phase System (DIAPOPS)' (Nalge Nunc International). To date there are no publications describing the use of NucleoLink to perform 1-tube RT-PCR-enzyme hybridisation assays (RT-PCR-EHA).

This study describes the development of RT-PCR-EHA in NucleoLink tubes to produce a sensitive, low cost, 1-tube protocol for detecting the presence of femtogram amounts of rRNA from 4 bacterial fish pathogens *Aeromonas salmonicida*, *Tenacibaculum maritimum* (formerly *Flexibacter maritimus*), *Lactococcus garvieae* and *Yersinia ruckeri*. The system is optimised using purified rRNA and then used to detect rRNA from bacteria grown in selective-enrichment media.

MATERIALS AND METHODS

Cultures. Unless otherwise specified, all bacteria were obtained from the culture collection held by the Fish Health Unit, Department of Primary Industries, Water and Environment, Tasmania.

Target organisms. Atypical *Aeromonas salmonicida* isolated from greenback flounder (Whittington et al. 1995), DPIWE Accession No. 93/956-2; *Tenacibaculum maritimum* NCIMB 2154^T; *Lactococcus garvieae* ATCC 49156^T; and *Yersinia ruckeri* Serotype O1b isolated from Atlantic salmon, DPIWE Accession No. 90/3988 were used in this study.

Specificity organisms. The specificity of the RT-PCR-EHA system was assessed at 2 levels: firstly, using phenotypically similar bacteria, near related species and bacteria likely to be isolated alongside the target bacterium; secondly, using bacteria identified as having some genotypic similarity in respect to the specific 16S rRNA primer sequence as identified by Carson (1998). The bacteria tested for each of the 4 bacteria are listed in Table 1.

16S rRNA primers and internal probes. 16S rRNA primer sets (Carson 1998) were used for the PCR assays. The properties of the resulting amplicons were as follows: *Aeromonas salmonicida* (261 bp, 55% G + C); *Tenacibaculum maritimum* (288 bp, 49% G + C); *Lactococcus garvieae* (145 bp, 46% G + C) and *Yersinia ruckeri* (247 bp, 54% G + C). For each RT-PCR-EHA the reverse primer was chosen as the solid-phase primer. Solid-phase primers were modified by the addition of a 10-base thymidine linker to the 5′-end of the primer and phosphorylation at the end of this linker, as recommended by Nalge Nunc International.

The internal biotin-labelled probes used for hybridisation to the solid-phase RT-PCR products were developed by Carson (1998), except the *Aeromonas salmonicida* probe, which was described by Høie et al. (1997).

RNA extraction. Pure and target RNA were extracted as folows:

Pure rRNA: Pure target RNA was obtained using an RNAAqueous™-4PCR (Ambion) extraction kit following the manufacturer's instructions. RNA concentration was measured at an A_{260}:A_{280} absorbance ratio using a Genequant DNA/RNA calculator (Pharmacia Biotech).

Target RNA from selective-enrichment media: Bacterial RNA from the selective-enrichment samples was extracted using the rapid, high-throughput DNA extraction method described by Wilson & Carson (2001) with some modifications for the extraction of RNA. Briefly: all extraction reagents were prepared with solvents treated with 0.2% (v/v) diethyl pyrocarbonate (DEPC). Lysis, DNA binding and elution were performed using a Polyfiltronics glass microfibre (Type GF/B) 800 µl, 96-well Uni-filter plate, and a UniVac vacuum manifold (Whatman). The filter wells were treated with water containing 0.2% (v/v) DEPC for at least 2 h prior to extraction. After treatment the wells were drained by applying a vacuum of about 20 kPa for about 1 min. A volume of 500 µl cold guanidinium isothiocyanate buffer (L6) with 1% (v/v) β-mercaptoethanol was added to each well, and 200 µl of the sample (selective-enrichment medium seeded with bacteria) was then added to the appropriate filter well. After 15 min, the lysate was removed from the filter wells by applying a vacuum of about 13.5 kPa. The wells were washed twice with 100 µl of buffer (L2) and

Table 1. Bacteria used for testing specificity of RT-PCR-EHA. Homology: % in respect to primer of closest match; FCLB: unidentified *Flexibacter-Cytophaga*-like normal flora isolated from Atlantic salmon

Species	Strain no.	Homology
***Aeromonas salmonicida*-specificity**		
A. salmonicida[a]	84/09062-B13	100%
A. sobria	ATCC 43979^T	100%
A. eucrenophila	ATCC 23309^T	94%
A. jandaei	ATCC 49568^T	94%
A. schubertii	ATCC 43700^T	94%
A. veronii bv *sobria*	ATCC 9071^T	94%
A. veronii bv *veronii*	ATCC 35624^T	94%
A. hydrophila	ATCC 7966^T	85%
A. hydrophila	ATCC 7965	85%
A. hydrophila[b]	UTS 67	85%
A. bestiarum	ATCC 14715	85%
A. caviae	ATCC 15468^T	76%
A. trota	ATCC 49657^T	76%
A. media	ATCC 33907^T	75%
Vagococcus salmoninarum	NCDO 2777^T	
Tenacibaculum maritimum	NCIMB 2154^T	
Hafnia alvei	95/6404	
Proteus rettgeri	96/5494	
Yersinia intermedia	92/4041	
Aeromonas sp.[d]	NF 1	
Aeromonas sp.[d]	NF 2	
Pseudomonas sp.[d]	NF 3	
Enterobacter sp.[d]	NF 4	
***Tenacibaculum maritimum*-specificity**		
Cytophage marinoflava	ACAM 75	65%
T. ovolyticum	NCIMB 13127^T	
FCLB mucoid	89/2244-9	
FCLB mucoid	89/2756-1	
FCLB mucoid	96/5171	
FCLB mucoid	CRC-2	
Flavobacterium columnare	NCIMB 2248^T	
F. johnsoniae	ATCC 17061^{Co-T}	
Vibrio splendidus bv I	ATCC 25914^T	
V. ordalii	ATCC 33509^T	
V. anguillarum	85/3475-1	

Species	Strain no.	Homology
***Lactococcus garvieae*-specificity**		
Carnobacterium piscicola	ATCC 35586^T	76%
Flavobacterium columnare	NCIMB 2248^T	71%
Streptococcus sp.[d]	CORT 1	
Streptococcus sp.[d]	CORT 2	
L. piscium	NCDO 2778^T	
S. iniae[c]	QDPI 95/41693-4a	
Enterococcus faecalis	ATCC 29212	
Vagococcus salmoninarum	NCDO 2777^T	
A. hydrophila	ATCC 7966^T	
***Yersinia ruckeri*-specificity**		
Y. pseudotuberculosis	96/5417-2	96%
Y. enterocolitica	96/5440-1B	87.5%
Haemophilus influenzae	ATCC 33391^T	68%
Citrobacter freundii	90/2624-18	87.5%
Y. intermedia	92/4041	
H. alvei	95/6404	87.5%
Proteus rettgeri	96/5494	
V. angullarum	85/3475-1	
Carnobacterium piscicola	ATCC 35586^T	
V. salmoninarum	NCDO 2777^T	
Pseudomonas sp.[d]	NF 3	
Enterobacter sp.[d]	NF 4	
Specificity: all systems		
Atypical *Aeromonas salmonicida*	93/0956-2	
Escherichia coli	ATCC 25922	87.5% (*Y. ruckeri*)
L. garvieae	ATCC 49156^T	
Y. ruckeri serotype O1b	90/3988	

[a]Atypical *A. salmonicida* isolated from goldfish in Victoria, DNA supplied by N. Gudkovs, Australian Fish Disease Laboratory, Australian Animal Health Laboratory, CSIRO, Australia
[b]DNA supplied by J. Oakey, University of Technology, Sydney, Australia
[c]DNA supplied by A. Thomas, Queensland Department of Primary Industries, Australia
[d]Fish normal flora isolated from rainbow trout or Atlantic salmon

then washed 5 times with 200 µl of 70% ethanol and once with acetone, at a vacuum pressure of 40 kPa. The vacuum was allowed to run until there was no visible trace of solvents in the wells or on the drip directors underneath the wells (usually 5 min); 30 µl of sterile DEPC-treated water, pre-heated to 90°C, was then added to each filter well. After 10 min, the nucleic acids were eluted from the glass microfibre-filter plate. A further 20 µl of pre-heated water was added to the wells and allowed to stand for 2 min, after which time the final elute was collected by vacuum and rehydrated overnight at 4°C.

Specificity bacteria. Organisms used for specificity testing were grown in pure culture and then suspended in 100 µl of DEPC-treated 18 Mohm water. Nucleic acids were extracted by boiling for 15 min and cooling rapidly to –20°C for 5 min followed by a 10 min centrifuge at 18 000 × *g*. The nucleic acids (supernatant) were then transferred to a clean sterile tube.

DNase treatment of RNA. Immediately prior to RT-PCR, 1.4 µl of DNase buffer (1 mM $MnCl_2$, 1 mM $CaCl_2$ and 0.1 M Tris-HCl pH 8.0) with 0.5 U DNase (Promega) was added to 5 µl of nucleic acid extract. The samples were incubated for 30 min at 37°C followed by denaturation at 75°C for 5 min, and cooled to 4°C for at least 1 min.

Determination of sensitivity. Test sensitivity was determined at 2 levels, firstly with pure target RNA and secondly with RNA extracted from selective-enrichment media: HK for *Aeromonas salmonicida;* POSI for *Tenacibaculum maritimum*; CORT for *Lactococcus garvieae;* and POST for *Yersinia ruckeri* (T. Wilson & J. Carson, proprietary formulations, Cooperative Research Centre for Aquaculture, Sydney) using the vacuum system described here. For the pure RNA, serial dilutions were prepared from RNA of known concentration and these dilutions were amplified by RT-PCR. For the RNA from selective-enrichment media, a 0.5 McFarland suspension of bacteria in log-phase of growth was prepared in sterile water. From this a 1:1000 dilution followed by 10 decimal dilutions using the selective-enrichment media as diluent were prepared. Just prior to RNA extraction, viable counts of the suspensions were determined by the Miles & Misra method (Miles et al. 1938) using Shiehs medium (Song et al. 1988) for the *T. maritimum* and blood agar base No. 2 (Oxoid) supplemented with 7% (v/v) defibrinated sheep blood (SBA) for the other bacteria; plates were incubated for 48 h at 25°C. Bacterial RNA from each of the decimal dilutions was immediately extracted as described above, and the RNA amplified by RT-PCR-EHA.

Binding of solid-phase primer to microwells. The modified reverse primers were covalently bound to the NucleoLink strips by the carbodiimide condensation reaction (CCR) as described for the Covalink NH BreakApart™ strips (Rasmussen et al. 1994). CCR reagent, sufficient for one 8-well strip, was prepared by adding 1.63 mg of 1ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) to 840 µl of 18 Mohm water, 8.5 µl of 1 M 1-methylimidazole (1-MeIm) and 0.85 µl of 1000 ng μl^{-1} of solid-phase primer. NucleoLink strips were placed into a Nunc Frame (Catalogue no. 249182), and a 100 µl aliquot of CCR reagent solution was added to each well. The strips were sealed with Nunc Tape 8 (Catalogue no. 249719) and incubated at 50°C for 5 h. After incubation, non-covalently bound primer was removed by washing 3 times with 150 µl of DEPC-treated pre-warmed 0.4 M NaOH with 0.25% Tween 20. The strips were incubated at 50°C for 15 min followed by 3 more washes. The strips were then washed 3 times in DEPC-treated reverse osmosis (RO) water (<2 µS), soaked for 5 min, and then washed 3 more times with water. The wells were emptied thoroughly by tapping sharply several times upside down on a paper towel. Once dry, the strips were stored for up to 2 mo at 4°C in a clip-seal plastic bag.

Reverse transcriptase technique. NucleoLink wells were hydrated and blocked with 200 µl of DIAPOPS buffer with 10 mg ml^{-1} fraction V BSA (prepared in DEPC-treated RO water [<2 µS] and filter-sterilised through a 0.2 µm filter) at room temperature for 1 h. The RT-PCR reaction mix contained 200 µM each of dNTPs, 1.375 mM $MgSO_4$ for *Aeromonas salmonicida* and 2 mM $MgSO_4$ in all other cases, 1 × RT-PCR buffer (Invitrogen), 0.1% DEPC-treated Tween 20, 3 µM of each primer for *Tenacibaculum maritimum*, 2 µM each primer for *Lactococcus garvieae*, and 2 µM forward primer and 0.25 µM reverse primer for *A. salmonicida* and *Yersinia ruckeri*, 0.2 µl Superscript™ 1-step RT-PCR with platinum *Taq* (Invitrogen), 3 µl DNase-treated RNA, and sufficient 18 Mohm water to bring the total reaction volume to 20 µl. Optimum conditions for PCR cycling were: cDNA from RNA at 50°C for 30 min followed by a 3 min denaturation at 94°C, followed by 35 cycles consisting of denaturation at 94°C (45 s), annealing at 62°C (45 s) for *T. maritimum* and *L. garvieae*; annealing at 60°C (45 s) for *A. salmonicida* and *Y. ruckeri*, extension at 72°C (45 s), with a final extension at 72°C for 5 min. A positive control, a no-sample and a no-RT enzyme-negative control were included in each RT-PCR run.

DIAPOPS amplification was assessed by gel electrophoresis of amplicon generated in the liquid phase of the biphase PCR reaction.

Denaturation of non-covalently bound amplicons. Non-covalently bound solid-phase amplicons were removed by washing 3 times, soaking for 5 min, and washing 3 more times with 0.2 M NaOH with 0.1% Tween 20 added just before use.

The wells were then washed 3 times, soaked for 5 min and washed 3 more times in DIAPOPS buffer (80 mM Tris-HCl, 20 mM Tris base, 150 mM NaCl, 0.1% Tween 20, pH 7.5).

Hybridisation with biotin-labelled probes. A final concentration of 50 nM denatured internal probe and 100 µg ml^{-1} salmon sperm DNA (Invitrogen) diluted in hybridisation solution (6 × standard saline citrate [SSC], 5 × Denhardt's solution [Amresco], 0.1% Tween 20) were added to each well. Hybridisation was carried out at 50°C for at least 1 h. Unbound probe was removed by washing 3 times in wash buffer (0.5 × SSC with 0.1% Tween 20), soaking for 15 min at 50°C and washing 3 more times. After washing, the wells were emptied thoroughly by tapping sharply several times upside down onto a paper towel.

Colorimetric detection of labelled probes. Streptavidin alkaline phosphotase (Promega) was diluted 1:2000 in DIAPOPS buffer and 100 µl was added to each well. The strips were incubated at 37°C for 1 to 2 h. After incubation the wells were washed 3 times, soaked for 5 min and washed 3 more times with DIAPOPS buffer. Then, 100 µl of 10 mg ml^{-1} *p*-nitrophenylphosphate (Sigma-Aldrich) diluted in 1 M diethanolamine with 1 mM $MgCl_2$ (pH 9.8) was added to each well. Color development was allowed to proceed for 60 min in the dark at room temperature and OD readings at 405 nm were taken in an ELISA plate reader. If borderline positive/negative OD readings were obtained (values about 1.2 times the negative control after 60 min), the reaction was allowed to proceed up to 18 h. After this time, samples with absorbance readings of at least 1.4 times the highest negative control reading corresponded to samples that contained target RNA. If necessary, the EHA reaction was stopped with 100 µl of 1 M NaOH.

RESULTS AND DISCUSSION

Sensitivity of RT-PCR-EHA

As demonstrated for *Yersinia ruckeri* in Fig. 1, a sensitivity of 4 fg or less was achieved for RT-PCR-EHA for each of the 4 bacteria from pure rRNA. This value was only possible when a conjugate ratio of 1:2000 was used; a lower conjugate ratio increased the background reading so that it was sometimes indistinguishable from weak positive reactions while, with a higher conjugate ratio, sensitivity was decreased. Primer concentration was critical in achieving optimum results. Primer concentration was optimised to give maximum sensitivity, so that agarose gel electrophoresis and EHA readings complemented each other. Reactions that gave a strong band and weak EHA readings or reactions that gave strong EHA and weak gel readings were considered sub-optimal. Optimum results were achieved for *Aeromonas salmonicida* and *Y. ruckeri* using a primer ratio of 1:8 as recommended for regular PCR-ELISA in NucleoLink tubes (Oroskar et al. 1996), however 4 times the recommended concentration of primer was required, that is 2 µM of the forward and 0.25 µM of the reverse primers (Fig. 2). This primer ratio resulted in inefficient RT-PCR-EHA when used with the other 2 bacteria. For *Tenacibaculum maritimum* and *Lactococcus garvieae*, a 1:1 ratio of the 2 primers at a concentration of 3 µM each for *T. maritimum* and 2 µM each for *L. garvieae* gave the best results.

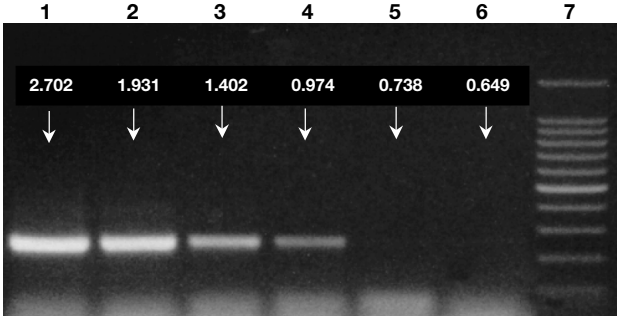

Fig. 1. Purified *Yersina ruckeri* RNA showing sensitivity of biphase PCR. Assessment by agarose gel electrophoresis and corresponding EHA readings. Lane 1: 4 pg RNA; Lane 2: 400 fg RNA; Lane 3: 40 fg RNA; Lane 4: 4 fg RNA; Lane 5: 0.4 fg RNA; Lane 6: negative control; Lane 7: Advanced Biotechnologies 100 bp ladder

The ability to distinguish weak positive results from negative background readings was tested by running 7 negative RT-PCR-EHA reactions alongside a weak positive (4 fg of template RNA) and repeating the experiment 2 times. In each case the weak positive gave a greater OD reading than the negative controls,

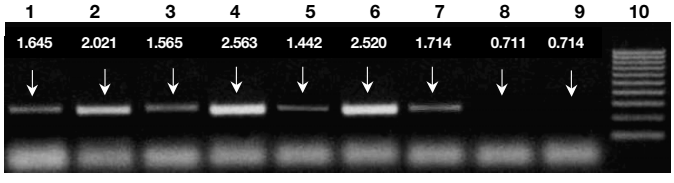

Fig. 2. *Aeromonas salmonicida* pure RNA. Effect of differing primer concentrations on agarose gel electrophoresis and EHA readings. Lanes 1 to 7: 1 pg RNA: Lane 1: 2 µM each primer; Lane 2: 2 µM forward primer, 1 µM reverse primer; Lane 3: 1 µM each primer; Lane 4: 1 µM forward primer, 0.5 µM reverse primer; Lane 5: 0.5 µM each primer; Lane 6: 2 µM forward primer, 0.25 µM reverse primer; Lane 7: 2 µM forward primer, 0.5 µM reverse primer; Lane 8: negative control with no RT enzyme; Lane 9: negative control with no RNA; Lane 10: Advanced Biotechnologies 100 bp ladder

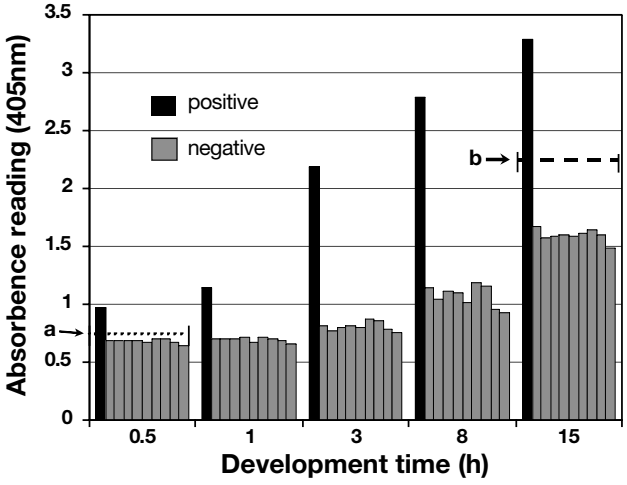

Fig. 3. *Yersinia ruckeri*. RT-PCR-EHA absorbance values showing difference between weak positive absorbance readings (4 fg of template RNA) and 7 negative absorbance readings over time. Line a: cut-off value 1.2 times the highest negative control (1.2 × neg = 0.830) after 0.5 h incubation; Line b: cut-off value 1.4 times the highest negative control (1.4 × neg = 2.339) after 15 h incubation

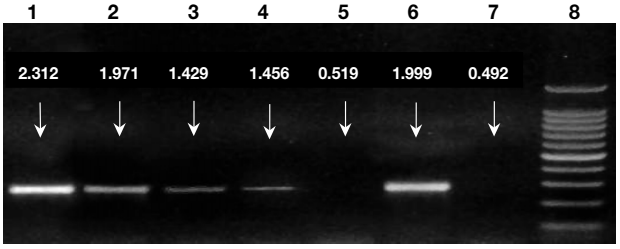

Fig. 4. *Tenacibaculum maritimum* RNA extracted from enrichment culture medium showing sensitivity of biphase PCR. Assessment by agarose gel electrophoresis and corresponding EHA readings. Lane 1: 1000 CFU per 200 µl sample volume; Lane 2: 100 CFU per 200 µl sample volume; Lane 3: 10 CFU per 200 µl sample volume; Lane 4: 1 CFU per 200 µl sample volume; Lane 5: 0.1 CFU per 200 µl sample volume; Lane 6: 10 pg positive control; Lane 7: negative control; Lane 8: Advanced Biotechnologies 100 bp ladder

with the positive reading usually 1.2 times greater than the highest negative value after 0.5 h, as shown for *Yersinia ruckeri* in Fig. 3. Occasionally, weak positive results were not easily differentiated from the negative controls even after 1 h developing time. In such cases, extending the color development period to 15 h was sufficient to separate the weak positive reactions from the negative values by a factor of at least 1.4.

Once optimised for pure RNA, the RT-PCR-EHA was tested on RNA extracted from selective-enrichment media using the vacuum system described. A sensitivity of 1 CFU per 200 µl sample volume was achieved for *Aeromonas salmonicida* and *Tenacibaculum maritimum* (Fig. 4), 9 CFU for *Lactococcus garvieae*, and 3 CFU for *Yersinia ruckeri*. The sensitivity achieved for *L. garvieae* was less than that achieved for the other bacteria. This was probably due to inefficient lysis during the RNA extraction procedure. Increasing the concentration of lysozyme or lengthening the incubation time did not improve sensitivity.

The sensitivity achieved using RNA extracted from the selective-enrichment media as RT-PCR template was slightly less than that achieved for the pure RNA, this was most likely due to limitations of the RNA extraction procedure. The main limitation to sensitivity in the procedure is the ability to add only one-quarter of the eluted RNA to a single RT-PCR reaction. During extraction, the bacterial RNA in a 200 µl sample of enrichment-culture medium is concentrated to about 35 µl of RNA elute, but to add this quantity to a single reaction, the total volume of the PCR mix would need to be significantly increased. While this is possible, the cost per test would be substantially increased. Were the total volume of eluted RNA to be used in 1 RT-PCR reaction, the sensitivity of the RT-PCR-EHA system for 200 µl of enrichment broth would be an estimated 1 CFU for each bacterium.

Specificity of the RT-PCR-EHA

The specificity of the PCR primers and corresponding internal probes were established previously (Carson 1998). For *Tenacibaculum maritimum*, *Lactococcus garvieae* and *Yersinia ruckeri* there is no reported evidence of cross-reaction with near-related species, as defined by phenotype or genotype. Similarly specificity is observed for *Aeromonas salmonicida*, with the exception that cross-reaction can occur with some strains of *A. hydrophila* and *A. bestiarum* (Carson 1998) which cannot be resolved by use of an internal *A. salmonicida* species probe (Høie et al. 1997). Due to this cross-reaction, positive *A. salmonicida* reactions are always confirmed by sequencing of the PCR amplicons or by performing biochemical tests (Carson et al. 2001) on the cultured organism. This high level of specificity of both primers and internal probes was observed using the NucleoLink RT-PCR-EHA system. This is demonstrated in Fig. 5, which shows the specificity results for *T. maritimum*. Clear, unambiguous reactions were evident, even with low concentrations of template RNA, and there was complete agreement between the presence of amplicon in the liquid-phase as detected by gel electrophoresis and the solid-phase amplicon as detected by EHA.

In conclusion, this study has described the development of RT-PCR-EHA in NucleoLink™ tubes. The system is optimised at every step to provide streamlined

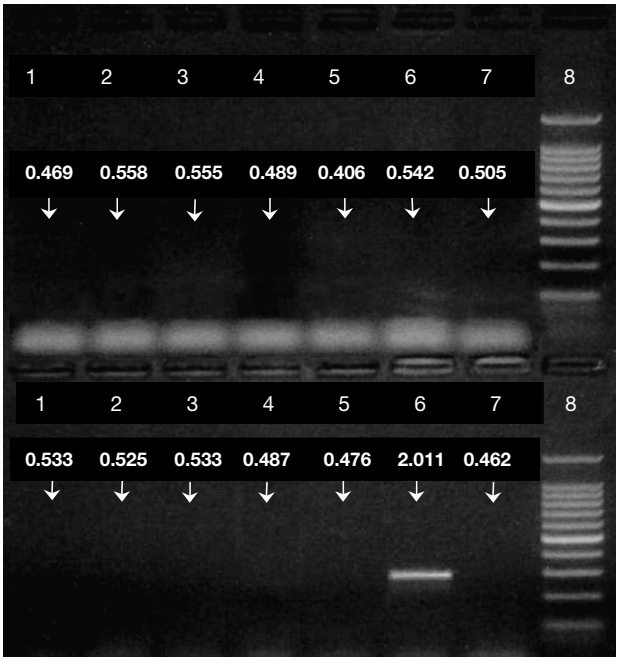

Fig. 5. Liquid-phase PCR results and corresponding EHA absorbance readings showing the specificity of the RT-PCR-EHA for *Tenacibaculum maritimum*. Top row, Lane 1: FCLB mucoid 89/2756-1; Lane 2: FCLB mucoid CRC-2; Lane 3: FCLB mucoid 89/2244-8; Lane 4: FCLB mucoid 89/3001-2; Lane 5: *T. ovolyticum* NCIMB 13127^{T}; Lane 6: *Flavobacterium columnare* NCIMB 2248^{T}; Lane 7: *Vibrio anguillarum* 85/3475-1; Lane 8: Advanced Biotechnologies 100bp ladder. Bottom row, Lane 1: *V. ordalii* ATCC 33509^{T}; Lane 2: *V. splendidus* I ATCC 25914^{T}; Lane 3: *Cytophaga marinoflava* ACAM 75; Lane 4: *F. johnsoniae* ATCC 17061^{Co-T}; Lane 5: atypical *Aeromonas salmonicida* 93/956-2; Lane 6: *T. maritimum* NCIMB 2154^{T}; Lane 7: negative control; Lane 8: Advanced Biotechnologies 100 bp ladder

high-throughput sampling. The use of 1 tube per sample from cDNA to EHA decreases the cost and time involved in sample transfer and decreases the risk of cross-contamination between samples. The system is also rapid with the 96-well RNA extraction to EHA results achieved in as little as 8 h. An sensitivity of 4 fg for pure rRNA or 1 to 9 CFU for selective-enrichment culture was achieved for the 4 bacterial fish pathogens *Aeromonas salmonicida*, *Tenacibaculum maritimum*, *Lactococcus garvieae* and *Yersinia ruckeri*. Work is in progress to validate this technology outside the laboratory to produce a powerful high-throughput system for surveillance and monitoring of pathogens in fish populations.

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