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Absolute quantitation of infectious salmon anaemia virus using different real-time reverse transcription PCR chemistries

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Routine laboratory diagnosis of infectious salmon anaemia virus (ISAV) infection is primarily by reverse transcription polymerase chain reaction (RT-PCR) because of the high sensitivity and rapid turnaround time of the test. This paper describes methods for highly reproducible absolute viral load measurements using external standard curves generated with either ISAV recombinant plasmid DNA (pDNA) standards or transcribed RNA standards prepared by *in vitro* transcription with T7 RNA polymerase, and using a two tube real-time or quantitative (q)RT-PCR with SYBR® Green I chemistry and a single tube qRT-PCR with TaqMan® probe chemistry. When applied to virus samples of known virus titer for the highly pathogenic ISAV strain NBISA01 and the low pathogenic ISAV strain RPC/NB-04-085-1, both methods showed a 100-fold lower detectable titer for RPC/NB-04-085-1 but with a higher number of viral RNA molecules compared to NBISA01. Overall, the SYBR® Green I method overestimated copy numbers in samples having equivalent Ct values with the TaqMan® probe method. Taken together, the findings suggest that the TaqMan® probe method with the *in vitro* transcribed RNA standard curve is the preferred method for reliable and rapid quantitation of ISAV in samples.

1. Introduction

Infectious salmon anaemia (ISA) is a highly fatal viral disease of marine-farmed Atlantic salmon caused by ISA virus (ISAV), an orthomyxovirus belonging to the genus *Isavirus* within the family *Orthomyxoviridae* (Kawaoka et al., 2005). The genome is composed of eight segments of linear, single-stranded (ss)RNA of negative sense ranging in length from 1.0 to 2.4 kb with a total molecular size of approximately 14.3 kb (Clouthier et al., 2002). The fish disease is reportable with the World Animal Health Organization (OIE). Virus can be detected in fish tissues by RT-PCR (Mjaaland et al., 1997), electron microscopy (Hovland et al., 1994), indirect fluorescent antibody test (IFAT), and by virus isolation using permissive fish cell lines (Dannevig et al., 1995; Bouchard et al., 1999; Kibenge et al., 2001). Comparison of the different methods showed the RT-PCR method to be the most sensitive for virus detection (Snow et al., 2003).

Data generated by real-time or quantitative (q) RT-PCR can be analyzed using either absolute or relative quantitation (reviewed by Bustin, 2005). Absolute quantitation requires construction of a standard curve using relevant standards such as a known copy number of plasmid DNA (pDNA) or *in vitro* transcribed RNA standards (Bustin, 2000; Wong and Medrano, 2005). Relative quantitation describes the change in expression of the target gene relative to some untreated reference sample and normalized to a reference gene, usually a housekeeping gene (reviewed in Giulietti et al., 2001; Livak and Schmittgen, 2001). It is becoming increasingly apparent that more than one reference gene is required for proper use of relative quantitation (a minimum of three reference genes is recommended), making it cumbersome to use let alone to compare test performance between different laboratories. Moreover, housekeeping genes are not necessarily appropriate references for qRT-PCR data normalization (Sellars et al., 2007). In contrast, absolute quantitation analysis is useful in determining absolute viral RNA copies based on a constant, allowing straight forward comparison of data from different PCR runs on the same day or on different days, and more importantly between different laboratories.

There are two chemistries used most commonly for detection of PCR products during qRT-PCR. These are the DNA binding fluorophore SYBR® Green I (Simpson et al., 2000), and the sequence-specific fluorescently labeled probes (Holland et al., 1991; Lay and Wittwer, 1997). Quantitation of ISAV by qRT-PCR first utilized the SYBR® Green I format, targeting RNA segment 8 (Munir and Kibenge, 2004), and then subsequently used TaqMan® probes initially targeting RNA segment 8 (Mjaaland et al., 2005) and then comparing RNA segments 7 and 8 (Snow et al., 2006). The authors found the segment 8 TaqMan® qRT-PCR assay to be more sensitive

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than the segment 7 TaqMan® qRT-PCR assay (Snow et al., 2006). All the previous ISAV quantitation reports have used relative quantitation of ISAV transcripts calibrated to housekeeping genes (Mjaaland et al., 2005; Kileng et al., 2006; Jørgensen et al., 2007; Snow et al., 2006), however, there has never been correlation with biological significance of the amount of viral RNA detected in a sample. Using expression of reference/housekeeping genes is relevant when studying gene expression, but has less relevance in viral quantitation except for estimating the quality of the RNA in a sample and detecting presence of inhibitory effects. This report describes the use of ISAV segment 8 pDNA and *in vitro* transcribed RNA standards for absolute quantification of ISAV RNA copy number equivalents in both a two tube qRT-PCR using SYBR® Green I and a single tube one-step qRT-PCR with a TaqMan® probe. Moreover, this study established the relationship of qRT-PCR cycle threshold (Ct) value to median tissue culture infectious dose ($TCID_{50}$) when used to assess viral load in a sample for ISAV isolates of differing pathogenicities. Considering the replication strategy of influenza viruses, ISAV replication is expected to generate viral mRNA and cRNA from the vRNA genome. When primed with gene specific primer or random hexamers, the total RNA from ISAV-infected cultures will have a population of cDNA generated from viral mRNA, cRNA, and vRNA. It is possible to specifically amplify ISAV vRNA by priming the non-coding UTR region in the RT step with sequence specific primer but this requires a two-step RT-PCR to allow RNase treatment before addition of a second gene specific primer in the PCR step. Thus, the idea of relating transcript copies to ISAV genome equivalents is limited when using cDNA primers that are not specific for vRNA, although this method was used for absolute quantitation of coronavirus (Vijgen et al., 2005), a non-segmented ssRNA virus of positive sense. When quantitating segmented RNA viruses the question would be how many individual genome segments are contained in an infectious virus particle? ISAV is not well studied in this respect; but influenza virions containing more than eight individual RNA segments have been isolated (Flint et al., 2004). Thus for the present study, in order to extrapolate the segment 8 ISAV copies as ISAV RNA copy number equivalents, an assumption was made that the genome in a single infectious ISAV particle has at least one molecule of each RNA segment.

2. Materials and methods

2.1. Viruses and virus culture

Two ISAV isolates of differing genotypes and pathogenicities were compared. NBISA01 is a highly pathogenic strain belonging to the North American genotype, whereas RPC/NB 04-085-1 is a low pathogenic strain of the European genotype found in Eastern Canada (Kibenge et al., 2006). The two isolates have variations in the amino acid sequence of the haemagglutinin-esterase (HE) protein, with deletions of 13 and 17 amino acids in the highly polymorphic region (HPR) for RPC/NB 04-085-1 and NBISA01, respectively (Kibenge et al., 2007). In an experimental trial using equal viral doses, NBISA01 induced very high mortality in Atlantic salmon (95%) and moderate mortality in rainbow trout (50%), whereas RPC/NB 04-085-1 induced very low mortality in Atlantic salmon (18.2%) and no mortality in rainbow trout (Kibenge et al., 2006). These ISAV isolates were propagated in the TO cell line (Wergeland and Jakobsen, 2001) and the cell lysates were titrated on TO cell monolayers as described previously (Kibenge et al., 2001). For serial sampling during virus replication, virus was propagated in 24 h-old TO cell monolayers (~80% confluent) in six-well tissue culture plates. Infected cells were incubated at 16 °C in maintenance medium. Sampling was done at days 0, 3 and 6 by freezing the whole plate at −80 °C prior to the total RNA extraction step.

2.2. Sample extraction

Total RNA was extracted from virus samples and fish tissue samples using 1.25 ml of TRIZOL Reagent (Invitrogen) and 375 μl of sample volume. For the fish tissue samples, each tissue was weighed and macerated to a 10% suspension (w/v) in PBS with 10× antibiotics. The extracted RNA was eluted in 20–50 μl of nuclease-free water and was treated with DNAse I using the Roche DNAse treatment kit following the manufacturer's procedure. RNA was quantitated by UV spectrophotometry.

2.3. First strand cDNA synthesis

For use in the two tube SYBR® Green I method, first strand cDNA synthesis was performed using the Transcriptor reverse transcriptase first strand cDNA synthesis kit (Roche). Different amounts of RNA were used in cDNA synthesis depending on the source of the RNA. cDNA synthesis of ISAV segment 8 transcribed RNA used 1 μl of RNA per reaction. cDNA synthesis of total RNA extracted from the different virus samples of known virus titer (in $TCID_{50}$) and ISAV-positive fish tissues used 1 μl of RNA per reaction. cDNA synthesis of total RNA extracted from serial sampling during virus replication in TO cells used 300 ng of RNA per reaction. Three different primers were used for cDNA synthesis; random hexamer primers and oligo-dT primers that come with the cDNA synthesis kit (Roche), and the gene specific F5/R5 primers. The F5/R5 primers were first described by Devold et al. (2000) to amplify 220 bp of the ISAV segment 8, and previously described for single tube one-step qRT-PCR (Munir and Kibenge, 2004). The cDNA synthesis master mix consisted of 4 μl of 5× RT reaction buffer, 2 μl of dNTP mix (200 μM), primer (2 μl of random hexamer (600 μM) or 2 μl of oligo-dT primer (0.8 μg/μl) or 1 μl of gene specific F5/R5 primer (20 μM)), 0.5 μl RNase inhibitor (40 U/μl), 0.5 μl of Transcriptor reverse transcriptase (20 U/μl), and nuclease-free water to adjust the 20 μl volume. The reactions were incubated at 25 °C for 10 min followed by 55 °C for 30 min with a final enzyme denaturation at 85 °C for 5 min (Workenhe et al., 2008).

2.4. Preparation of plasmid DNA standards

The pDNA standard was obtained by cloning the 878 bp genomic RNA of ISAV segment 8 RT-PCR product (Cunningham and Snow, 2000) into the pCRII-TOPO vector (Invitrogen); the clone was designated pCRIITOPODNA-NBISA01-S8. The recombinant plasmid was purified using the High Pure Plasmid Purification kit (Roche). The plasmid DNA concentration was determined in triplicate by UV spectrophotometry. The mass of a single pDNA molecule was calculated using the formula 1 bp ~ 660 g/mol and the 4880 bp size of the recombinant plasmid, following the method in the ABI Manual of absolute real-time RT-PCR quantification (Anon., 2003).

2.5. In vitro transcription of ISAV RNA segment 8

The pCRIITOPODNA-NBISA01-S8 clone was also used for *in vitro* transcription with T7 RNA polymerase in the sense direction in order to generate *in vitro* transcribed RNA. For this, 200 ng of recombinant plasmid was linearized by digestion with BamHI enzyme (New England Biolabs) in a 20 μl reaction volume following the manufacturer's protocol. The linearized DNA was then purified using the QIA quick PCR purification kit (Qiagen), and was recovered in 30 μl of elution buffer. *In vitro* transcription was carried out in a 40 μl volume using 20 μl of linearized plasmid DNA, 1× T7 RNA polymerase buffer, 2 μl of 100 mM DTT, 16 μl of 10 mM NTPs (Invitrogen), 1 μl RNase OUT (40 U/μl) (Invitrogen), and 1 μl of T7 RNA

polymerase (50 U/μl) (Invitrogen). The reaction was incubated for 2 h at 37 °C. RNA purification was carried out using RNeasy kit (Qiagen), and was eluted in 30 μl of nuclease-free water. Nucleic acid concentration was determined by UV spectrophotometry. DNase treatment was done using 1 unit of RQ1 RNase-free DNase I (1 U/μl) (Promega) per μg of RNA following the manufacturer's procedure. This treatment was performed twice to ensure complete elimination of any residual plasmid DNA (which could potentially yield a positive result in two-step RT-PCR even in the absence of RT). RNA was cleaned up using RNeasy kit and eluted in 30 μl of nuclease-free water and the concentration was again determined by UV spectrophotometry. The ISAV segment 8 *in vitro* transcribed RNA was analyzed using a native 1% agarose gel to check the integrity of the RNA before use.

2.6. Construction of ISAV segment 8 in vitro transcribed RNA standards

The concentration of the ISAV *in vitro* transcribed RNA was determined by UV spectrophotometry in triplicate. The copy number of the *in vitro* transcribed RNA per microliter was calculated as described by Fronhoffs et al. (2002). Serial 10-fold dilutions of the RNA transcripts were prepared starting with the highest concentration of 2.79×10^{11} copies/μl. For use in the two tube SYBR® Green I method, cDNA synthesis was carried out using 1 μl of each *in vitro* transcribed RNA serial dilution. The single tube one-step qRT-PCR TaqMan® method used 8 μl of each *in vitro* transcribed RNA serial dilution per reaction.

2.7. Two tube qRT-PCR with SYBR Green I chemistry, and standard curves and quantitation

QPCR was performed on the first strand cDNA using the LightCycler (LC) 1.2 instrument (Roche) with Fast Start DNA Master SYBR® Green I (Roche) and the ISAV segment 8 primer pair F5/R5 amplifying 220-bp product (Devold et al., 2000; Munir and Kibenge, 2004). Briefly, the 20 μl reaction consisted of 2 μl of cDNA and 18 μl of the master mix prepared using 0.3 μl of the 20 μM of the forward and reverse primers (final concentration of 0.3 μM), 2 μl SYBR® Green I, 3.2 μl of the 25 mM stock $MgCl_2$ (a final concentration of 0.005 μM), and 12.2 μl of nuclease-free water. The cycling conditions consisted of 10 min denaturation at 95 °C to activate the hot start polymerase followed by 50 cycles of 95 °C for 5 s, 59 °C for 10 s, 72 °C for 10 s, and detection at 80 °C for 2 s. Melting curve analysis was performed from 70 to 95 °C in 0.1 °C/s increments to assess the specificity of the RT-PCR products. For generation of the standard curves, the pDNA and *in vitro* transcribed RNA standards were run in triplicates. In order to use standard curves to calculate the ISAV segment 8 genome copies, pDNA and *in vitro* transcribed RNA standards were run alongside the unknown samples. For calculating viral genome copy numbers/ml of unknown sample, the viral genome equivalents/20 μl PCR reaction was multiplied by a factor of $20/11 \times 1000/375$. The factor is based on cDNA synthesis using 11 μl of the total 20 μl RNA eluted from 375 μl of virus lysate, and the use of 2 μl of cDNA from the 20 μl cDNA synthesis reaction. The Ct values were used to generate a standard curve plot of cycle number (*Y*-axis) versus log concentration (*X*-axis). The quality of standard curves was judged by the slope of the standard curve and the correlation coefficient (r). The slope of the line was used to estimate the efficiency of the target amplification using the equation $E = (10^{-1/\text{slope}}) - 1$. In case of the SYBR® Green I qRT-PCR, melting curve analysis was used to check the specificity of the RT-PCR product. In some cases, the RT-PCR products were resolved in 1% agarose gel electrophoresis.

2.8. Single tube one-step qRT-PCR with TaqMan® chemistry, and standard curves and quantitation

The single tube one-step qRT-PCR with TaqMan® primers and probe targeting ISAV segment 8 is a modification of the TaqMan® qRT-PCR assay for the detection of ISAV described by Snow et al. (2006), which uses relative quantitation methods. The modifications made in this study included use of a single tube with a one-step RT-PCR kit (Roche) and TaqMan probe in a 96-well plate in the LC 480 instrument (Roche) with absolute quantitation methods. Briefly, 8 μl of RNA are added to 12 μl of master mix consisting of 9.28 μl LC 480 RNA Master hydrolysis probe, 1.88 μl of activator $Mn(OAc)_2$ (50 mM), 1 μl of enhancer (20×), 1.13 μl of ISAV Segment 8 Forward primer and Reverse primer (Snow et al., 2006) (20 mM each) and 1.04 μl of ISAV segment 8 probe (Snow et al., 2006) (6 μM). The primers and probe binding sequences are identical for both of the virus isolates used in the present study. The cycling conditions consisted of 1 cycle of RT for 3 min at 63 °C followed by denaturation at 95 °C for 3 s, and 45 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 1 min and amplification and detection at 72 °C for 1 s. For generation of the standard curve, the *in vitro* transcribed RNA standards were run in 5 replicates. The standard curve was constructed automatically with LC software version 4.0 (Roche) using the Ct values obtained when the serial 10-fold dilutions of the *in vitro* transcribed RNA samples with known numbers of RNA transcripts were used as templates. The standard curve obtained was then used as an external standard curve in all subsequent TaqMan® qRT-PCR assays on LC480. For calculating ISAV RNA copy number equivalents per ml of unknown sample, the ISAV RNA copy number equivalents/20 μl RT-PCR reaction was multiplied by a factor of $20/8 \times 1000/375$ based on the use of 8 μl of the total 20 μl RNA eluted from 375 μl of virus lysate used for RT-PCR reaction.

2.9. Construction of a standard curve for estimating $TCID_{50}$ from Ct values

To construct a standard curve for relating the virus titer of a sample expressed as $TCID_{50}$ with the Ct values obtained in qRT-PCR, total RNA extracted from 10-fold dilutions of virus lysates of ISAV strains NBISA01 and RPC/NB-04-0851 was tested with both the two tube qRT-PCR with SYBR® Green I chemistry and the single tube one-step qRT-PCR with TaqMan® chemistry. The $TCID_{50}$ (*X*-axis) of each sample was then plotted against the respective Ct value (*Y*-axis), and a linear fit was constructed as described in Falsey et al. (2003).

3. Results and discussion

3.1. Generation of ISAV RNA segment 8 recombinant plasmid DNA standards and absolute quantitation of ISAV with SYBR® Green I chemistry

In order to develop a qRT-PCR method for absolute quantitation of ISAV segment 8 RNA transcripts, the full segment 8 genomic RNA sequence was cloned into a pCRII-TOPO vector for use as the standard. The copy numbers of the pDNA standards prepared ranged from 3×10^1 to 3×10^9. Preliminary qRT-PCR analysis showed that copy numbers below 3×10^2 gave inconsistent Ct values within the triplicates. Thus pDNA standards of 3×10^2 to 3×10^7 copies were used to generate the standard curve. The curve had a PCR amplification efficiency of 2.0 with high linearity (correlation coefficient $r = 0.9979$). The pDNA standard curve was used to quantify the ISAV segment 8 cDNA copy number/ng

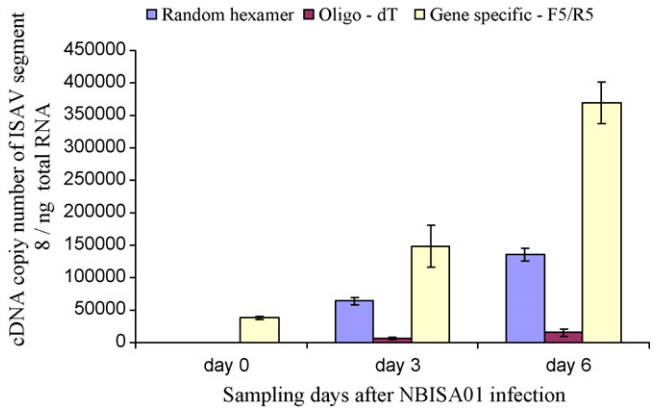

Fig. 1. cDNA copy number of ISAV segment 8 from unknown samples of cDNA generated using RNA extracted from TO cells infected with ISAV strain NBISA01 and three priming strategies (random hexamer, oligo-dT and gene specific primer) (data are average ± S.D. of three separate triplicates). The qRT-PCR used SYBR® Green I chemistry with absolute quantitation based on the external standard curve of the ISAV RNA segment 8 pDNA standards.

of total RNA extracted from ISAV-infected TO cells and primed for cDNA synthesis using three different priming strategies (oligo-dT, random hexamers, and gene specific F5/R5 primers). In all the cDNA priming strategies the 0 h samples showed the lowest copy number compared to the 3-day and 6-day samples, indicating specific increase in ISAV transcripts due to virus replication. cDNA generated using the gene specific primer showed an overall highest absolute copy number of the ISAV segment 8 ng^{-1} of total RNA, followed by that of the random hexamer and then the oligo-dT primer (Fig. 1). The agarose gel electrophoresis of the RT-PCR products showed the expected 220 bp PCR product for the three different priming strategies (data not shown), confirming specificity of the PCR reactions. Each primer has a different way of priming cDNA synthesis from RNA of ISAV-infected TO cell lysates. The oligo-dT primer binds to the poly(A) tails of mRNA to generate first strand cDNA, whereas the gene specific F5/R5 primer anneals specifically to ISAV segment 8 genomic RNA, cRNA or mRNA to generate first strand cDNA. Random hexamers are capable of priming cDNA at many points along the viral RNA, cRNA, or mRNA template as well as cellular RNA templates, generating fragmentary copies of entire populations of RNA molecules (Sambrook and Russel, 2001).

As expected from the limiting RNA populations that can be primed using oligo-dT primers, this priming strategy had the lowest copy number of ISAV transcripts at all the sampling points. The process of mRNA synthesis from the ssRNA genome of ISAV is not well studied. However, influenza A virus which belongs to the *Orthomyxoviridae* family similarly to ISAV has been well studied in this respect. Influenza A virus negative-strand RNA (vRNA) serves as a template for the synthesis both of capped, polyadenylated viral mRNA and of full-length positive-strand RNA or complementary RNA (cRNA) (Cros and Palese, 2003). The poly(A) tail of influenza virus mRNA is synthesized by reiterative copying of a 5–7 nt long U sequences of 16 nt from the 5′ end of the viral RNA template. The cRNA is associated with the same viral proteins as the vRNA and serves as a template for the synthesis of new vRNA molecules, which in turn serve as templates for mRNA and cRNA, particularly early in the infection (Robertson et al., 1981; Fodor and Smith, 2004; Amorim and Digard, 2006). Even though there is no detailed characterization of molecular replication strategy of ISAV, sequencing of 3′ and 5′ ends of segments 7 and 8 has revealed that ISAV mRNA is polyadenlyated (Sandvik et al., 2000).

3.2. Generation of ISAV RNA segment 8 in vitro transcribed RNA standards and absolute quantitation of ISAV with SYBR® Green I chemistry and TaqMan® probe chemistry

Since ISAV has a ssRNA genome, it was necessary to use *in vitro* transcribed RNA of the full segment 8 coding sequence to construct a standard curve. It was considered that *in vitro* transcribed RNA templates would estimate more accurately template amounts in the RNA inputs and therefore give a more accurate quantitation as they would be subjected to the same RT reaction (unlike the pDNA standards for qPCR). For initial calibration, first strand cDNA synthesis used the gene specific F5/R5 primer from 10-fold diluted *in vitro* transcribed RNA of 10^1–10^{10} copies. The F5/R5 primer showed non-specific amplification signals in transcribed RNA preparations with $\leq 10^5$ copies. Thus, for comparison of the F5/R5 with the random hexamer priming, *in vitro* transcribed RNA standards were prepared in serial 5-fold dilutions with copies ranging from 3.2×10^6 to 1×10^9. The qPCR was carried out under the same conditions as for the pDNA standard curve. The F5/R5 primed cDNA had a higher amplification efficiency (E) of 2.14 compared to that for random hexamers primed cDNA ($E = 1.94$), which was mainly a result of the lower dilutions of the *in vitro* transcribed RNA standards, which generated closer Ct values between dilutions. These low template reactions in the F5/R5 primed cDNA were associated with primer-dimers. Oligo-dT priming was not attempted on the *in vitro* transcribed RNA templates since they were not polyadenylated.

From the standard curves obtained using *in vitro* transcribed RNA standards with the SYBR® Green I chemistry, we selected one default method for estimating the viral load in the unknown samples. For this, the utility of the two primers (random hexamers and gene specific F5/R5; Oligo-dT primers were excluded since they would not be specific for T7 *in vitro* transcribed non-polyadenylated RNA standards) to prime cDNA synthesis from all ISAV templates (vRNA, cRNA, and mRNA) and optimality of the PCR amplification efficiency were compared. The F5/R5 showed non-specific primer-dimers in reactions using $\leq 10^5$ *in vitro* transcribed RNA copies whereas the quantitation limit with random hexamer primers was 10^3 *in vitro* transcribed RNA copies; the standard curves generated with random hexamer primers also had a better PCR efficiency compared to those with the gene specific F5/R5 primers. Thus, the random hexamer cDNA priming-based two step method was selected as the default for absolute quantitation with the SYBR® Green I chemistry.

In order to provide a method for absolute quantitation of ISAV using TaqMan® probe chemistry, the segment 8 TaqMan® probe-based qRT-PCR assay developed and validated by Snow et al. (2006) was modified for use in a single tube with a one-step RT-PCR kit (Roche) in a 96-well plate in the LC 480 instrument (Roche) with absolute quantitation methods. Preliminary qRT-PCR analysis showed that the *in vitro* transcribed RNA preparations with $\geq 2.2 \times 10^{11}$ copies (or ≥15.16 ng/μl cRNA) inhibited the RT-PCR, giving Ct values >34.0 whereas for preparations with 2.2×10^{10} copies, the Ct value was <5.0 and increased in proportion to the dilution of the *in vitro* transcribed RNA preparation with a detection limit of 2.2×10^1 *in vitro* transcribed RNA copies. Based on these observations, serial 10-fold dilutions were prepared, and those in the range from 2.2×10^9 to 2.2×10^2 were used to establish a standard curve for ISAV segment 8 RNA transcripts with 2–5 replicates per dilution point. The standard curve had an amplification efficiency of 1.965 and error of 0.00866.

Table 1 summarizes a comparison between the two methods (SYBR® Green I-based two tube qRT-PCR versus TaqMan® probe-based single tube one-step qRT-PCR) when applied to RNA extracted from eight serial 10-fold dilutions of virus lysates of NBISA01 and RPC/NB 04-085-1 in terms of their dynamic range

Table 1
Comparison of the dynamic range of ISAV segment 8 two tube qRT-PCR with SYBR Green I chemistry and single tube one-step qRT-PCR with TaqMan probe chemistry

	Two tube qRT-PCR with SYBR Green I		Single tube one-step qRT-PCR with TaqMan probe	
	NBISA01 ($10^{8.75}$ $TCID_{50}$/ml)	RPC/NB 04-085-1 ($10^{5.75}$ $TCID_{50}$/ml)	NBISA01 ($10^{8.75}$ $TCID_{50}$/ml)	RPC/NB 04-085-1 ($10^{5.75}$ $TCID_{50}$/ml)
Dynamic range ($TCID_{50}$/ml)	$10^{0.75}$	$10^{0.75}$	$10^{1.75}$	$10^{1.75}$
Reliable detection[a] limit ($TCID_{50}$/ml)	$10^{4.75}$	$10^{2.75}$	$10^{4.75}$	$10^{2.75}$
Reliable detection[a] limit (ISAV RNA copy number equivalents/ml)	5956.4 ± 24.3	18568.1 ± 539.1	27.7 ± 1.8	227.2 ± 55.8

[a] Reliable detection is defined as the dilutions run in triplicate giving similar Ct values.

relative to the virus titres expressed as $TCID_{50}$/ml. Both methods had the same $TCID_{50}$ detection limits for NBISA01 and RPC/NB-04-085-1, $10^{4.75}$ $TCID_{50}$/ml and $10^{2.75}$ $TCID_{50}$/ml, respectively. For the same titers of the two isolates the SYBR® Green I method showed 215- and 81-fold higher copy numbers for NBISA01 and RPC/NB-04-085-1, respectively. The data suggest that there is 100-fold lower detectable virus titer of the low pathogenic ISAV strain RPC/NB-04-085-1, which was accompanied by 3- (in the SYBR® Green I method) and 8-fold (in the TaqMan® method) higher copy number of RPC/NB-04-085-1 compared to NBISA01. The difference in the $TCID_{50}$ detection limit for the two virus strains is probably related to the fact that qRT-PCR also detects viral RNA in non infectious or defective virus particles which are probably more in the lower pathogenic ISAV. This would imply that the molecular basis for the virulence difference between the two viruses occurs at the post-transcription steps of virus replication, probably resulting in a higher production of non-infectious virus particles by the low pathogenic ISAV strain RPC/NB-04-085-1.

While using the same *in vitro* transcribed RNA standards for quantitation of ISAV RNA copy equivalents in both chemistries, the SYBR® Green I-based system reported higher RNA copies per ml of virus lysate for the same Ct value in the TaqMan® probe chemistry (data not shown). The difference can be partly explained by the sequence-specific detection chemistry of the TaqMan probe chemistry (Holland et al., 1991; Lay and Wittwer, 1997), compared to the non-specific dsDNA binding of SYBR® Green I fluorophore (Simpson et al., 2000). Moreover, the SYBR® Green I method loses reliability in reactions with low templates amounts (Ct values ≥35.0). The inconsistency of SYBR® Green I readings for low template reactions was previously reported by Walters and Alexander (2004).

3.3. Correlation of $TCID_{50}$ with Ct values

Using the reliable detection limit (reliable detection is defined as the dilutions run in triplicate giving similar Ct values) for the virus titrations a standard curve was constructed to estimate $TCID_{50}$/ml from Ct values for the two ISAV strains, NBISA01 and RPC/NB-04-085-1. The standard curve plots of Ct versus $\log_{10}$ $TCID_{50}$/ml for the SYBR® Green I and TaqMan chemistries are shown in Fig. 2A and B. Both plots have a linear model fit and have small slope difference between the isolates manifested by the parallel nature of the two lines. Both the SYBR® Green I and TaqMan reaction linear fits suggest that for a certain Ct value NBISA01 will have higher $\log_{10}$ $TCID_{50}$ compared to the RPC/NB-04-085-1 for the range of dilutions considered. This is consistent with NBISA01 being highly pathogenic (Kibenge et al., 2006) where with lower viral genome copies, it can give a higher titer $TCID_{50}$ compared to the less pathogenic RPC/NB-04-085-1. Similarly, using the NBISA01 dilutions that have 5 points on the standard curve, the linear fits generated using the TaqMan® one tube one-step method were compared with the linear fit generated using the SYBR® Green I two

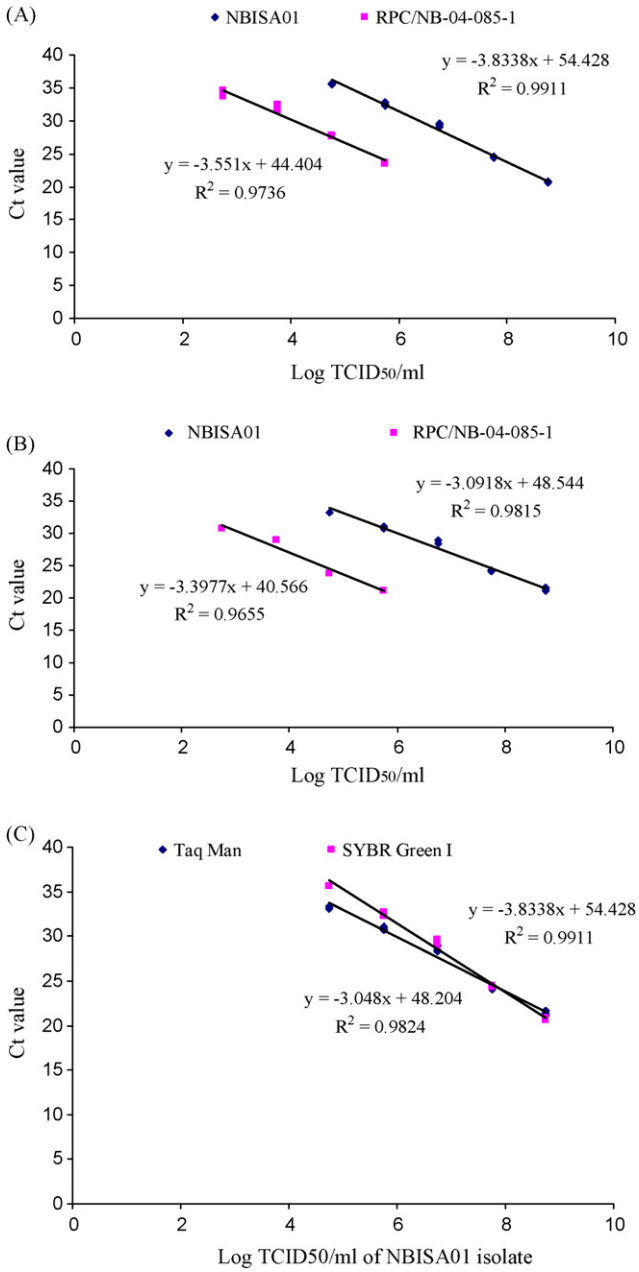

Fig. 2. Standard curve relating $TCID_{50}$ to Ct value from RT-PCR using 10-fold dilutions of virus lysates of known titre (A) SYBR Green I two tube method, Ct values vs. $\log_{10}$ $TCID_{50}$ for NBISA01 and RPC/NB-04-085-1, (B) TaqMan one tube method, Ct values vs. $\log_{10}$ $TCID_{50}$ for NBISA01 and RPC/NB-04-085-1, (C) SYBR Green I two-tube and TaqMan one-tube method, Ct values vs. $\log_{10}$ $TCID_{50}$ for NBISA01.

tube method (Fig. 2C). The linear fits show slight differences in the two methods in that the SYBR® Green I two tube reactions show a slightly higher Ct value for a specified $TCID_{50}$ below $10^{7.92}$ ml^{-1} (the $TCID_{50}$ value where the two lines cross). The difference can be explained by the fact that the TaqMan® probe method involves a single step that uses all the cDNA from the RT-step whereas the SYBR® Green I method uses 2 μl of cDNA generated in 20 μl reaction, introducing a 10-fold dilution of the cDNA template. Thus, the slightly lower Ct values of the SYBR® Green I two tube method are related directly to the template cDNA amounts available for the PCR stage.

The standard curve for NBISA01 constructed using the TaqMan® single tube one-step method was used to estimate the $TCID_{50}$ in tissue samples of experimentally infected fish. The standard curve estimated the fish tissue samples to have virus titers ranging from $10^{4.87}$ to $10^{6.23}$ $TCID_{50}$/ml. Thus, the method can be used to estimate ISAV loads in fish tissues based on RNA copy numbers, and to estimate the viral titers ($TCID_{50}$) without use of the time-consuming virus titration in fish cell lines.

In conclusion, this report describes methods for absolute quantitation of ISAV genome copies using external standard curves generated with either ISAV pDNA standards or *in vitro* transcribed RNA standards, and for the first time report a correlation of Ct values to viral titers expressed as $TCID_{50}$/ml using two ISAV isolates of differing pathogenicities and two detection chemistries. Both SYBR® Green I and TaqMan® probe chemistries showed a 100-fold lower detectable titer for RPC/NB-04-085-1 but with a higher number of viral RNA starting molecules compared to NBISA01, indicating that the low pathogenic ISAV produces more non-infectious or defective particles than the highly pathogenic ISAV. Overall, the SYBR® Green I method overestimated ISAV RNA copy number equivalents in samples having equivalent Ct values with the TaqMan® probe method. Thus, the TaqMan® probe method with the *in vitro* transcribed RNA standard curve is the better method for reliable and rapid quantification of ISAV in samples.

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