Development of a quantitative Light Cycler real-time RT-PCR for detection of avian reovirus

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Abstract

A robust, ultrasensitive, and accurate quantitative assay was developed for avian reovirus (ARV) with the Light Cycler SYBR Green-based real-time reverse transcription-PCR (real-time LC RT-PCR). The assay exhibited high specificity as all negative controls and other avian pathogens, such as Newcastle disease virus (NDV), infectious bronchitis virus (IBV), infectious bursal disease virus (IBDV), avian influenza virus (AIV), and mycoplasma synovia (MS), failed to show any positive detection. A minimum of 39 copies/l of ARV genomic RNA could be detected by the assay. By dilution analysis, the real-time LC RT-PCR developed in this study was 3-log more sensitive than the conventional RT-PCR for the detection of ARV. The vaccine and field isolates of ARV were detected by the real-time LC RT-PCR. As a result of the high sensitivity and specificity of the assay with a relatively rapid and simple procedure, the real-time LC RT-PCR will be useful as a routine assay for the clinical diagnosis of ARV infection.

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1. Introduction

Avian reovirus (ARV) is an important cause of diseases in poultry. In particular, reovirus-induced arthritis, chronic respiratory diseases, and malabsorption syndrome (Fahey and Crawley, 1954) provoke considerable economic losses. ARV and mammalian reovirus (MRV) belong to the genus Orthoreovirus. Both share physical–chemical and morphological characteristics, that include segmented genomes consisting of 10 genome segments of double-stranded (ds) RNA. The RNA is packaged into a non-enveloped icosahedral double capsid (Spandidos and Graham, 1976). All ARV-encoded proteins, that include at least 10 structural proteins (λA, λB, λC, μA, μB, μBC, μBN, σC, σA, and σB), and four nonstructural proteins (μNS, P10, P17, and σNS) have been demonstrated (Varela and Benavente, 1994; Bodelon et al., 2001). The genome S1 contains three open reading frames which translated P10, P17, and σC proteins. Protein σC, encoded by the third open reading frame of S1 genome segment (Shapouri et al., 1995), shows noticeably higher divergence than other σ-class proteins (Liu et al., 2003). σC is not only a cell attachment protein (Martinez-Costas et al., 1997) but also an apoptosis inducer (Shih et al., 2004). Protein σC is also the target for type-specific neutralizing antibodies while antibodies against σB are group-specific (Wickramasinghe et al., 1993). Protein P10 is a viroporin (Bodelon et al., 2002) and responsible for ARV-encoded cell fusion (Bodelon et al., 2001; Shmulevitz and Duncan, 2000). A recent report suggests that P17 retards cell growth by activation of P53 pathway (Lin et al., 2005). ARV is encoded by the genome segment S2 (Yin et al., 2000), has been identified as a double-stranded RNA (dsRNA) binding protein (Yin et al., 2000). In addition, ARV is an inhibitor of the double-stranded RNA-dependent protein kinase

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Another protein of ARV, σNS, encoded by the genome segment S4 (Chiu and Lee, 1997), has been reported for its single-stranded RNA (ssRNA) binding activity (Yin and Lee, 1998; Touris-Otero et al., 2005). More recently, an epitope on the σNS required for ssRNA binding has been mapped (Huang et al., 2005).

The polymerase chain reaction (PCR) as a diagnostic technique is generally known as a very sensitive, specific, and rapid tool for detection of viruses. It has been shown that reverse transcription (RT)-PCR can be used in the detection of avian reovirus (Lee et al., 1998; Liu et al., 1999a, 2004; Bruhn et al., 2005). However, conventional PCR tests can be hampered by the high risk of contamination by previously amplified materials. To date, no published data exist on the applicability of real-time RT-PCR protocols for ARV detection. This study was aimed at developing a rapid, sensitive, and specific real-time RT-PCR assay for the detection of ARV σA-encoding gene with SYBR Green I using a Light Cycler (LC).

2. Materials and methods

2.1. Virus purification and RNA extraction from ARV-infected cell cultures

A total of 11 ARV isolates, including S1133, 916, R2, 1017-1, 2408, 750505, T6, 601G, ReoV A, OS161, and 601SI were used in this study. A previous study indicated that S1133, 2408, R2, and 750505 could be grouped into serotype 1, while 916 and T6 represented two additional different serotypes 2 and 3 (Lee et al., 1992). The other six virus strains still remain unclassified. The genotypes of each strain have been described in our previous studies (Liu and Huang, 2001). ARV isolates were propagated in primary chicken embryo fibroblast (CEF) cells. Upon development of 70–80% cytopathic effect (CPE), the cell cultures were frozen and stored at −70 °C. The viral dsRNA was further purified by LiCl fractionation precipitation (Huang et al., 2005). The viral RNA was then washed twice with 70% ethanol to remove LiCl and resuspended in diethylpyrocarbonate (DEPC)-treated water.

2.2. Primer design

A set of primers was designed according to the sequences of sigma A-encoding gene of ARV S1133 strain (GeneBank accession no. AF104311) by LC probe design software (Roche Molecular Biochemicals, Mannheim, Germany). The primer sequences were as follows: forward primer σA1, 5′-ATTACGCAGAGGCATTT3′ (covering nucleotides 799–815); and the reverse primer σA2, 5′-CCCACCTGGCAGAATAACA3′ (complementary to nucleotides 1039–1024). The amplified cDNA fragment using primer pair σA1/σA2 was expected to be 241 base pairs (bp) in length.

2.3. Real-time LC PCR and conventional RT-PCR

PCRs were performed on a Light Cycler (Roche Molecular Biochemicals). Each reaction was carried out in 1 μl of purified RNA and 19 μl reaction mixtures, which were 0.2 U of RNase inhibitor, 2 mM MgCl2, 1 U of FastStart DNA Master Mix SYBR Green I (containing Taq DNA polymerase, SYBR Green I, and deoxynucleoside triphosphate mix), 0.5 μM of the primers (forward and reverse). Reverse transcription was carried at 50 °C for 10 min. PCRs were subjected to 10 min of 95 °C hot-start enzyme activation, and 45 cycles of 95 °C denaturation for 3 s, 54 °C annealing for 30 s, and extension at 72 °C for 1 min with one final extension cycle at 72 °C for 7 min. The amplified cDNA fragment using primer pair σA1/σA2 was expected to be 241 base pairs (bp) in length.

PCR products were checked by 1.5% agarose gel electrophoresis in Tris-borate-EDTA buffer.

2.4. Reproducibility of real-time LC RT-PCR

An assay for quantification analysis of ARV S1133 cDNA was developed using plasmid pUC18-α-A as a standard. The plasmid includes the full-length α-A-encoding gene of ARV S1133 strain as described previously (Liu and Huang, 2001). The plasmids made from PCR product were used as the source of DNA template. The concentration of plasmid pUC18-α-A was measured spectrophotometrically. Copy number was calculated by the following formula:

\[
6 \times 10^{23} \text{(copies/mol)} \times \text{concentration (g/μl)} = \text{amount (copies/μl)}
\]

After quantification, plasmid pUC18-α-A was serially diluted and used as a standard control. The sensitivity of real-time RT-PCR assays were determined by running 10-fold serial dilutions of plasmid DNA (pUC18-α-A). The cDNA mass of plasmid ARVα-A was determined by spectrophotometrically. Input target copies were calculated based on the size of the plasmid and its corresponding DNA mass. For quantification of ARV genomic RNA, a series of 10-fold dilutions were made with concentrations ranging from 10 to 10^7 copies per reaction. Water was used as dilution solvent. To generate a standard curve, the threshold cycle (Ct) of these standard dilutions was plotted against the number of plasmid copies used as input. The precision of real-time LC RT-PCR for detecting ARV was expressed by a coefficient of variation.

2.5. The sensitivity and specificity of real-time LC RT-PCR

To test the specificity of the assay, negative controls and some avian pathogens, including Newcastle disease viruses (NDV-B1 & V302), infectious bronchitis viruses (IBV-Mass & V348), infectious bursal disease virus (IBDV-2512), avian influenza virus (AIH16), and mycoplasma synovia (MS) were tested. The sensitivity of the real-time LC RT-PCR assay was tested by limiting dilution assay (Taswell, 1981). ARV genomic RNA was then diluted into 100, 20, 5, and 1.25 copies per assay tube. Ten tubes at each concentration were assayed using the forward and reverse primers. The percent of negative tubes was plotted against the input copy number in a semilog plot.

3. Results

3.1. The optimal conditions of the real-time LC RT-PCR

Based on our previous reports (Liu and Huang, 2001; Liu et al., 2003), a set of highly conserved sequences were selected from the α-A-encoding gene of ARV and used for primer design. The copy number of plasmid pUC18-α-A was determined spectrophotometrically. A series of 10-fold dilutions with concentrations ranging 10^6 to 10^2 copies per reaction were used to evaluate the real-time LC RT-PCR. The assay was linear over this range with a sensitivity of at least 10 copies (Fig. 1; upper panel). The real-time PCR products (241 bp) were separated on an 1.5% agarose gel stained with ethidium bromide (Fig. 1; lower panel).

In the present study, various PCR reaction parameters were examined such as cycling time and temperature and Mg^2+ concentration in order to get the optimal working conditions. Conditions were chosen such that the Ct values were the lowest possible and the fluorescence acquisition curves were robust and parallel to each other at various template concentrations. The cycling time was 40 s/cycle and the Mg^2+ concentration was 4 mM. The relationship between the Ct and log_{10} ARV copy number showed good correlation (r = −1.00). All serially diluted standards were duplicated and amplified by PCR.

3.2. Reproducibility

The plasmid pUC18-α-A diluted serially from 10^6 to 10^2 copies/μl was detected by real-time PCR assay (Fig. 1). A standard curve was then constructed plotting threshold cycle (Ct) values against the known copy number of the standard sample. The standard curve showed good correlation between copy number and Ct values (r = −1.00) (data not shown).

ARV genomic RNA diluted serially from 10^6 to 10^2 copies/μl was detected by real-time PCR assay (Fig. 2; upper panel). PCR products (241 bp) were separated on a 1.5% agarose gel stained with ethidium bromide (Fig. 2; lower panel). A standard curve (Fig. 3) was created with 10-fold dilutions of ARV genomic RNA (10^2 to 10^7 genome copies) to quantify ARV. The mean melting point of PCR products from ARV S1133 in within run tests were 87.46 ± 0.08 °C and in different run tests were 87.45 ± 0.14 °C (Fig. 4). Linear regression of the Ct values and the quantity of RNA revealed a good negative linearity (r = −1, error = 0.0935; slope = −3.508, and intercept = 38.03). The slope corresponding to the efficiency of RT-PCR were −3.4 cycles/log_{10} unit with ARV genomic RNA. The results shown in Table 1 indicates that repeated testing of our assay reveals 100 copies/μl detected in 100% of runs while no positive fluorescence signal was detected at 10 copies/μl. The coefficient of variation (CV) for the assay crossing points (Ct values) for the different concentrations of ARV genomic RNA are shown in Table 1. The assays were extremely reproducible with CVs ranging between 1.4 and 0.6%. Intra-assay CV was 0.6–1.3% while inter-assay CV was 0.6–1.4%. A linear range from 10^2 to 10^7 copies of target RNA was noted with both assays. From 10 runs of PCR
in the same condition, the average slope was 3.503 ± 0.094 and the average PCR efficiency was 1.929 ± 0.033. The concentrations indicated in Table 1, fall within the linear range of the assay.

3.3. Sensitivity and specificity

To determine the end point of sensitivity of the assay, serial dilutions at concentrations between 100 and 1.25 copies were
analyzed. Extrapolation of the 37% negative point on the y-axis intersected at about 39 copies on the x-axis (Fig. 5). This was in excellent agreement with fluorometric determination of the copy number, implying the scorpion assay is both sensitive and robust (that is no appreciable false negatives or false positives).

The specificity of the real-time LC RT-PCR was 100% since negative control or some avian viruses showed no detectable fluorescent signals. Vaccine and field isolates were amplified in the real-time PCR and all were positive with the melting points around 85.69–87.7°C (Fig. 6; upper panel). According to the results of melting curve analysis, ARV strains S1133, 2048, 750505, T7, OS161, 601SI, and ReoVA, that were classified into genotype I, were in the range of 87.17–87.73 while ARV strains 916, R2, and 601G grouped into genotype II were in the range of 85.69–86.70. The PCR products amplified from all tested ARV isolates were 241 bp in length as expected (Fig. 6; lower panel). However, the PCR products were sequenced to confirm the specificity of the assay. No cross-reactions were found with non-ARV isolates, containing Newcastle disease virus, infectious bronchitis virus, infectious bursal

Table 1

<table>
<thead>
<tr>
<th>Copy number</th>
<th>Mean crossing point</th>
<th>Intra-assay CV (%)</th>
<th>Inter-assay CV (%)</th>
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<tr>
<td>10&lt;sup&gt;2&lt;/sup&gt;</td>
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<td>1.4</td>
</tr>
<tr>
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<td>0.6</td>
<td>1.6</td>
</tr>
<tr>
<td>10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>24.23</td>
<td>0.63</td>
<td>1.2</td>
</tr>
<tr>
<td>10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>20.57</td>
<td>0.6</td>
<td>0.7</td>
</tr>
<tr>
<td>10&lt;sup&gt;7&lt;/sup&gt;</td>
<td>17.20</td>
<td>0.6</td>
<td>0.6</td>
</tr>
</tbody>
</table>

Fig. 3. Standard curve of for real-time LC RT-PCR. Serially diluted ARV S1133 genomic RNA was amplified and analyzed in real-time. The threshold cycle (C<sub>t</sub>) values were plotted against copy number to construct the standard curve, r = 1.00. ARV RNA copy number was determined spectrophotometrically.

Fig. 4. The mean melting point of PCR products from ARV S1133 within run tests and different run tests. The x-axis indicates the melting temperatures while the y-axis indicates the fluorescence intensity over the background.

Fig. 5. The sensitivity of real-time LC RT-PCR by limit dilution assay. ARV S1133 genomic RNA was diluted into 100, 20, 5, and 1.25 copies per assay tube. Ten tubes at each concentration were assayed. The percent of negative tubes were plotted against the input copy number in a semi-log plot.
disease virus, avian influenza virus, and mycoplasma synovia
(Fig. 7).

3.4. Sensitivity comparisons of conventional RT-PCR and
real-time LC RT-PCR

The sensitivity of the conventional RT-PCR was compared
with the real-time LC RT-PCR. The dynamic range of the con-
ventional RT-PCR was narrower, with positive detection of $10^3$
to $10^7$ genomic copies of ARV (Fig. 8) while the real-time LC
RT-PCR had a wide dynamic range, detecting 39 to $10^7$ copies
of ARV genomic RNA. By dilution analysis, the real-time LC RT-
PCR was 100–1000 times more sensitive than the conventional
RT-PCR.

4. Discussion

In recent years, several attempts have been made to detect
ARV in chicken tissues or cultured cells by conventional RT-
PCR (Lee et al., 1998; Liu et al., 1999a, 1999b, 2004; Bruhn
et al., 2005). However, most of these available reports indicate
that the technique has low sensitivity. To date, there is no pub-
lished study of real-time RT-PCR assay for the detection of avian
reovirus. We defined PCR conditions for efficient amplification
and quantification of nucleic acid sequences of σA-encoding
gene of ARV using real-time fluorescence measurement. The
main difficulty in developing a molecular assay for ARV is the
design and construction of the primers because of the genetic
diversity of ARV (Liu et al., 2003, 2004). This observation imply
that it would be a challenge to use a single set of primers for
detection of ARV isolates. However, a single conserved primer
pair in our real-time LC RT-PCR was highly specific to the target gene of ARV since no cross-reactions with related avian pathogens were observed. The assay does, however, rely on a highly specialized equipment, the Light Cycler instrument. In this thermocycler, PCR analysis is carried out in capillary glass reaction cuvettes allowing rapid heat exchange. This results in small intrasample temperature variation despite the fast 45-cycle PCR assays. Since the reaction is carried out in a single step in a closed system, the risk of carryover contamination of following PCR amplification is considerably reduced. Using this real-time LC RT-PCR technique, less than 2 h were required to complete setting up and performing a 45-cycle amplification and data analysis. Therefore, the assay developed in this study may be an excellent diagnostic tool with high sensitivity and specificity and fast turnaround time. This technique also provides great flexibility because no target-specific probes are required and yet specific products can be distinguished from non-specific products by studying melting peak profiles generated from the amplified PCR end-products after the assay.

The assay was highly reproducible with relatively small intra- and inter-assay variability in the study. In this report, measures for mean inter- and intra-assay variation were similar ranging from 0.6–1.4% and 0.6–1.3%, respectively. Reproducibility of all assays with regards to Ct value appears very good. When tested against a range of ARV strains and related avian viruses, all ARV isolates showed highly specific results and all unrelated avian viruses exhibited no signals. The real-time LC RT-PCR resulted in high sensitivity with the capability of quantifying ARV genomic RNA templates to as few as 39 copies per reaction. Assay sensitivity facilitates measurement of ARV genetic elements in biological material available in very limited quantity or where ARV is present at low levels. Because the assay was linear over five orders of magnitude, analytic levels should very rarely be outside of the measurement range. The results also indicated that the assay developed in this study showed more sensitivity than the conventional RT-PCR for the detection of ARV. The real-time LC RT-PCR has several advantages over conventional RT-PCR, such as short working time, high sensitivity, specificity, and low risk of contamination. It is possible to differentiate different genotypes as well as variant or vaccine strains of ARV using melting curve analysis. The preliminary results demonstrated that different genotypes of ARV could be discriminated by means of distinct Tm values. In addition, formation of specific products can easily be detected by using melting curves. Melting curve analysis in conjunction with real-time PCR was first introduced in 1997 (Ririe et al., 1997; Lay and Wittwer, 1997). Using this technique, detection of amplification products and analysis of the melting curves can be performed by applying the non-specific DNA binding fluorescent dye SYBR Green I. SYBR Green I is less expensive and can be used in an economical manner. Additionally, this simpler method can be performed with any established PCR primers with only minor modifications of the described protocols. Using the Light Cycler system, amplification products can be detected by the dye being intercalated in minor groove of dsDNA, and genotype determination can be performed by analysis of the melting curves. As shown in this study, the Tm values are directly related to genotypes based on the αA-encoding gene of ARV (Liu and Huang, 2001), if other external and internal factors such as MgCl2 concentration and the length of the PCR products are controlled (Wittwer et al., 1997). Moreover, the Tm values of corresponding ARV strains were highly reproducible with relatively small intra- and inter-assay variability in this study.

In conclusion, the quantitative assay described in this work can be used to improve the early and rapid detection of ARV infections or disease outbreaks and to study the role of genetic elements in ARV infection and pathogenesis. The assay is an excellent diagnostic tool with high sensitivity and specificity and fast turnaround time.

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