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Article

Evaluation of SYBR Green Based Real-Time Polymerase Chain Reaction (qPCR) in Detection of Reovirus

Faizul Fikri Mohd Yusop[1], Syamsiah Aini Ahmad Shohaimi[1], Leow Bee Leng[1], Redzwan Sidik[1], and Ong Chon Pin[2]

[1]*Veterinary Research Institute, Department of Veterinary Services, 59 Jalan Sultan Azlan Shah, 31400 Ipoh, Perak, Malaysia.*
[2]*Taylor's University, No 1, Jalan Taylor's, 47500 Subang Jaya, Selangor, Malaysia.*

Correspondence should be addressed to:
Faizul Fikri Mohd Yusop; faizulfikri@dvs.gov.my

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***Abstract*— Avian reovirus (ARV) belongs to the Orthoreovirus genus and Reoviridae family and contains icosahedral symmetry with a double-shelled arrangement of surface protein. Avian reoviruses infection can often be seen in a broad range of avian species, including chickens, ducks, pigeons, and turkeys. These viruses can cause tenosynovitis, causing acute lameness due to swelling of one or both hock joints, and can be associated with malabsorption, hepatitis, myocarditis, respiratory disease, and enteritis. This study objective was to use the BRYT Green® based real-time RT-PCR assay in targeting the S4 segment gene for the detection of AVR, thus improving the efficiency and cost of the diagnosis. Thirty-one (n=31), including known ARV-positive samples consisting of pool organs, liver, tendon, and allantoic fluid, were selected to be tested using BRYT Green®-based real-time RT-PCR. The finding of this study showed the one-step BRYT Green®-based RT-qPCR protocol for the detection of the Reovirus S4 genome segment is a success and has advantages compared with conventional RT-PCR. This assay is time-efficient, cost-effective, user-friendly, sensitive, and highly specific. There is no doubt that this protocol can be used in many laboratory diagnoses for avian reovirus infection.**

***Keywords*— Reovirus, real-time RT-PCR, BRYT Green**

I. INTRODUCTION

Avian reovirus (ARV) belongs to the Orthoreovirus genus and Reoviridae family [1]. Their virus particles are approximately 70 to 80 nm in size and are non-enveloped. They contain icosahedral symmetry with a double-shelled arrangement of surface protein [2]. According to the Baltimore classification of viruses, reovirus belongs to the Class III double-stranded RNA (dsRNA) viruses. It has 10 dsRNA genome segments and can be separated into three sizes based on its electrophoretic mobility: large (3L), medium (3M), and small (4S) [3].

Avian reovirus infection can often be seen in a broad range of avian species, including chickens, ducks, pigeons, and turkeys [4 - 8]. Reovirus was initially found to cause tenosynovitis, causing acute lameness due to swelling of one or both hock joints [2]. It was also shown to be associated with malabsorption, hepatitis, myocarditis, respiratory disease, and enteritis [8]. Furthermore, the various strains of the virus complicate diagnosis and control due to its different tissue tropism [9, 10].

Avian reoviruses can be spread via vertical and horizontal transmission routes, although egg transmission was also viable [11- 13]. Most congenital infected chicks most likely spread

the virus via the fecal-oral route and occasionally the respiratory tract. Additionally, reovirus can be introduced into the feet of chicks through open wounds and maintained at the hock joints [14, 15]. They can be cultured into embryonated chicken eggs via the intra-yolk route. Six days of incubation will induce embryonic haemorrhage and the appearance of yellowish-green foci on the liver, and ultimately death [16]. Numerous primary chick embryo and chicken cell cultures are also used to cultivate avian reovirus, including lung, liver, fibroblasts, and kidney of the chicken embryo and chick kidney cells. The typical cytopathic effect in the infected cell culture is syncytia formation [17, 18].

The traditional isolation and identification of reoviruses from tissues or fluid samples were deemed time-consuming [14]. Thus, molecular diagnostics, including dot-blot hybridisation, PCR, and PCR combined with RFLP, have created a more sensitive and rapid alternative [19-21]. However, these molecular tests still require comparison with the 'gold standard' for avian reovirus diagnosis – virus isolation [2].

Recently, there has been a rise in using real-time PCR for RNA quantification and genetic analysis. Real-time PCR has two types of fluorescent reporters: SYBR green and Taqman probes [22]. The Taqman approach of real-time PCR is the most precise and easily optimised, but its dual-labeled probes are relatively expensive [22]. Another approach, SYBR Green-I fluorescent dye binds to the PCR product, was less reliable but is cost-effective. It was known that the SYBR Green-based qPCR was used to detect many viruses, but the establishment of SYBR Green-based qPCR for reovirus is yet to be found. Here, we report our findings and research on the detection of reovirus (S1133 strain and other field sample strains). Lastly, we have employed BRYT Green®-based assay in this study. Both assays have similar principles, but it was shown that BRYT Green® based exhibits better fluorescence enhancement compared to the usual SYBR Green-I based kit. In this study, we aim to use the BRYT Green® based real-time RT-PCR assay in targeting the S4 segment gene for the detection of avian reovirus. This assay will improve the efficiency and cost of the diagnosis.

II. MATERIAL AND METHOD

A. Viruses

Reovirus was propagated in 5-6 days old embryonated minimum pathogen-free chicken eggs. The allantoic fluid was harvested 24 hours post-inoculation and stored at -80°C until RNA extraction.

B. Clinical standard samples

To validate the reliability of BRYT Green®-based real-time RT-PCR, the Reovirus S1133 strain was used as the standard control. The virus-containing allantoic fluid was also subjected to 10-fold dilution for further tests.

C. Organ samples from ARV-infected chickens

Sixteen (n=16) organ samples consisting of pool organs, liver, and tendon were obtained from the Pathology Unit, Veterinary Research Institute (Ipoh), Perak, Malaysia. These samples were identified during routine diagnostic efforts. All 16 samples have been confirmed to be ARV-positive by virus isolation and conventional RT-PCR. For this study, all samples were tested by BRYT Green®-based real-time RT-PCR. All organ samples were processed and filtered through 0.22 μM Millex-GP PES membrane filter (Merck, Kenilworth, New Jersey, USA). The supernatants were stored at -80°C prior to viral RNA extraction.

D. RNA extraction

ARV RNA was extracted from 200 μL of supernatant from the allantoic fluid using the TRIzol® reagent (Thermofisher Scientific, Waltham, Massachusetts, USA) according to the manufacturer's instructions. Each RNA fraction was eluted in 30 μL of RNase-free water.

E. BRYT Green®-based real-time RT-PCR

BRYT Green®-based real-time RT-PCR was performed using a primer pair (Reo2F & Reo2R) as described in Table 1 below. All PCR amplifications are conducted under the same conditions. To minimise primer-dimer formation, primer set concentration and thermocycling conditions were both optimised (Appendix 1 & 2).

TABLE 1. BRYT-Green® Based RT-qPCR Primer Sequences.

Primers	Sequence	Length	Positions	Tm (°C)
Reo2F	5'-GACGCGCTGGCG GACGAGCG-3'	20	819-838	71.17
Reo2R	5'-TGGTCACCCTCAC GGATCAT-3'	20	1071-1090	60.62

RT-PCR reaction was performed using GoTaq® 1-Step RT-qPCR System (Promega, Madison, Wisconsin, USA), according to the manufacturer's instruction with minor adjustments. Extracted RNA was mixed with 50 μL of nuclease-free water and chilled at -20°C. Subsequently, 10 μL of 2X GoTaq® qPCR Master Mix, 0.2 μL of each primer, 0.4 μL of 50X GoScriptTM RT Mix for 1-Step RT-qPCR, 7.2 μL of nuclease-free water and 2 μL of RNA template were added to each tube. BRYT Green real-time RT-PCR was performed in QuantStudio 3 Real-Time PCR System (Thermofisher Scientific, Waltham, Massachusetts, USA) with QuantStudioTM Design and Analysis Software v1.4.3. Real-time RT-PCR was carried out at 95°C for 15 mins to activate HotStart Taq polymerase, and 40 cycles at 95°C denaturation for 10 sec, 61°C annealing for 30 sec, and 72°C extension for 30 sec. A single fluorescence signal acquisition was set at the end of each extension step. Amplified cDNA products were detected by melting curve analysis consisting of 60°C annealing for 60 sec and then the temperature was increased by 0.15°C/s till it reached 95°C. Real-time RT-PCR was set to the continuous acquisition mode to record fluorescence signal density changes during the temperature increase. Following amplification, a melting curve analysis was performed with the same software. Each test was performed in duplicate.

F. Sensitivity of BRYT Green®-based real-time RT-PCR

The sensitivity of the assay was tested by the limiting dilution assay. ARV S1133 strain RNA was reverse-transcribed into cDNA and diluted from neat concentration up to 10-7 dilution. The tubes at each concentration were assayed using the forward and reverse primers. Samples with Ct values of less than 36 were considered positive. The primer amplification efficiency is calculated using the formula E = -1 + $10^{(-1/slope)}$ x 100%.

G. Sensitivity of BRYT Green®-based real-time RT-PCR

Conventional reverse transcriptase PCR was performed in a thermal cycler (Bio-Rad, Hercules, California, USA) according to the manufacturer's instructions with minor modifications using SuperScript® III One-Step RT-PCR System with Platinum® Taq DNA Polymerase (Invitrogen, Carlsbad, California, USA). The thermal profile of the one-step conventional RT-PCR was performed in a final volume of 20 µL. The mixture contains 10 µL of 2X reaction mix (containing 0.4 mM of each dNTP and 3.2 mM MgSO4), 0.5 µL SuperScript III RT/Platinum Taq Mix, 0.2 µL of each primer (20 µM), 7.1 µL of RNase-free water and 2.0 µL of RNA or cDNA.

The assay was performed at 48°C for 30 min and 94°C for 5 min. The cDNA was amplified through 40 cycles of PCR consisting of 94°C denaturation for 1 min, 61°C annealing for 1 min, 68°C extension for 1 min, with the final extension of 68°C for 5 min. The amplified PCR products were evaluated using 1.5% agarose gel electrophoresis in Tris-borate-EDTA (TBE) buffer stained with SYBR® Safe DNA gel stain (Invitrogen, Carlsbad, California, USA). The amplification has resulted in an amplicon size of 272 bp.

H. Specificity of BRYT Green®-based real-time RT-PCR

The specificity of BRYT Green®-based RT-qPCR was assessed using the RNA generated from Newcastle disease virus (NDV), avian influenza virus (FLUAV), and infectious bronchitis virus (IBV) that display similar clinical signs and symptoms as avian reovirus. The one-step BRYT Green®-based RT-qPCR was performed as stated above, and the products were verified by gel electrophoresis using 1.5% agarose gel (data not shown).

I. Evaluation of Assay Validity and Reliability

To evaluate the validity and reliability of BRYT Green®-based RT-qPCR, 18 known ARV-positive (including 2 Reovirus S1133 strains) and 13 known ARV-negative samples were analysed. Ct values of the 18 known ARV-positive samples were less than 36 cycles (data not shown) with a melting temperature of 85 ± 1°C. The real-time assay was compared with the results that were carried out and verified by the Veterinary Research Institute (Ipoh) through virus isolation and conventional RT-PCR for all 31 known and unknown field samples.

J. Reproducibility of BRYT Green®-based RT-qPCR

To validate the reproducibility of the assay, the ARV cDNA was developed using the S1133 strain and was serially diluted (up to 10^{-7} from neat concentration) and used as standard control. Three separate dilution series were assayed in a single run to evaluate its intra-assay variations, whereas the same test was carried out in three distinct runs to evaluate its inter-assay variations. The standard deviation (SD) was obtained using QuantStudio™ Design and Analysis Software v1.4.3. The coefficient of variation was calculated using the formula CV = (SD [C_t value]/overall mean [C_t value]) x 100.

III. RESULTS

A. Sensitivity

1) BRYT Green®-based real-time RT-PCR

To determine the sensitivity endpoint of the assay, serial dilutions of the cDNA template at a dilution range of neat concentration to 10-7 were analysed. The amplification plot fluorescence curve (Figure 1) derived from a serially diluted cDNA template indicates a sensitivity of detection up to the 10-5 dilution.

To determine the linearity of the reaction and the RT-PCR primer amplification efficiency, the cycle threshold (Ct) values of individual dilutions were analysed. The assay has a detection range that spanned across 10-5 dilutions of ARV cDNA. A strong inverse linear relationship was observed between the cDNA dilution and Ct values over 6 dilutions, as indicated by a simple linear regression plot (Figure 2). The amplification efficiency of the primer is 83.22%.

2) Conventional gel-based RT-PCR

The primer pair (Reo2F & Reo2R) targets the S4 segment genome of ARV, resulting in PCR products of 272 bp in length being separated on 1.5% agarose gel stained with SYBR® Safe DNA gel stain. PCR products were detectable at a dilution of up to 10-5 (Figure 3).

A. Specificity

Melting curve analysis on the RT-qPCR products has exhibited the absence of primer-dimer formation and nonspecific products (Figure 4). Specific amplification of the ARV target sequence was identified at a melting temperature within the range of 85 ± 1°C. The red peak (Reo^{-4}) was the sole detectable fluorescent signal. The specificity of the primer set and the BRYT Green®-based RT-qPCR was 100% since no detectable fluorescent signals were present with avian influenza virus, infectious bronchitis virus, Newcastle disease virus RNA, and no-template control.

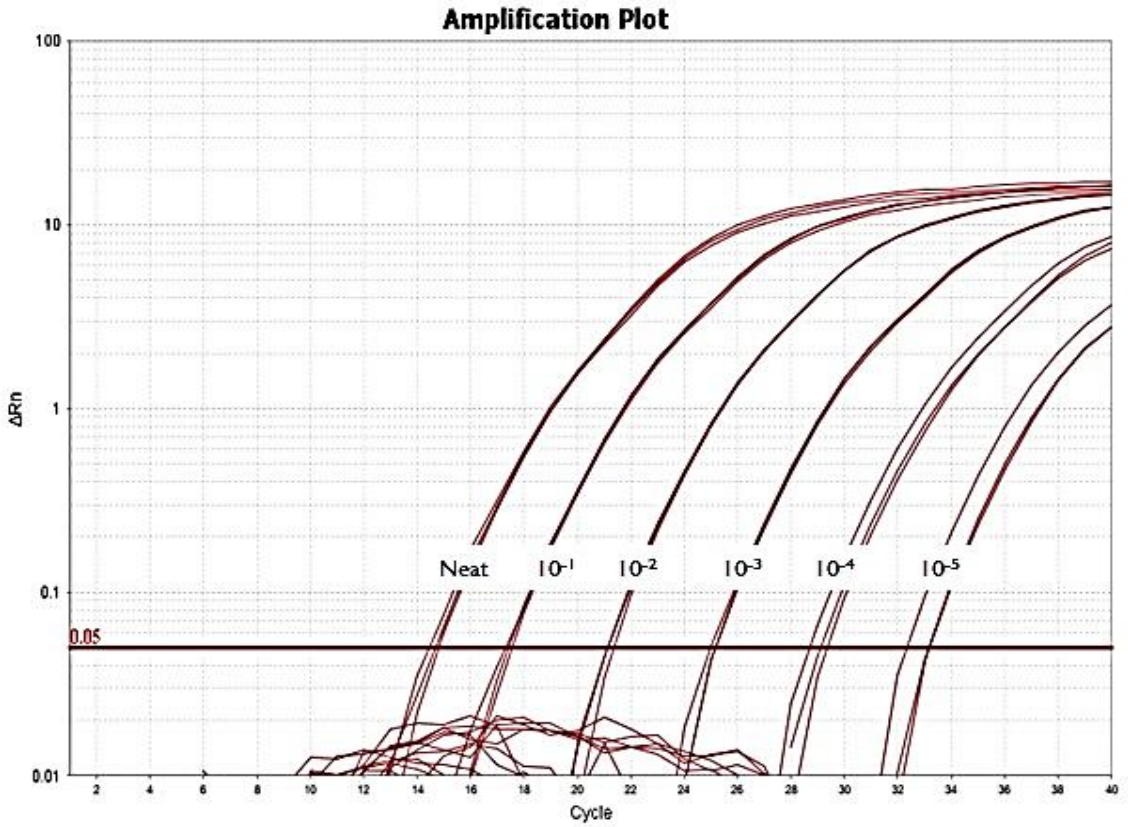

Figure 1: Real-time RT-PCR for the detection of avian reovirus. cDNA templates derived from ARV S1133 strain genomic RNA were diluted serially from neat concentration to 10-7 and assayed with real-time PCR. The x-axis indicates the cycle number, whereas the y-axis indicates fluorescence intensity.

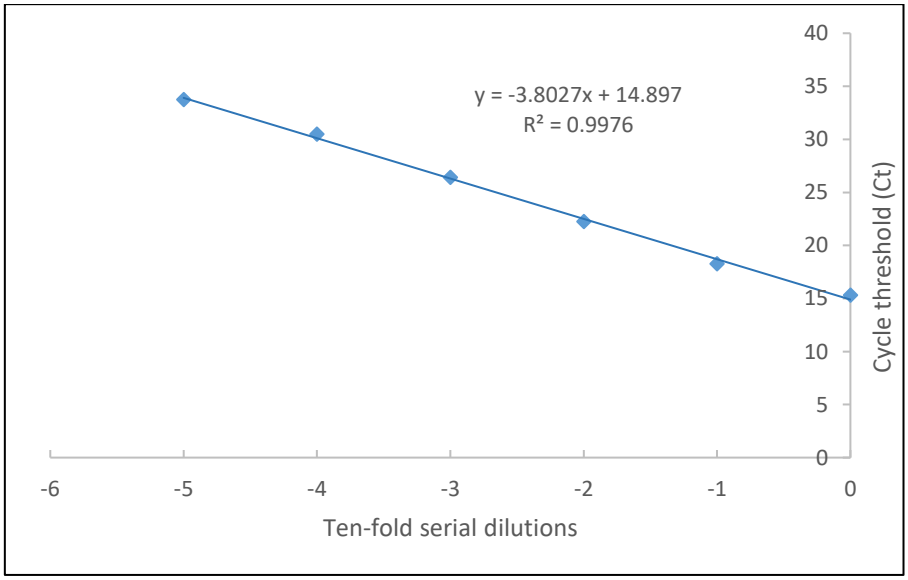

Figure 2: Simple linear regression line of Ct values vs ten-fold serial dilutions of the standard RNA genome of cDNA of Reovirus S1133 strain.

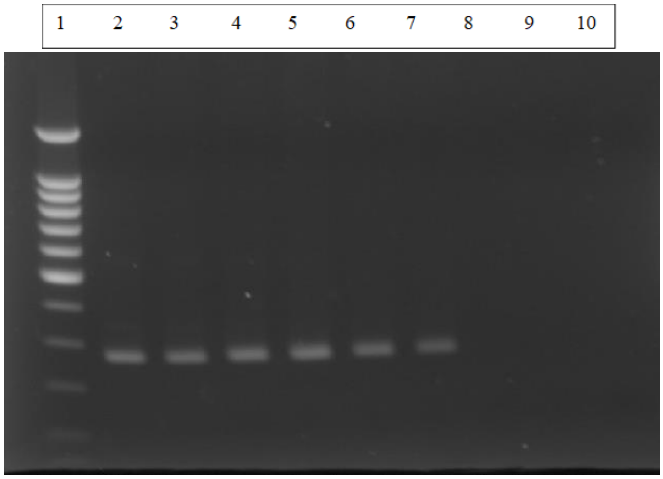

Figure 3. Agarose gel electrophoresis of conventional RT-PCR amplified products from ARV S4 genome segment. The cDNA template was serially diluted from a neat concentration to 10-7 and underwent gel electrophoresis using 1.5% agarose gel stained with SYBR® Safe DNA Gel stain. Lane 1 100-bp DNA ladder; Lane 2 neat concentration of ARV cDNA; Lane 3 10-1 dilution; Lane 4 10-2 dilution; Lane 5 10-3 dilution; Lane 6 10-4 dilution; Lane 7 10-5 dilution; Lane 8 10-6 dilution; Lane 9 10-7 dilution; Lane 10 no-template control.

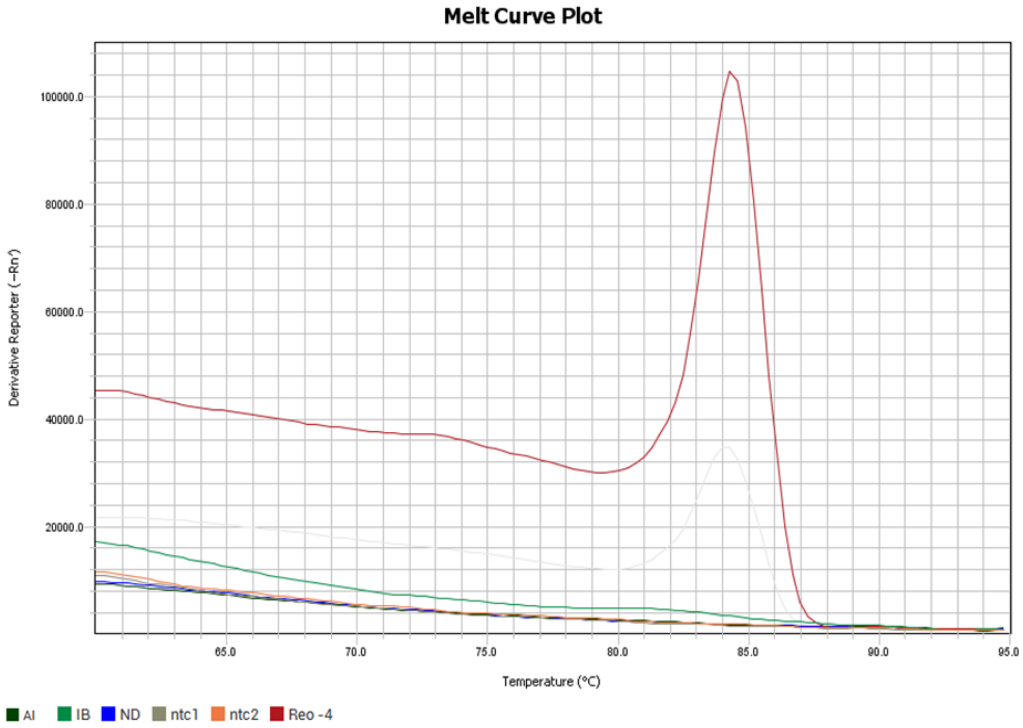

Figure 4: Melting curve analysis for reovirus cDNA of 10-4 dilution (Reo 10-4), avian influenza (AI) virus, infectious bronchitis (IB) virus, Newcastle disease (ND) virus, and no-template control (NTC).

B. Validity and Reliability of Assay

The optimised real-time RT-PCR assay was used in 31 known positive and negative clinical samples originating from pool organs, tendon, ceacal tonsil, and liver samples. From 31 clinical samples tested, 16 out of 18 positive controls were positive for ARV targeting the S4 genome segment, whereas 13 out of 13 negative controls were shown negative for ARV.

C. Reproducibility of BRYT Green®-based RT-qPCR

The assay displayed high reproducibility with the coefficient of variation within each run (intra-assay variability) and between each distinct run (inter-assay variability) ranging from 0.57–2.27% and 3.26–6.33%, respectively (Table 2).

TABLE 2. Intra- And Inter-Assay Reproducibility Of Bryt Green®-Based Real-Time Rt-Pcr

Dilution	**Mean C_t value**	**Intra-assay CV (%)**	**Inter-assay CV (%)**
Neat	15.30	1.08	5.75
10^{-1}	18.25	0.90	5.79
10^{-2}	22.25	0.57	6.33
10^{-3}	26.40	0.39	6.05
10^{-4}	30.48	2.27	6.30
10^{-5}	33.75	0.99	3.26

CV coefficient of variation

IV. DISCUSSIONS

Avian reovirus causes financial loss in many agricultural industries, including farms and hatcheries. In the Veterinary Research Institute of Ipoh, viral isolation remains the gold standard for the diagnosis of viral diseases. However, conventional RT-PCR was used for emergency cases or confirmation tests from other labs around Malaysia. Presently, the emergence of real-time PCR has sparked an interest in many lab personnel and researchers due to its advantageous sensitivity, reproducibility, rapidity, and minimal risk of contamination [23]. In this present study, we have reported the establishment of BRYT Green®-based real-time RT-PCR assay, known to exhibit higher fluorescence than the traditional SYBR Green I-based assay in detecting avian reovirus for diagnostic purposes.

Based on the optimisation tests that we have conducted, it was shown that the annealing temperature of 61°C exhibited the highest prominent band compared to other annealing temperatures used. Thereon, we have determined that a final primer concentration of 200 nM for Reo2F and Reo2R primers displayed the highest relative fluorescence unit and lowest CT value compared to 100 and 150 nM concentrations of primers.

Using this information, we conduct our future experiments using the stated conditions in our sensitivity and specificity analysis. Based on the sensitivity test for the RT-qPCR assay results, the limit of detection can be determined. We have concluded that the detection limit of this assay spans towards 10-5 dilution from its original concentration (neat). Unfortunately, we had to conduct this study without quantifying its actual nucleic acid concentration due to the lack of equipment, NanoDrop. However, this study serves to establish a rapid diagnostic method to ideally replace the time-consuming conventional RT-PCR. Our conventional RT-PCR assay has also revealed a detection limit of 10-5 dilution, similar to the BRYT Green®-based assay. Even so, conventional RT-PCR is prone to error and time-consuming, emphasising the importance of the replacement of the conventional method [24-26]. According to Figure 2, the R2 value was 0.9976, which indicated a good correlation between viral copy numbers and Ct values. On the other hand, the primer amplification efficiency was found to be 83.22%. In Zhang et al.'s study, they stated that the MIQE guideline for primer amplification efficiency has an acceptable range from 80% to 120% [27]. This suggests that our primer efficiency was within the acceptable range and remains feasible.

Additionally, we have conducted a specificity test for our RT-qPCR assay. The absence of positive reactions from other RNA viruses, including AI virus, ND virus, and IB virus, have displayed substantial specificity of the test. The optimal melting peak of ARV amplicon strains is 85 ± 1°C. There are no primer-dimer and nonspecific products detected.

The BRYT Green®-based RT-qPCR was applied to 31 known positive and negative clinical samples obtained from avian species in the Veterinary Research Institute of Ipoh, specifically avian species that exhibit similar clinical signs and symptoms of ARV. The gene amplification of the S4 genome segment of ARV for the clinical samples was assessed: 16 out of 18 known positive samples, while 13 out of 13 known negative samples were verified via RT-qPCR assay. The reason for the lack of agreeability for the known positive clinical samples could be due to the degradation of the RNA. Reference [28] has suggested that RNA degradation may take part even at storage temperature -70°C. It was found that lyophilisation of the genome components may help preserve the RNA for long-term storage [29]. Overall, the BRYT Green®-based real-time RT-PCR is time-effective, cost-effective, sensitive, specific, and user-friendly.

In the Veterinary Research Institute of Ipoh, the go-to method was always conventional PCR. However, this establishment of BRYT Green®-based RT-qPCR was due to a few reasons: (1) The assay is user-friendly in terms of primer design and optimisation procedures [24]. (2) The assay utilises a low-cost fluorochrome, and no requirement for probe design which was deemed expensive, and (3) artifacts commonly observed in specific probes, specifically at amplification cycles beyond 30th round, are minimal and can be ruled out by the melting curve analysis [30].

V. CONCLUSION

In conclusion, we report that the establishment of a one-step BRYT Green®-based RT-qPCR protocol for the detection of the Reovirus S4 genome segment is a success. This assay is time-efficient, cost-effective, user-friendly, sensitive, and highly specific. There is no doubt that this protocol can be used in many laboratory diagnoses for avian reovirus infection.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this paper.

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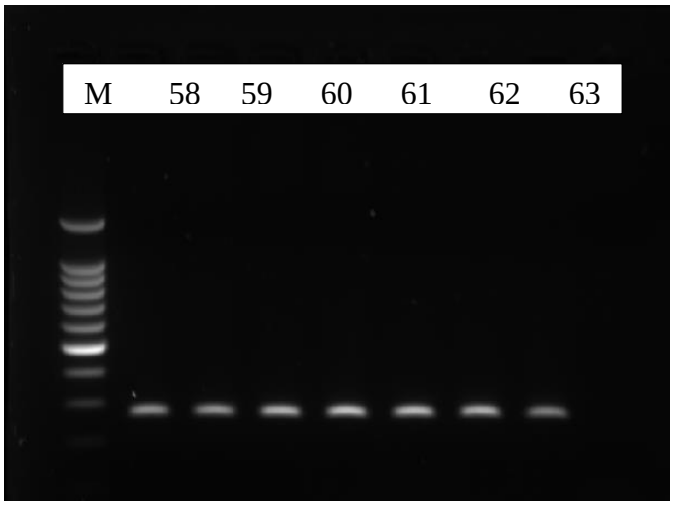
APPENDIX

Annealing Temperature Optimisation

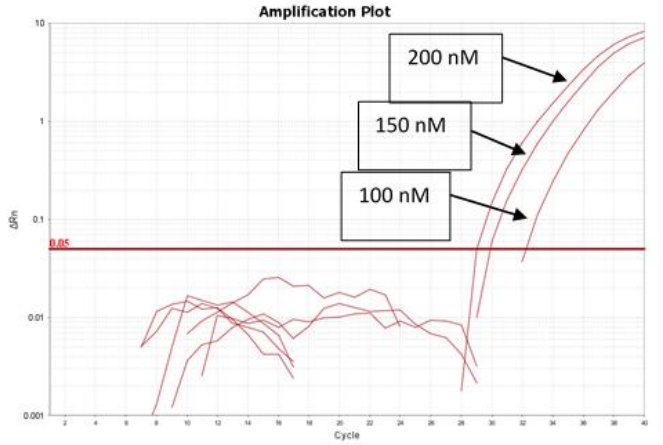
From the Appendix 1 below, it was shown that annealing temperature of 61°C has the most prominent band compared to other temperatures.



Appendix 1: Agarose gel electrophoresis of conventional RT-PCR amplified products for Reovirus S4 genome segment. Lane 1: 100 bp DNA ladder; Lane 2: 58°C; Lane 3: 59°C; Lane 4: 60°C; Lane 5: 61°C; Lane 6: 62°C; Lane 7: 63°C; Lane 8: 64°C.

Primer concentration optimisation

Based on Appendix 2, it was shown that a primer final concentration of 200 nM has the lowest CT value while exhibiting the highest relative fluorescent unit compared to 100 and 150 nM.



Appendix 2: Amplification plot of BRYT Green® Based RT-qPCR for reovirus using a primer concentration range of 100 – 200 nM.