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

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Abstract

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 NPC/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., 1995; Dannevig 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 straightforward 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...
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; Jorgensen 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 (TCID50) 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 quantifying 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 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 ºC in maintenance medium. Sampling was done at days 0, 3 and 6 by freezing the whole plate at −80 ºC 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 TCID50) 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/RS primers. The F5/RS 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/RS 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 ºC for 10 min followed by 55 ºC for 30 min with a final enzyme denaturation at 85 ºC 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 pCRII-TOPODNA-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 pCRII-TOPODNA-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 x 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 nuclelease-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 nuclelease-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 × 1011 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 MgCl2 (a final concentration of 0.005 μM), and 12.2 μl of nuclelease-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 20/8 × 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.

2.8. 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 (20X), 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 × 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 TCID50 from Ct values

To construct a standard curve for relating the virus titer of a sample expressed as TCID50 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 TCID50 (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 × 103 to 3 × 109. Preliminary qRT-PCR analysis showed that copy numbers below 3 × 102 gave inconsistent Ct values within the triplicates. Thus pdNA standards of 3 × 102 to 3 × 107 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
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 $10^3$–$10^6$ copies. The F5/R5 primer showed non-specific amplification signals in transcribed RNA preparations with $\leq 10^2$ 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 $3.2 \times 10^5$ to $1 \times 10^9$. 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 $< 10^5$ in vitro transcribed RNA copies whereas the quantitation limit with random hexamer primers was $10^5$ 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 $\geq 15.16 \, \text{ng/\muL}$ cRNA) inhibited the RT-PCR, giving Ct values $> 34.0$ whereas for preparations with $2.2 \times 10^{10}$ 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 $2.2 \times 10^6$ in vitro transcribed RNA copies. Based on these observations, serial 10-fold dilutions were prepared, and those in the range from $2.2 \times 10^9$ to $2.2 \times 10^5$ 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.

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Table 1: Comparison of 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^{9.75} TCID_{50}/ml)   | RPC/NB-04-085-1 (10^{7.75} TCID_{50}/ml)        |
| Dynamic range (TCID_{50}/ml) | 10^{7.75}                         | 10^{7.75}                                     |
| Reliable detection limit (TCID_{50}/ml) | 10^{4.75}                         | 10^{4.75}                                     |
| Reliable detection limit (ISAV RNA copy number equivalents/ml) | 5956.4 ± 24.3                     | 18568.1 ± 539.1                               |

\(^a\) 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 differences 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
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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 TCID50/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 titre 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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