Vannier P, Espeseth D (eds): New Diagnostic Technology: Applications in Animal Health and Biologics Controls. Dev Biol (Basel). Basel, Karger, 2006, vol 126, pp 133-145.

Development, Application and Validation of a Taqman® Real-Time RT-PCR Assay for the Detection of Infectious Salmon Anaemia Virus (ISAV) in Atlantic Salmon (*Salmo salar*)

M. Snow[1], P. McKay[1], A.J.A. McBeath[1], J. Black[1], F. Doig[1], R. Kerr[1], C.O. Cunningham[1], A. Nylund[2], M. Devold[2]

[1] FRS Marine Laboratory, Aberdeen, U.K.
[2] Department of Biology, University of Bergen, Bergen, Norway

Key words: Infectious salmon anaemia virus, ISAV, real-time PCR, qRT-PCR, Taqman.

Abstract: Infectious salmon anaemia (ISA) is a disease of cultured Atlantic salmon (*Salmo salar*) which was successfully eradicated from Scotland following its emergence in 1998. The rapid deployment of sensitive diagnostic methods for the detection of ISA virus (ISAV) was fundamental to the swift eradication of ISA disease in Scotland and continues to be of crucial importance to surveillance of the aquaculture industry. This study reports the development, validation, application and interpretation of two independent, highly sensitive and specific semi-quantitative Taqman® real-time RT-PCR (qRT-PCR) methods for the detection of ISAV. Such technology offers considerable advantages over conventional RT-PCR methods in current routine use for ISAV surveillance. These include an increased sensitivity, enhanced specificity, semi-quantification using endogenous controls, a lack of subjectivity in results interpretation, speed of processing and improved contamination control.

Introduction

Infectious salmon anaemia virus (ISAV) is a pathogen of cultured Atlantic salmon (*Salmo salar*) which has caused significant production losses in Norway, Scotland, Canada, the U.S.A., and the Faroes. Emergence of ISA disease in Scotland in the late 1990s prompted a swift and apparently successful eradication programme [1]. The disease has, however, become established in Norway and Canada where in 1999 alone it was estimated to be responsible for losses totalling $11 and $14 million, respectively [2].

The rapid deployment of sensitive diagnostic methods for the detection of ISAV

was fundamental to the swift eradication of ISA disease in Scotland and remains equally important in managing the impact and spread of this virus in counties where it is endemic. Three diagnostic assays are in widespread use for ISAV diagnosis, which include virus isolation on permissive cell lines such as SHK-1 [3], indirect fluorescent antibody testing (IFAT) [4] and reverse transcriptase polymerase chain reaction (RT-PCR) [5]. Of these assays, the RT-PCR is generally regarded as the most sensitive [6-8] although a recent study highlighted a lack of reproducibility between laboratories and emphasized a need to standardize inter-laboratory protocols [9]. Much of the variability was attributed to the use of different external controls and methods applied to interpret and confirm results [9].

Recent developments in real-time PCR technology offer the potential to overcome many of the limitations of conventional PCR including an improved sensitivity of ISAV diagnostics. Indeed, a sensitivity increase of 100-fold over conventional RT-PCR was recently reported using a SYBR green-based assay for the detection of ISAV [10]. Further advantages of Taqman® real-time PCR chemistry include improved specificity of amplification, since signal generation is reliant on the annealing of a highly specific secondary probe to the primary amplification product. In addition the development of an endogenous control allows the confirmation of target integrity as well as facilitation of relative quantification of viral load and subsequent comparison between samples. Real-time PCR thus offers considerable advantages over conventional RT-PCR-based protocols and has the potential to reduce problems in the interpretation and reproducibility of results between laboratories.

In this report we describe the development, validation and application of Taqman®-based assays targeting two independent genes of ISAV coupled to an assay targeting the constitutively expressed endogenous control translation elongation factor 1 alpha (ELF1α) [11]. The comparative efficiencies of these assays were determined and a method for the relative quantification of viral target-level developed. Assays were compared with the conventional RT-PCR in widespread use for ISAV diagnostics [5] throughout an experimentally induced clinical outbreak of ISAV and in samples analysed for routine ISAV diagnostics in our laboratory in 2004.

Materials and Methods

Development of Taqman Real-Time RT-PCR method

Primers and probe design

RT-PCR primer pairs and Taqman® MGB probes targeting ISAV segments 7 and 8 were selected from alignments of all available sequences using published sequence data and the Primer Express software (Applied Biosystems), and are detailed in Table 1. Both primers and probes were targeted to conserved gene regions to ensure detection of all documented variants of ISAV. Primers designed for amplification of the salmon ELF1α gene were those recently published by Moore et al. (2005). The ELF probe was designed to target mRNA specifically by spanning two exons such that it would not amplify contaminating salmon genomic DNA.

Table 1: Real-time PCR primers a

Organism	Gene target	Upst prim
ISAV	Segment 7	CAC TCC AA
ISAV	Segment 8	CTA GG GT
Salmo salar	ELF1α mRNA	CC AC A

RNA extraction

Tissues were stored in 1 ml field samples, 50-100 mg tissue v subsequently extracted according in DEPC-treated H_2O, quantifi spectrophotometer (Amersham 10mg tissue was homogenised i (Qiagen). RNA was subsequer protocol for the extraction of R in 200 µl DEPC-treated H_2O a (designated A control) were co subsequent RT and PCR steps.

Reverse transcription

For field diagnostic samples, containing 2 µg RNA in 9.5 µ 10min and then placed on ice. contained 9.5 µl RNA prepare 1mM each dNTP, 1Uµl^{-1} RNas Reactions were incubated at 37 performed using the Taqman® 9.125 µl RNA & 1.25 µl of 50 µ were placed on ice and mixed RNase Inhibitor and 1.25U for 90 min followed by 95°C f performing reactions contain subsequent PCR steps.

Real-time RT-PCR (qRT-P

Assays were performed o to conduct the following cyclir 95°C for 10 min (AmpliTaq Gol and 60°C for one min (anne containing 1 µl cDNA temp 250 nM Taqman® probe. Cont (designated C control).

Table 1: Real-time PCR primers and Taqman® MGB probes.

Organism	Gene target	Upstream primer	Downstream primer	Taqman® trobe	Amplicon size (bp)
ISAV	Segment 7	CAGGGTTGTA TCCATGGTTG AAATG	GTCCAGCCCT AAGCTCAACT C	FAM-CTCTCTCATTG TGATCCC-MGB	155
ISAV	Segment 8	CTACACAGCA GGATGCAGAT GT	CAGGATGCCG GAAGTCGAT	FAM-CATCGTCGCT GCAGTTC-MGB	104
Salmo salar	ELF1α mRNA	CCCCTCCAGG ACGTTTACAA A	CACACGGCCC ACAGGTACA	FAM-ATCGGT#GGTA TTGGAAC	57

RNA extraction

Tissues were stored in 1 ml RNALater (Ambion) and stored at -20°C before RNA extraction. For field samples, 50-100 mg tissue was homogenised in 1ml TRIZOL using a disposable pestle and RNA subsequently extracted according to the manufacturer's protocol (Invitrogen). RNA was finally resuspended in DEPC-treated H_2O, quantified by measuring the OD at 260 and 280 nm on a Genequant Pro spectrophotometer (Amersham Biosciences) and stored at -80°C. In the case of experimental samples, 10mg tissue was homogenised in 600 µl RLT buffer (Qiagen) for 1min using the TissueLyser system (Qiagen). RNA was subsequently extracted using the RNeasy kit according to the manufacturer's protocol for the extraction of RNA from animal tissues (Qiagen). Total RNA was finally resuspended in 200 µl DEPC-treated H_2O and stored at -80°C. For routine diagnostics, negative extraction controls (designated A control) were conducted by performing a blank extraction. Controls were taken through subsequent RT and PCR steps.

Reverse transcription

For field diagnostic samples, reverse transcription reactions were conducted by first preparing dilutions containing 2 µg RNA in 9.5 µl DEPC-treated dH2O. RNA samples were incubated at 55-60°C for 10min and then placed on ice. Reverse transcription was conducted in a total volume of 20 µl which contained 9.5 µl RNA prepared as above, 5 $ng\mu l^{-1}$ random hexamers (Promega), 1x 1st strand buffer, 1mM each dNTP, $1U\mu l^{-1}$ RNase OUT and $10U\mu l^{-1}$ MMLV reverse transcriptase (all from Invitrogen). Reactions were incubated at 37°C for 1h. In the case of experimental samples, reverse transcription was performed using the Taqman® RT Kit (Applied Biosystems) in a final reaction volume of 25 µl. Firstly, 9.125 µl RNA & 1.25 µl of 50 µM oligo $d(T)_{16}$ were mixed and denatured at 70°C for 10 min. Reactions were placed on ice and mixed with 1x reverse transcriptase buffer, 5.5 mM $MgCl_2$, 2 mM dNTPs, 0.4U RNase Inhibitor and 1.25U Multiscribe reverse transcriptase. Reactions were incubated at 48°C for 90 min followed by 95°C for five min. Negative reverse transcription controls were conducted by performing reactions containing no target (designated B control). Controls were taken through subsequent PCR steps.

Real-time RT-PCR (qRT-PCR) reagents and cycling

Assays were performed on an Applied Biosystems 7000 Sequence Detection System programmed to conduct the following cycling profile: 50°C for two min (AmpErase uracil N-glycosylase incubation), 95°C for 10 min (AmpliTaq Gold polymerase activation) followed by 45 cycles of 95°C for 15s (denaturation) and 60°C for one min (annealing/extension). Reactions were conducted in a final volume of 25 µl containing 1 µl cDNA template, 1x Taqman® Universal PCR mastermix, 900nM each primer and 250 nM Taqman® probe. Controls were conducted for each primer and probe set containing no target (designated C control).

Validation of reaction efficiencies and relative quantification

To determine whether the salmon host cell endogenous ELF1a assay control might permit the relative quantification of ISAV and ensure efficient performance of each assay, a validation experiment was conducted to determine whether the efficiency of all assays was comparable. For each assay, triplicate reactions were conducted on each dilution of a 10-fold serial dilution of cDNA prepared from concentrated standards. Standard curves were generated by plotting the relative dilution of RNA versus the cycle number required to elevate the fluorescence signal above the threshold for the ISAV Segment 7, 8 and ELF assays. Reaction conditions with respect to probe and primer concentrations were independently evaluated as described in the Applied Biosystems literature. The effect of reducing the total reaction volume from 50 µl to 25 µl was also determined.

Sensitivity

The same 10-fold dilution series of ISAV cDNA was used to evaluate the comparative sensitivity of both Taqman® qRT-PCR methods with that of conventional RT-PCR. Triplicate individual reactions were conducted for each test using the methods described above.

Specificity

ISAV is the only orthomyxovirus from fish to be characterised at the molecular level, and as such has been designated within the genus Isavirus. Nucleotide sequence of probes was used to search GenBank using BLAST X [12] to identify any known organisms with which they might cross-react. Segment 7 and 8 probes and primer sets were tested against cDNA derived from a range of other RNA viruses capable of infecting Atlantic salmon including Atlantic salmon paramyxovirus, viral haemorrhagic septicaemia virus (VHSV), infectious haematopoietic necrosis virus (IHNV), infectious pancreatic necrosis virus (IPNV) and pancreas disease virus (PD). To ensure the detection of all forms of ISAV, segment 7 and 8 primers and probes were designed from sequence alignments containing representatives of all known subtypes of ISAV, which are classified according to the sequence of a highly polymorphic region (HPR) of the haemagglutinin esterase (HE) gene. Such alignments included the HPR0 subtype from which all other HPR subtypes of the virus which are associated with clinical disease in aquaculture may have been derived [13,14].

Application of relative quantification to the detection of ISAV in experimentally infected fish

Pathogen-free fish

Atlantic salmon post smolts (mean weight +/- SE, 89.4 +/- 14.69 g) were reared at the FRS Marine Research Unit, Aultbea, Ross-shire, Scotland. Before the experimental challenge, four fish were screened for the presence of ISAV using RT-PCR and qRT-PCR as described above. Fish were allowed to acclimatize for seven days and starved for 24 h before experimental infection. Water temperatures were maintained at 11°C for the duration of the experimental infection. Seawater was extracted from the North Sea and passed through a sand filter and UV disinfection treatment before entering fish tanks.

Experimental infection with ISAV

Atlantic salmon post smolts (n=75) were anaesthetised by immersion in methane tricaine sulphonate (3-aminobenzoic acid ethyl ester of MS-222; Sigma) at a concentration of 100 mg l-1, infected intra-peritoneally with a dose of 2×10^6 $TCID_{50}$ $fish^{-1}$ in an inoculum volume of 100 µl, and distributed between two 360 l aquaria (Tanks 1 & 2). Controls consisted of 75 fish similarly anaesthetised and injected with an equivalent volume of culture medium containing no virus, and distributed between a further two tanks (Tanks 3 and 4). Two additional tanks were similarly set up, each of which contained 25 virus infected and non-infected fish respectively. These tanks were used to monitor the efficacy of the viral challenge. All fish were monitored for the duration of the experimental infection period (20d) and all dead fish were recorded and removed twice daily.

Sampling and comparative PCR analysis

From each set of virus-infected (Tanks 1 and 2) and control tanks (Tanks 3 and 4), a total of four fish were sacrificed and sampled on each of days 1,2,3,4,5,6,7,9,11,15 and 19 post infection. Kidney tissue was excised, sampled into RNALater (Ambion) and stored at -80°C for analysis by PCR. RNA extraction and Taqman® qRT-PCR was performed on experimental kidney samples using the methods described above for amplification of ISAV segment 7, 8 and ELF1a. Samples were also processed for conventional RT-PCR detection of ISAV segment 8 which was previously described [5] and is in routine diagnostic use within our laboratory.

Application of qR
ISAV surveillance

Field samples intenc
conducted by FRS in Scot
both the RT-PCR method
of ISAV was suspected ir
During this investigation a
by both qRT-PCR and cc

RESULTS

Development of

Validation of re

The amplification
fish tissue are indica
efficiencies with that
(ΔS) of <0.1 [16]. This
for the relative quan
in this study. For eac
number and dilution f
theoretical value of
probe concentration
in sensitivity was rec

Sensitivity

The segment 8
higher dilution that
detected ISAV at a
addition, very faint
conventional RT-PC

Specificity

Both segment 7
derived from viruse
proved to be of the
ISAV [15].

Application of
mentally infect

Experimental ir

The mortality ob
experimental infecti
infection and all fisl
showed clinical sign
livers and the prese
throughout this peri

Application of qRT-PCR to the detection of ISAV in field samples during routine ISAV surveillance in Scotland

Field samples intended for ISAV diagnosis during the routine ISA surveillance programme conducted by FRS in Scotland were processed in parallel throughout a nine month period in 2004, using both the RT-PCR method and qRT-PCR methods reported in this study. During this period the presence of ISAV was suspected in Scotland, although it was not attributed as the cause of clinical disease [15]. During this investigation a total of 279 kidney and 87 gill tissue samples were processed for ISAV diagnosis by both qRT-PCR and conventional RT-PCR.

RESULTS

Development of Taqman real-time RT-PCR method

Validation of reaction efficiencies and relative quantification

The amplification efficiency graphs obtained from serial dilutions of ISAV-positive fish tissue are indicated in Figure 1. Each of the ISAV assays exhibited comparable efficiencies with that of ELF1α as indicated by them exhibiting a difference in slope (ΔS) of <0.1 [16]. This indicates that the ELF1α endogenous control is a suitable reference for the relative quantification of ISAV over the range of starting concentrations used in this study. For each reaction series, there was also a high correlation between cycle number and dilution factor (R^2 =1.0 in all cases) and slope values approached the optimum theoretical value of -3.32, indicating efficient amplification of all assays. Primer and probe concentrations were optimised at 900 nM and 250 nM respectively and no loss in sensitivity was recorded by reducing the total reaction volume to 25 µl.

Sensitivity

The segment 8 Taqman® qRT-PCR assay consistently detected ISAV at a log higher dilution that the segment 7 Taqman® qRT-PCR assay which itself consistently detected ISAV at a log higher than the conventional RT-PCR (data not shown). In addition, very faint products were observed nearing the endpoint of detection for conventional RT-PCR which required experience in their interpretation.

Specificity

Both segment 7 and 8 assays yielded no detectable product when using cDNA derived from viruses other than ISAV. Virus identified in field samples during 2004 proved to be of the HPR0 type, which demonstrated efficient detection of variants of ISAV [15].

Application of relative quantification to the detection of ISAV in experimentally infected fish

Experimental infection of salmon with ISAV

The mortality obtained in the unsampled tanks used to monitor the efficacy of the experimental infection is shown in Figure 2. Mortality commenced on day 11 post-infection and all fish in the ISAV infected group were dead by day 20. All dead fish showed clinical signs consistent with ISA disease which included pale gills, darkened livers and the presence of ascites. No mortalities were recorded in the control tanks throughout this period.

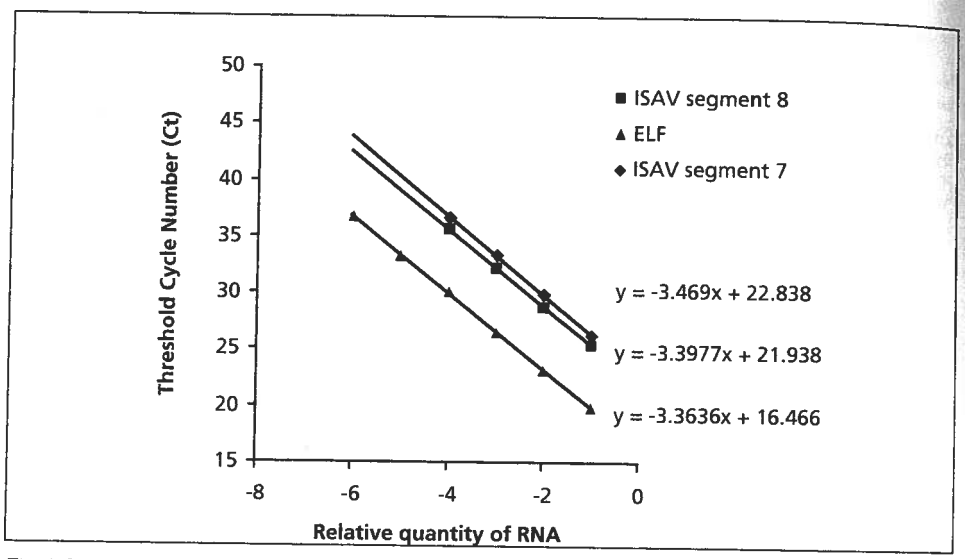

Fig. 1: Standard curves obtained by plotting the relative dilution of RNA versus the cycle number required to elevate the fluorescence signal above the threshold for the ISAV Segment 7, 8 and ELF assays. Equations were derived from linear regression.

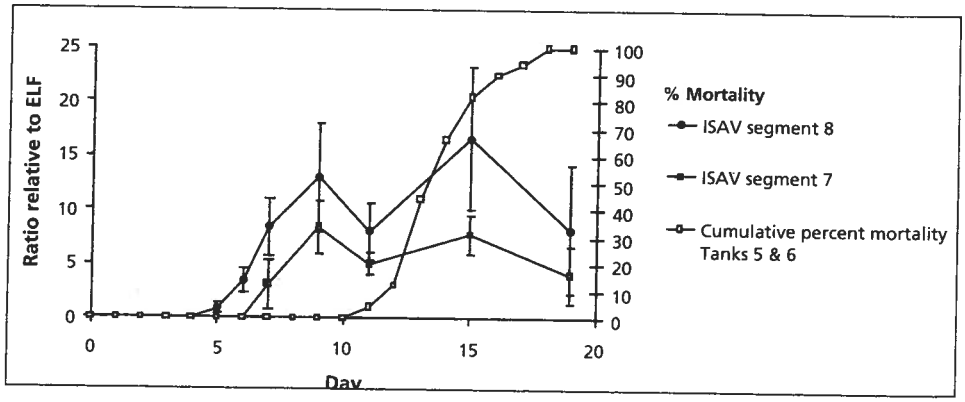

Fig. 2: **Graph indicating the mean level of ISAV segment 7 and 8 detected at different time-points following the experimental infection of Atlantic salmon with ISAV.** Mean levels were derived from four fish sampled at each time-point and represent the level (in arbitrary units) of each ISAV gene expressed as a proportion of the endogenous ELF-1α control signal detected in the same sample. Error bars indicate the standard error of the mean. The mean cumulative percent mortality obtained in un-sampled ISAV challenge reference tanks is indicated.

Sampling and cor

Results obtained fr
signal corresponding t
ELF1α endogenous co
calculation was perfor
literature supplied wit
positive for ELF1α ind
cases. All fish testing pc
7. At day 1 post infecti
all subsequent time-p
segment 8, however, w
assay, when expressed
in any of the control fi
cription (B), real-time
Results obtained o
ISAV segment 8 corre
the same two fish testi
products were observ
interpretation. In som
expected 155bp produ

**Application of rel
samples during r**

Two hundred and s
screened for the pre
segments 7 and 8 in a
Six samples were ide
samples were also po
assays. qRT-PCR assa
samples, respectively (
by conventional RT-P
five and six samples
respectively. Higher
in samples testing pc
negative for RT-PCR
3). Interestingly, som
magnitude to those ob
conditions.

During surveillanc
total of 87 fish samp
RT-PCR and both se
were positive using o
both segment 7 and
RT-PCR assay. Three
assay only and a furt
Taqman® qRT-PCR.
Samples which were
virus. For those which

ɜment 8

ɜment 7

ɜx + 22.838

77x + 21.938

36x + 16.466

sus the cycle number required
Segment 7, 8 and ELF assays.

Mortality

ISAV segment 8

ISAV segment 7

Cumulative percent mortality
Tanks 5 & 6

at different time-points following
vels were derived from four fish
ts) of each ISAV gene expressed
the same sample. Error bars indi-
nortality obtained in un-sampled

Sampling and comparative PCR analysis

Results obtained from the qRT-PCR analysis are detailed in Figure 2. The relative signal corresponding to target ISAV RNA was normalized to that obtained with the ELF1α endogenous control in each case. Values were expressed in arbitrary units. This calculation was performed according to the comparative Ct method described in the literature supplied with the ABI 7000 Sequence Detection System. All samples tested positive for ELF1α indicating efficient RNA extraction and reverse transcription in all cases. All fish testing positive for ISAV segment 8 also tested positive for ISAV segment 7. At day 1 post infection, two of the four fish sampled tested positive for ISAV, but at all subsequent time-points all fish tested positive. The mean values obtained for segment 8, however, were consistently higher that those obtained for the segment 7 assay, when expressed as a ratio to all ELF1α (Fig. 2). No contamination was recorded in any of the control fish sampled or in any of the RNA extraction (A), reverse transcription (B), real-time or conventional PCR controls (C) performed as described above.

Results obtained on the same sample set using a conventional RT-PCR targeting ISAV segment 8 corresponded with those obtained with the qRT-PCR methods, with the same two fish testing negative. In many of the positive cases, however, very weak products were observed on conventional agarose gels, which required experienced interpretation. In some cases non-specific products were also observed alongside the expected 155bp product.

Application of relative quantification to the detection of ISAV in field samples during routine ISA surveillance in Scotland

Two hundred and seventy nine kidney samples (each a pool of up to five fish) were screened for the presence of ISAV using Taqman® qRT-PCR assays targeting segments 7 and 8 in addition to conventional RT-PCR which also targets segment 8. Six samples were identified as positive using the conventional RT-PCR. All six samples were also positive using both ISAV segment 7 and 8 Taqman® qRT-PCR assays. qRT-PCR assays for segment 7 and 8 identified a further 11 and 12 positive samples, respectively (corresponding to 17 individual samples), which tested negative by conventional RT-PCR. Of these, six samples were positive using both assays, but five and six samples were positive using only the segment 7 or segment 8 assay respectively. Higher relative quantities of ISAV segment 7 and 8 were identified in samples testing positive for conventional RT-PCR as opposed to those testing negative for RT-PCR and positive by either one or both of the Taqman® assays (Figure 3). Interestingly, some of the values obtained from field samples were of a similar magnitude to those obtained during the course of clinical infection under experimental conditions.

During surveillance for ISAV, gill samples were also taken from some fish. Of a total of 87 fish samples processed, 41 yielded positive results using conventional RT-PCR and both segment 7 and segment 8 Taqman® qRT-PCR assays. No samples were positive using only conventional PCR. Seventeen samples were positive using both segment 7 and segment 8 assay but were negative using the conventional RT-PCR assay. Three samples were positive using the segment 7 Taqman® qRT-PCR assay only and a further 12 samples were positive using only the ISAV segment 8 Taqman® qRT-PCR. Relative quantities of virus detected are indicated in Figure 4. Samples which were positive using all methods yielded high relative quantities of virus. For those which tested negative using conventional RT-PCR, but positive using

both Taqman® qRT-PCR assays, low relative values were obtained for both segment 7 and segment 8 assays. Similarly samples testing positive using only one of the qRT-PCR assays also yielded low relative values.

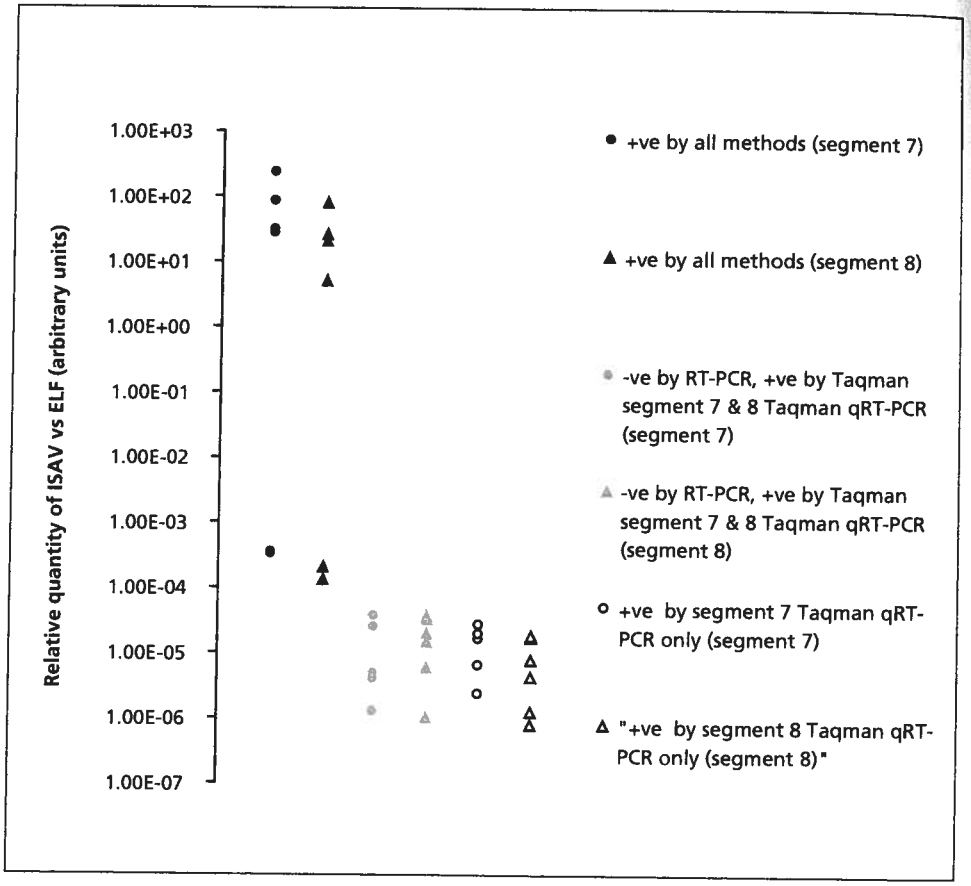

Fig. 3: **Relative levels of ISAV segment 7 and 8 detected in field kidney samples analysed during routine surveillance for ISAV in Scotland during 2004.** ISAV Levels obtained using Taqman® segment 7 (indicated by circles) and segment 8 (indicated by triangles) semi-quantitative qRT-PCR assays and expressed as a proportion of the endogenous ELF-1α control signal detected in each sample are detailed. Results are indicated according to the number of the three diagnostic tests (RT-PCR, Taqman® qRT-PCR segment 7, Taqman® qRT-PCR segment 8) generating positive results in each case.

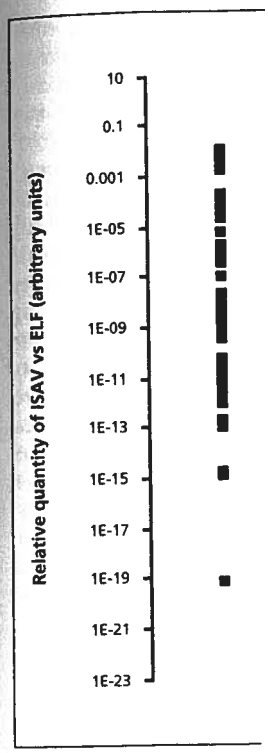

Fig. 4: **Relative levels of IS veillance for ISAV in** by squares) and segn as a proportion of the are indicated accord segment 7, Taqman®

Discussion

The results preser
step Taqman® qRT-PC
Both methods provec
routine use for ISAV
Taqman® qRT-PCR
with this technology i
expression against en
results, speed of proc

The segment 8 qF
7 assay during initial
the consistent detect
during a clinical outt

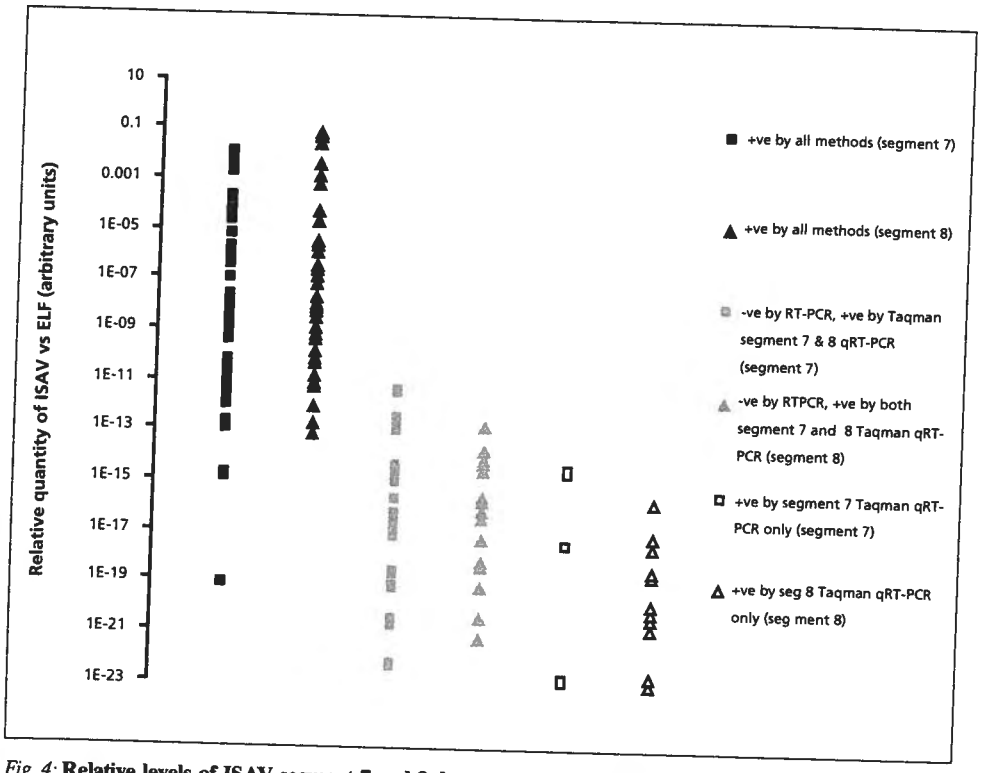

Fig. 4: **Relative levels of ISAV segment 7 and 8 detected in field gill samples analysed during routine surveillance for ISAV in Scotland during 2004.** ISAV Levels obtained using Taqman® segment 7 (indicated by squares) and segment 8 (indicated by triangles) semi-quantitative qRT-PCR assays and expressed as a proportion of the endogenous ELF-1α control signal detected in each sample are detailed. Results are indicated according to the number of the three diagnostic tests (RT-PCR, Taqman® qRT-PCR segment 7, Taqman® qRT-PCR segment 8) generating positive results in each case.

Discussion

The results presented describe two independent highly sensitive and specific two-step Taqman® qRT-PCR methods for the detection of ISAV in Atlantic salmon tissues. Both methods proved to be more sensitive than the conventional RT-PCR method in routine use for ISAV diagnosis [5]. In addition, the application of semi-quantitative Taqman® qRT-PCR to ISAV diagnostics offers a number of advantages associated with this technology including an increased sensitivity, enhanced specificity, quantitative expression against endogenous controls, a lack of subjectivity in the interpretation of results, speed of processing and contamination control.

The segment 8 qRT-PCR assay was shown to be more sensitive than the segment 7 assay during initial experimental dilution series experiments. This was supported by the consistent detection of higher levels of segment 8 in experimentally infected fish during a clinical outbreak of ISA disease and the fact that more field kidney and gill

samples were positive by the segment 8 assay only than by the segment 7 assay only. Since the assays displayed similar theoretical efficiencies, such a finding is most likely related to the differential expression of segment 7 and 8 segments throughout the viral infection cycle. Both segments 7 and 8 of ISAV have been shown to encode two proteins, based on removal of an intron from the primary transcript [17] or from overlapping open reading frames, respectively [18]. Segment 8 has been shown to encode a major structural protein [19], while segment 7 has recently been demonstrated to encode at least one protein which mediates the host interferon defence system [20]. An increased abundance of segment 8 mRNA generated during the viral life cycle may thus explain the apparent improved sensitivity of the Taqman® segment 8 assay.

Both Taqman® assays performed at least as well as the RT-PCR segment 8 assay in the analysis of experimentally infected fish. This was perhaps not surprising since the fish had been injected with a relatively high dose of ISAV which, based on the severity of the challenge, was actively replicating. Analysis of serial dilutions, however, suggested an increased sensitivity of the Taqman® assays. This increased sensitivity may be related to the specificity and sensitivity of Taqman® chemistry which permits additional cycles of PCR to be conducted (n=45) when compared to conventional PCR (n=35). Furthermore, the generation of the very small amplicons in both ISAV Taqman® assays may contribute to the improved sensitivity observed. Improved sensitivity of both Taqman® assays over conventional RT-PCR was also demonstrated during evaluation of both kidney and gill samples collected as part routine diagnostic surveillance of Scottish salmon aquaculture during 2004. Indeed, many samples testing negative by conventional PCR tested positive by both segment 7 and segment 8 Taqman® assays. However, the relative quantity of ISAV detected was lower than in those samples which tested positive by conventional RT-PCR. In these cases, the agreement of the independent Taqman® assays, coupled to the detection of signals beyond the apparent detection threshold for conventional RT-PCR strongly supports the fact that these were indeed true positive samples. In some cases, samples were negative by conventional RT-PCR and positive by only one of the segment 7 or 8 Taqman® assays. In all these cases, relative levels of ISAV detectable were also very low. The demonstrated difference in sensitivity of the segment 7 and 8 assays, which is probably related to differences in viral gene expression could account for such differences and supports the view that the results observed are not due to a lack of assay specificity.

The specificity of detection using Taqman® chemistry is significantly improved as compared to either conventional RT-PCR or real-time PCR based on chemistry such as SYBR green. Indeed, detection of Taqman® PCR products is dependent on the annealing of highly specific probes. Experience in our laboratory and in other studies has shown the method to be capable of the absolute discrimination of PCR products based on the presence of a single nucleotide mismatch in the probe binding region. Such specificity raises the prospect of false negatives using a single probe, should mutation occur within this region. To reduce the likelihood of false negatives, probes in this study were designed from absolutely conserved regions based on alignments of all known ISAV sequence types. The parallel use of two independent assays in this study provides further confidence in the characterisation of true negatives and adds significant power to the detection of rapidly mutating organisms such as RNA viruses.

The development of a quantitative method for ISAV diagnosis using the ELF1α endogenous control provides a significant advantage over the conventional RT-PCR method in ensuring quality control of RNA and subsequently generated cDNA quality and quantity. The elongation factor 1 alpha gene has been previously demonstrated to

be stably expressed be
calibrator for semi-qua
of ELF1α is thus a meas
times. In a diagnostic
levels in positive result
(e.g. conventional RT-
might also be used to
understanding of the r
Such a method thus off
interpretation of PC
positive/negative resu
current study of ISAV
of signals expected in
from kidney samples c
a similar magnitude to
lower. Such knowledg
and those in which clini
of detected virus iden
development of ISA c

Taqman® qRT-PCI
does not require the s
gels. As such, interpr
suited to implementa
processing also reduc
to five h) and minir
contamination. The T
amplification. Subsequ
(UNG) which specifi
order to reduce the ri

In conclusion, two
ISAV segment 7 and
endogenous control
levels. The applicatio
significant advantages
within a routine viral

REFERENCES

1 Stagg RM, Bruno
Epizootiological inv
Aberdeen: FRS Mar

2 Cipriano RC, Miller
O, Cipriano RC (eds
eradication. New C
Inspection Service, I
Commerce, Nationa

3 Dannevig BH, Falk
in a long-term cell li

be stably expressed between different individuals, and as such represents a suitable calibrator for semi-quantitative real-time PCR [21]. ISAV load expressed as a function of ELF1α is thus a measure which can be compared across individuals sampled at different times. In a diagnostic context such information can be used to express confidence levels in positive results obtained and as a basis to predict the likelihood of other tests (e.g. conventional RT-PCR or virus culture) also yielding positive results. Results might also be used to predict the clinical status of fish once a more comprehensive understanding of the relationship between viral RNA levels and pathology emerges. Such a method thus offers considerable scope both for improving the understanding and interpretation of PCR results, generating more information than a simple positive/negative result obtained using conventional RT-PCR. Indeed, within the current study of ISAV experimentally infected fish, we have demonstrated the range of signals expected in kidney tissues throughout a clinical disease outbreak. Results from kidney samples obtained in field samples during 2004 [15] were in some cases of a similar magnitude to these values, although in others relative values were significantly lower. Such knowledge might in future assist us in identifying carrier fish populations and those in which clinical disease is expected. In this case, however, further characterization of detected virus identified it as an HPR0 ISAV subtype, the relevance of which to development of ISA disease is not fully understood.

Taqman® qRT-PCR does not require any post-PCR processing and in particular does not require the subjective interpretation of weak products visualised on agarose gels. As such, interpretation of its results is more objective and less variable and suited to implementation in routine viral diagnostic testing. The lack of post-PCR processing also reduces the time taken to complete the method (approximately four to five h) and minimises the potential for obtaining false positives due to cross contamination. The Taqman® method also replaces dTTP with dUTP during PCR amplification. Subsequent PCR mixes are treated with the enzyme uracil N-glycosylase (UNG) which specifically degrades PCR products from previous amplifications in order to reduce the risk of amplicon carry-over resulting in sample contamination [22].

In conclusion, two highly sensitive and specific Taqman® qRT-PCR assays targeting ISAV segment 7 and segment 8 have been developed and evaluated. Use of an internal endogenous control ELF1α allows semi-quantitative expression of viral transcript levels. The application of Taqman® technology to ISAV diagnosis offers a number of significant advantages over conventional RT-PCR and lends itself well to implementation within a routine viral diagnostic environment.

References

1 Stagg RM, Bruno DW, Cunningham CO, Raynard RS, Munro PD, Murray AG, et al: Epizootiological investigations into an outbreak of infectious salmon anaemia (ISA) in Scotland. Aberdeen: FRS Marine Laboratory; 2001 April 2001, Report No 13/01.

2 Cipriano RC, Miller O: Infectious Salmon Anaemia: The Current State of Our Knowledge, in Miller O, Cipriano RC (eds): International response to Infectious salmon anemia: prevention, control and eradication. New Orleans, LA, U.S. Department of Agriculture, Animal and Plant Health Inspection Service, U.S. Department of the Interior, U.S. Geological Survey; U.S. Department of Commerce, National Marine Fisheries Service, 2003, pp 1-11.

3 Dannevig BH, Falk K, Namork E: Isolation of the causal virus of infectious salmon anaemia (ISA) in a long-term cell line from Atlantic salmon head kidney. J Gen Virol 1995;76(6):1353-1359.

4 Falk K, Namork E, Dannevig BH: Characterization and applications of a monoclonal antibody against infectious salmon anaemia virus. Dis Aquat Organ 1998;34(2):77-85.

5 Mjaaland S, Rimstad E, Falk K, Dannevig BH: Genomic characterization of the virus causing infectious salmon anemia in Atlantic salmon (*Salmo salar* L.): an orthomyxo-like virus in a teleost. J Virol 1997;71(10):7681-7686.

6 Devold M, Krossoy B, Aspehaug V, Nylund A: Use of RT-PCR for diagnosis of infectious salmon anaemia virus (ISAV) in carrier sea trout Salmo trutta after experimental infection. Dis Aquat Organ 2000;40(1):9-18.

7 Opitz HM, Bouchard D, Anderson E, Blake S, Nicholson B, Keleher W: A comparison of methods for the detection of experimentally induced subclinical infectious salmon anaemia in Atlantic salmon. Bulletin of the European Association of Fish Pathologists 2000;20(1):12-22.

8 Snow M, Raynard RS, Murray AG, Bruno DW, King JA, Grant R, et al: An evaluation of current diagnostic tests for the detection of infectious salmon anaemia virus (ISAV) following experimental water-borne infection of Atlantic salmon, *Salmo salar* L. J Fish Dis 2003;26(3):135-145.

9 Nerette P, Dohoo I, Hammell L, Gagne N, Barbash P, Maclean S, et al: Estimation of the repeatability and reproducibility of three diagnostic tests for infectious salmon anaemia virus. J Fish Dis 2005;28(2):101-110.

10 Munir K, Kibenge FS: Detection of infectious salmon anaemia virus by real-time RT-PCR. J Virol Methods 2004;117(1):37-47.

11 Moore LJ, Somamoto T, Lie KK, Dijkstra JM, Hordvik I: Characterisation of salmon and trout CD8alpha and CD8beta. Mol Immunol 2005;42(10):1225-1234.

12 Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ: Basic local alignment search tool. J Mol Biol 1990;215:403-410.

13 Cunningham CO, Gregory A, Black J, Simpson I, Raynard RS: A novel variant of the infectious salmon anaemia virus (ISAV) haemagglutinin gene suggests mechanisms for virus diversity. Bull Eur Ass Fish Pathol 2002;22:366-374.

14 Nylund A, Devold M, Plarre H, Isdal E, Aarseth M: Emergence and maintenance of infectious salmon anaemia virus (ISAV) in Europe: a new hypothesis. Dis Aquat Organ 2003;56(1):11-24.

15 Anon: Epizootiological investigation into a case of suspicion of infectious salmon anaemia (ISA) in Scotland in 2004. Aberdeen: FRS Marine Laboratory; 2005 June 05, Report No 11/05.

16 Gut M, Leutenegger CM, Huder JB, Pedersen NC, Lutz H: One-tube fluorogenic reverse transcription-polymerase chain reaction for the quantitation of feline coronaviruses. J Virol Methods 1999;77(1):37-46.

17 Ritchie RJ, Bardiot A, Melville K, Griffiths S, Cunningham CO, Snow M: Identification and characterisation of the genomic segment 7 of the infectious salmon anaemia virus genome. Virus Res 2002;84(1-2):161-170.

18 Clouthier SC, Rector T, Brown NE, Anderson ED: Genomic organization of infectious salmon anaemia virus. J Gen Virol 2002;83(2):421-428.

19 Biering E, Falk K, Hoel E, Thevarajan J, Joerink M, Nylund A, et al: Segment 8 encodes a structural protein of infectious salmon anaemia virus (ISAV); the co-linear transcript from Segment 7 probably encodes a non-structural or minor structural protein. Dis Aquat Organ 2002;49(2):117-122.

20 McBeath AJA, Collet B, Paley R, Duraffour S, Aspehaug V, Biering E, et al: Identification of an interferon antagonist protein encoded by segment 7 of infectious salmon anaemia virus. Virus Research 2006;115(2):176-184.

21 Ingerslev HC, Petters validation of referenc (*Salmo salar* L.). Mol

22 Longo N, Berninger NS in polymerase chain r

Dr. M. Snow, FRS Marine
E-mail: M.Snow@marlab.

21 Ingerslev HC, Pettersen EF, Jakobsen RA, Petersen CB, Wergeland HI: Expression profiliing and validation of reference gene candidates in immune relevant tissues and cells from Atlantic salmon (*Salmo salar* L.). Molecular Immunology 2006;43(8):1194-1201.

22 Longo N, Berninger NS, Hartley JL: Use of uracil DNA glycosylase to control carry-over contamination in polymerase chain reactions. Gene 1990;93:125-128.

Dr. M. Snow, FRS Marine Laboratory, 375 Victoria Rd, Aberdeen, AB11 9DB, U.K.
E-mail: M.Snow@marlab.ac.uk