DEVELOPMENT OF REAL-TIME PCR ASSAY FOR DUCK VIRAL ENTERITIS (DVE)

SYAMSIAH A.S.[1], SENG S.C.[2], KHAIRUL A.M[1], ONG G.H[1] AND RAMLAN M.[1]

[1] Veterinary Research Institute, Ipoh, Perak. [2] Universiti Malaysia Sabah, Kota Kinabalu, Sabah.

Corresponding author: syamsi_aini@yahoo.com

ABSTRACT. Duck Virus Enteritis (DVE) is a viral disease which causes an acute contagious herpesvirus infection of waterfowl such as ducks, swans and geese and is a pathogen of economical concern. DVE diagnosis depends on histopathologic studies and serology, which are not effective in the early phase of the disease as it is laborious and time-consuming. As a result, a more effective and sensitive test was preferred. Although rapid methods such as conventional PCR and nested PCR are available, PCR-based methods are not carried to end point detection. Hence, a more rapid and sensitive end point assay is required. There are real time PCR methods which use Taqman and fluorogenic probe. Two qPCR systems were compared using GoTaq qPCR and PCR with SYBR green I based on melting temperatures discriminations. One specific melting peak was generated at temperatures 88.0±0.5°C represent DVE gene products. The real time PCR assay developed was sensitive where 10 DNA copies can be detected and DVE gene was detected specifically with no signal for other viruses such as AI, ND, CAV and EDS. Viral DNA could still be detected in infected cell cultures and field samples which were collected few years ago.

Keywords: Duck Viral Enteritis (DVE), real-time PCR, melting temperature, sensitive, specific.

INTRODUCTION

Duck Virus Enteritis (DVE) is a viral disease which causes an acute contagious herpesvirus infection of waterfowl such as ducks, swans and geese (Leibotviz, 1984). The first DVE outbreak was reported in the western hemisphere in 1967 from a concentrated commercial duck producing area in Suffolk County, N.Y. (Leibotviz, 1984). In Malaysia, the first DVE outbreak occurred in Penang in 1992 and had caused mortality up to 100% in the infected ducks. Since then, DVE outbreaks have been reported in Perlis, Perak, Johor and Sabah. DVE still persist in the duck farms (Maizan *et al.,* 2000) DVE infection of the domestic ducks (*Anasplatyrhynchos domesticus*), swans and geese are characterized by mucosal eruptions of the gastrointestinal tract, internal bleeding (Leibotviz, 1984). This disease causes severe mortality in all ages of birds (Maizan *et al.,* 2000 and Leibotviz, 1984). The adult ducks

are tend to experience greater mortality than the young duck (Leibotviz, 1984). DVE has been limited to the members of the *Anatidae* family of ducks, geese, and swans of the order of Antiseriforms. A study documented that inoculation of DVE in embryonating chicken eggs caused chickens to adapt and grow up to 2 weeks of age (Leibotviz, 1984). DVE has not been reported in other avian species and other organisms such as mammals (Leibotviz, 1984).

Gross lesion is the presumptive diagnosis of the DVE disease where the histopathologic studies can support the findings. Isolation and identification of the DVE can confirm the diagnosis in the absence of the lesions. DVE virus can be collected from the liver, cloaca, spleen and bursa for the virus isolation (Shawky and Sandhu, 2003). Primary virus isolation also can be made by inoculation of susceptible duck's or chicken's embryo fibroblast tissue culture or on the chorioallantoic membrane (CAM) of 9-14 days old embryonating duck eggs, in duck embryo liver or kidney cells (Shawky and Sandhu, 2003). Laboratory confirmation of DVE can be done traditionally through the examination of the gross lesion at necropsy, however, it is time consuming (Maizan *et al.,* 2000). Although the serological methods are available through DVE virus antigen detection in tissues by immunoperoxidase staining (Islam *et al.,* 1993) and antibody test (IFA), the effectiveness in detecting the DVE virus needs to be improved. As a result, a rapid and more sensitive method for the DVE virus detection is needed for better diagnosis management. Recently, polymerase chain reaction (PCR) assay has been developed to be used as a rapid detection method of DVE. The use of the PCR assay gave a greater understanding of the pathogenesis and diagnosis of DVE. Studies have shown the successful detection of DVE (PCR) in cell culture and tissues from infected ducks using species specific primer set (Pritchard *et al.,* 1999). This method was able to differentiate DVE from other herpesviruses such as Marek's disease and goose herpesviruses (Pritchard *et al.,* 1999). Nested PCR assay, has also been reported for the diagnosis of DVE. This assay enable the detection of DVE virus directly from the small numbers of clinical and field samples, and results can be produced within 24 hours (Maizan *et al.,* 2000). However, in order to analyze the PCR products, electrophoresis is still needed and PCR assay is not the end-point assay. As a result, more rapid tests are needed to provide detailed information on virus spread and species identification.

Real-time PCR has many advantages over the conventional PCR, as it is more sensitive and is an automated, computer-based assay. It does not require gel based analysis at the end of PCR. The amplification detection is associated with fluorescence at the end of each PCR cycle and the time required including the sample processing, is less than four hours. This method would be useful in disease surveillance as it can provide early warning of a possible outbreak of the disease. There

were reports on DVE virus detection from the infected duck using quantitative time-course study of Taqman Polymerase Chain Reaction and also the real time PCR using the fluorogenic probe (Yang *et al.,* 2005). In the present study, we describe the development of Real-Time PCR (qPCR) assay for detection of DVE virus using the DVE specific primer and GoTaq PCR with SYBR green I.

MATERIALS AND METHODS

Virus strains

DVE Jensen strain (vaccine) was propagated in the chicken embryo fibroblast (CEF) tissue culture and incubated at 39.6-41.5°C for 1-2 days depending on the multiplicity of the infections index (MOI). These samples were used for DNA extraction and the evaluation of sensitivity and optimization of the real-time PCR assay. Positive DVE cases from 1996 (668/96) and 1998 (2381/98) were also used in this study. One sample each of Avian influenza (AI), Newcastle disease (ND), Chicken Anemia Virus (CAV) and Egg Drop syndrome Adenovirus (EDS) were obtained from Avian Virology Unit of Veterinary Research Institute, Ipoh, Malaysia and were used to evaluate the specificity of the assay.

Oligonucleotides

Specific primer pairs used in this study were DVEMF (5' GTTCGAAAGCG-GCGCGGTTTCCG 3') and DVEMR (5' ATCTTCGGCAGTCGTGATAA 3') with PCR product of 459bp. This primer was designed based on the UL6 and UL7 gene (Maizan *et al.,* 2000).

Viral DNA extraction

Maxwell Tissue LEV total RNA Purification kit (Promega) was used to extract DNA using automated Maxwell 16 Extractor (Promega) onto the homogenized tissues and the CEF tissue culture according to the manufacturer's procedure.

Conventional PCR

The purity of extracted DNA was determined initially through conventional PCR using Peltier thermal cycler. Briefly, a standard 25 µl reaction mixture contained 1x GoTaq buffer (Promega, USA), 0.2 mM dNTP mix, 1.5 mM $MgCl_2$, 5 U/µl AMV reverse transcriptase, 0.1 U GoTaq DNA polymerase, 0.4 pmol/µl of DVEM primer set (VRI), 2 µl of DNA and RNase free water. The amplification was carried out in Peltier thermal cycler. The cycling program consists of pre- denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 1 min, annealing temperatures at 58 °C for 1 min, extension at 72°C for 1 min and final extension at 72°C for 7 min. PCR product was analyzed on 1.5% agarose gel and visualized under UV light.

Real-time PCR (qPCR)

Different concentration of primer and DNA template, annealing temperature and the amplification cycles were set for experimental trial to determine the optimal conditions of the GoTaq qPCR and the SYBR green I qPCR for DVE. Briefly, for the GoTaq qPCR reaction, a standard 25 µl reaction mixture contained 1x GoTaq qPCR master mix, 0.3 µM of DVEM primer set (VRI) and nuclease free water. The amplification was carried out in BioRad i-cycler. The cycling program consists of hot start activation at 95°C for 2 min followed by 40 cycles of denaturation at 94°C for 15 s and annealing/extension temperatures at 60°C for 1 min. Dissociation at 95°C for 1 min, 55°C for 1min followed by 80 cycles at 55°C where +0.5°C per 10 s for melting curve analysis. A standard 25 µl reaction mixture contained 1x GoTaq buffer, 0.2mM dNTP mix, 1.5 mM $MgCl_2$, 5 U/µl AMV reverse transcriptase, 0.1 U GoTaq DNA polymerase, 0.4 pmol/µl of DVEM primer set (VRI), 0.5 µl Sybr Green 1dye diluted $1:10^3$ in RNase free water (Molecular Probe, USA) 2µl of DNA and nuclease free water. No template control (NTC) was used as negative control. The amplification was carried out in BioRad i-cycler. The cycling program consists of hot start activation at 95°C for 2 min followed by 40 cycles of denaturation at 94°C for 15 s and annealing/extension temperatures at 60°C for 1 min. Dissociation at 95°C for 1 min, 55°C for 1 min followed by 80 cycles at 55°C where +0.5°C per 10 s for melting curve analysis. qPCR with the optimal condition for DVE was used as the qPCR method for the following test.

Assay specificity

Specificity qPCR assay was established by carrying out melting peak analysis. Performance of the qPCR assay on one strain of Chicken embryonate fibroblast of DVE and other avian viruses such as Avian influenza (AI), Newcastle disease (ND), Chicken Anemia Virus (CAV) and adenovirus EDS (Egg drop syndrome) were included in specificity evaluation.

Assay sensitivity

The sensitivity of qPCR assay was determined using 10 fold serial dilution of extracted DNA from DVE samples ranging from neat to 10^{-4}.

Detection of the DVE gene in pool organs of DVE infected samples

200 µl of the homogenized tissues of positive DVE cases was used for conventional DNA extraction and DNA extraction kit. This is followed by qPCR amplification for DVE detection.

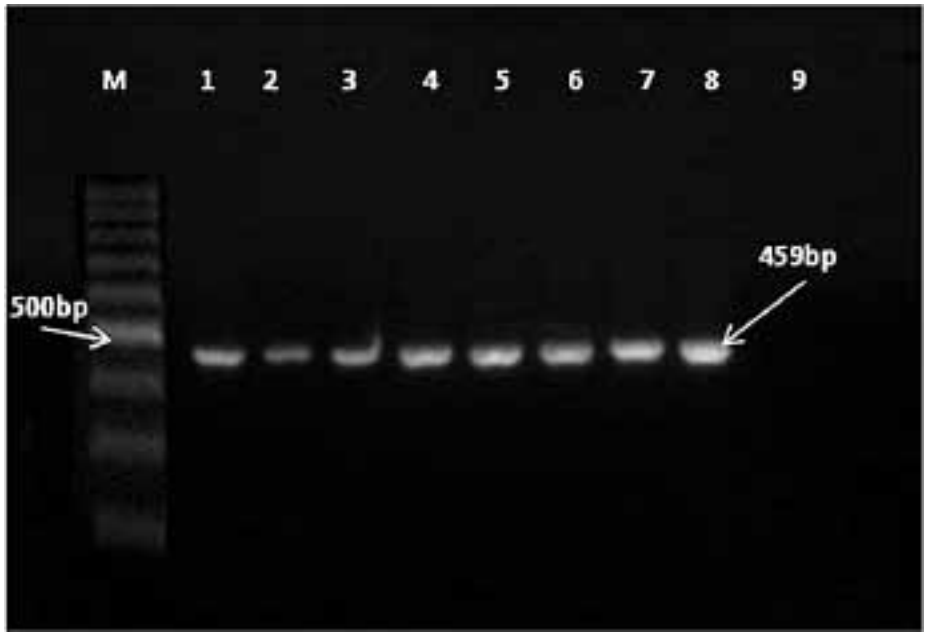

FIGURE 1. Gel profile for detection of DVE virus (CEF) and pooled organs, extracted using Promega Maxwell 16. 1: 668/96, 2: 2381/98, 3: DVE CEF-1, 4: DVE CEF-2, 5: DVE CEF-3, 6:DVE CEF-4, 7: DVE CEF-5 , 8:DVE CEF-6, 9: negative control, M: 100bp ladder

RESULTS

Conventional PCR

The band patterns generated at 459 bp corresponded to the DVE gene regions (Figure 1).

Interpretation of the melt curve from the real-time PCR (qPCR)

Melting peak was generated at temperatures of 88.0±0.5°C for DVE gene products using GoTaq qPCR (Figure 3) and SYBR green I qPCR (Figure 2). There was the primer dimer generated using GoTaq qPCR (Figure 3). This indicates that GoTaq qPCR failed to specifically amplify DVE gene and the SYBR green I qPCR successfully generated a specific melting curve for DVE detection. As a result, SYBR green I qPCR was used for the following qPCR reaction for the specificity and sensitivity evaluation.

Assay specificity

SYBR green I qPCR assay was able to detect the DVE gene in chicken embryonate fibroblast tissue culture. The melting temperatures was at 88±0.5°C, representing the melting temperatures of DVE gene. There was no specific peak

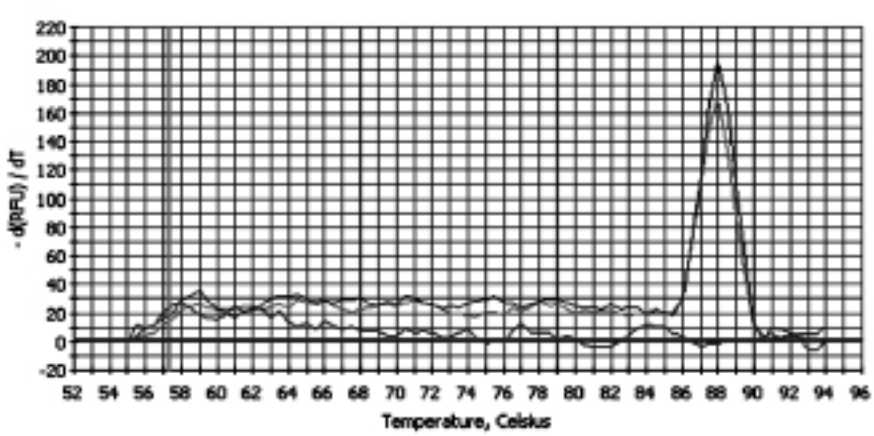

FIGURE 2. Melting temperature profile of DVE gene using SYBR green I qPCR.

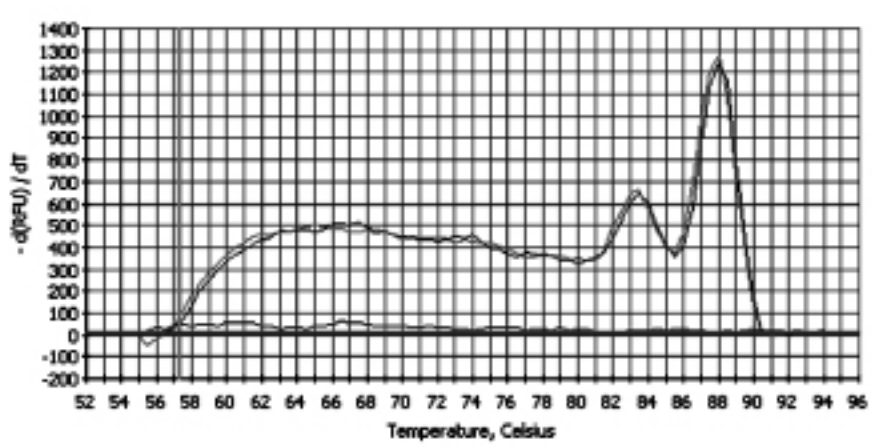

FIGURE 3. Melting temperature profile of DVE gene using GoTaq qPCR (Promega)

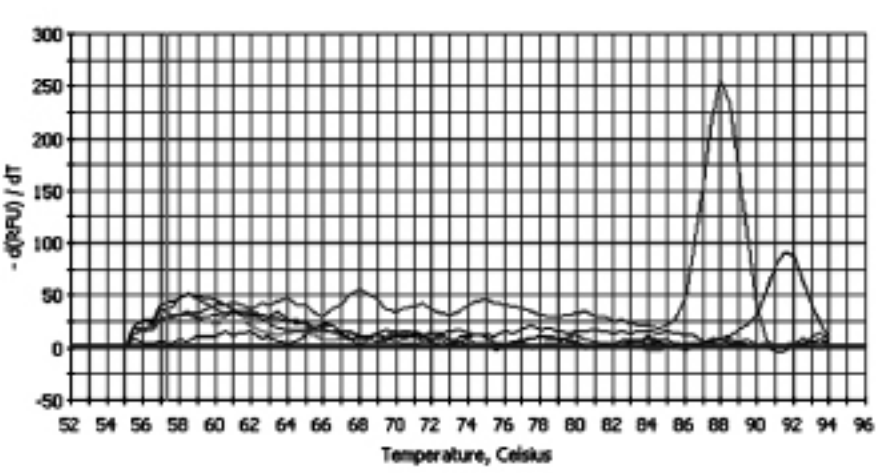

FIGURE 4. Specificity of the qPCR assay where the melting temperature profiles of each samples were display as: 1: DVE (CEF), 2: AI , 3: ND, 4: CAV, 5: EDS, 6: Negative control.

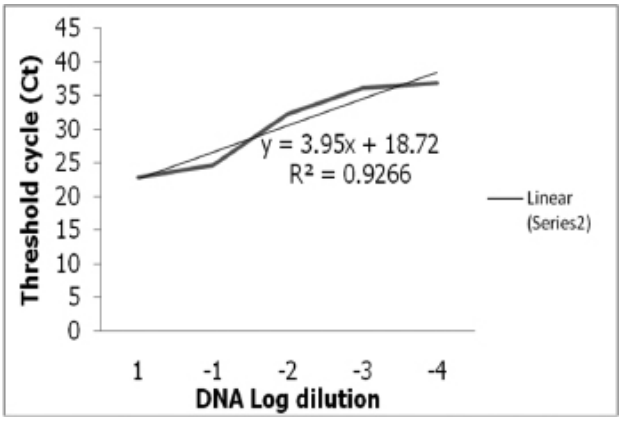

FIGURE 5. A linear relationship between threshold cycle and the 10-fold serially diluted in DNA (DVE virus) [Neat (10^5 copies) to 10^{-4} (10 copies)]. PCR efficiency was 92.66% as indicated by slope (m = −3.95). Standard curve was generated from amplification of DVE gene.

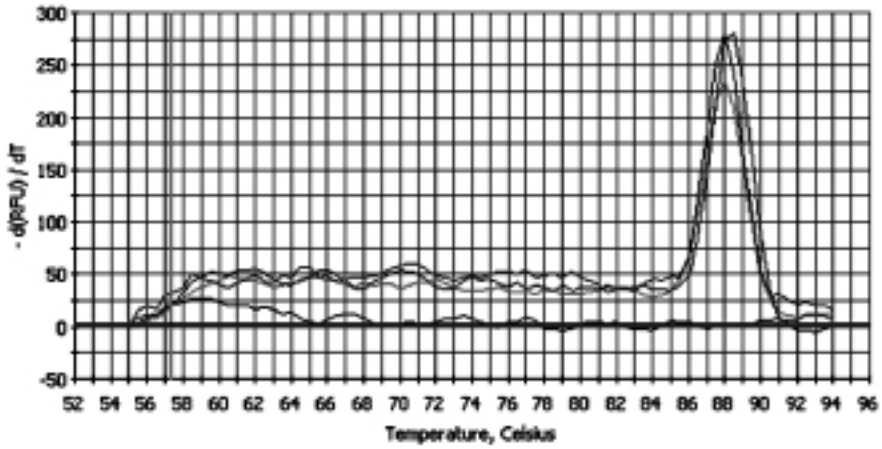

FIGURE 6. Melting temperature profile of qPCR assay of each DVE positive samples (pooled organs) extracted using DNA extraction kit (Maxwell 16) were displayed as: 1: 668/96, 2: 2381/98 3: Positive control DVE CEF-4, 4: negative control.

generated for other avian viruses such as AI, ND, CAV and EDS.

Sensitivity of the qPCR assay

Sensitivity of the assay was evaluated by the number of DNA copies. Ten-fold serial dilutions of the DNA (DVE) were done to determine the sensitivity of the assay. The assay had a linear response over a $5\log_{10}$ serial dilution range, from neat to 10^{-4}. The minimum DNA copies that could be detected were 10 copies of DNA molecules per reaction as shown in the standard curve (Figure 5).

Detection of the DVE gene in pooled organs of DVE infected samples

For evaluating the effectiveness of the assay using real field samples, two DVE positive isolates from year 1996 and 1998 respectively were applied in SyBr Green qPCR. The samples were successfully amplified and were detected using qPCR assay under the same condition where the melting temperature for the DVE gene was slightly varied at 88±0.5°C.

DISCUSSION

This study addresses the development of a sensitive, rapid and economical molecular diagnostic method for DNA virus during DVE disease outbreaks. This developed assay was able to amplify DVE genes using SYBR green I qPCR methods with the specific primer set DVEMFR and DVEMR through melting temperatures. The specificity of the primer set for DVE was proved through conventional PCR where only one band was observed and the amplicon size was specific for the DVE gene with the size 459 bp. Through several trial and error processes, it was found that GoTaq qPCR failed to amplify DVE gene specifically (Figure 3), but, SYBR Green I was found to be the optimized qPCR condition in DVE detection (Figure 2). As a result, SYBR green I qPCR was used for DVE detection because of the specificity and lower cost compared to GoTaq qPCR. Optimized condition have been determined where 60°C is the optimized annealing temperature for the DVE genes through 40 amplification cycles, and the melting temperature for the DVE was determined to be 88.0±0.5°C (Figure 2). For the assay specificity test (Figure 4) one observable melting peak correlated with DVE gene's melting temperature was obtained when the developed assay was applied on DVE (CEF). This indicated that DVEM primer pair and the developed assay was able detect the DVE virus gene specifically. No amplification was determined for the other avian virus which consists of AI, ND, CAV and EDS. This again highlights the specificity of the primer pair for DVE gene used in this study and the specificity of the developed qPCR assay.

SYBR Green I fluorescence is greatly increased upon binding to double stranded DNA during the extension step, more and more flourescent molecules will bind to the PCR products and produce higher fluorescence intensity. For this developed assay, it was found that SYBR Green I based real-time PCR provides a sensitive and rapid method for DVE detection. This developed assay has a dynamic detection limit over a 5 log10 serial dilution range. The slope (m = -3.95) of the 10-fold serial dilution standard curve generated from the amplification of DVE genes indicated an amplification efficiency of 92.66%. The correlation coefficient (R2 = 0.9266), suggested that there was a correlation between Ct values and template concentrations. The Slope -3.95, more negative than –3.32 indicated that reactions were slightly less than 100% efficient (ideal

efficiency). Less than ideal real-time PCR data can occur regardless of the stringent control of experimental conditions, but, it may due to other factors. The amplification of qPCR reaction for DVE of less than 100% efficiency may be attributed the degradation of SYBR green I due to the pH, freeze-thawing and light sensitivity. It was reported that SYBR green I remained stable for as long as 18 days. After 21 days, the degraded SYBR green I may exhibits inhibitions to PCR (Karsai *et al.,* 2001). The qPCR efficiency may declined when as the larger fragments (more than 400bp) were used in the reaction (Karsai *et al.,* 2001).

Although traditional and serological methods such as IFA test can be used for the DVE detection, it is time consuming, laborious and less sensitive. Traditional and serological assay for DVE detection need to be improved. GoTaq qPCR failed to amplify DVE gene specifically, but SYBR green I qPCR was able amplify DVE gene specifically. This real-time PCR assay developed using SYBR green I in this study is a useful tool for differential diagnosis of DVE disease with other diseases like DVH, AI, CAV, ND and so on. The ability of qPCR assay to sensitively and specifically detect DVE gene in infected tissue culture and the field samples collected a few years ago is an advantage in such situations.

REFERENCES

1. Hofstad M.S., Barnes H.J., Calnek B.W., Reid W.M. and H.W. Yoder Jr 1984. *Disease of Poutry. 8th Ed. : Leibovitz, L. Duck Plague (Duck Virus Enteritis).* Lowa State University Press, Ames, Iowa, USA.
2. Islam M.R., Nessa J. and Halder K.M. 1993. Detection of duck plague virus antigen in tissues by immunoperoxiodase staining. *Avian Pathology*. **22** (2), pp. 389-393.
3. Karsai A., Muller S., Platz S. and Hauser M.T. 2002. Evaluation of a Homemade SYBR® Green IReaction Mixture for Real-Time PCR Quantification of Gene Expression. *BioTechniques.* **32,** pp. 790-796.
4. Logan J., Edwards K. and Saunders N. 2009. *Real-time PCR: current technology and applications.* Horizon Scientific Press.
5. Maizan M., Aziz H., Ong G.H., Suraini M.H., Kamil W.N. and Sharifah S.H. 2000. Development of nested Polymerase Chain Reaction for the diagnosis Duck Viral Enteritis. *Proceeding of 12th Veterinary Assoc. Malaysia, Kuantan, 1-4 Sept 2000*. pp. 154-155.
6. Ong W.T., Omar A.R., Ideris A. and Hassan S.S. 2007. Development of a multiplex real-time PCR assay using SYBR Green 1 chemistry for simultaneous detection and subtyping of H9N2 influenza virus type A. *Journal of Virological Methods*. **144,** pp. 57–64.
7. Pritchard L.I., Morrissy C., Phuc K.V., Daniels P.W. and Westbury H.A. Development of a polymerase chain reaction to detect Vietnamese isolates of duck virus enteritis. *Veterinary Microbiology*. **68** (1-2), pp. 149-156.
8. Qi X.F., Yang X.Y., Cheng A.C., Wang M.S., Zhu D. and Jia R.Y. 2008. The pathogenesis of duck virus enteritis in experimentally infected ducks: a quantitative time-course study using Taqman polymerase chain reaction. *Avian Pathology*. **3** (3), pp. 307-310.
9. Saif Y.M. 2003. *Disease of Poutry. 11th Ed*.: Alexander D.J. *Newcastle disease.* Iowa State Press, A Blackwell publishing company.
10. Saif Y.M. 2003. *Disease of Poutry. 11th Ed*.: Cavanagh D. and Naqi S.A. *Infectious Bronchitis.* Iowa State Press, A Blackwell publishing company.
11. Saif Y.M. 2003. *Disease of Poutry. 11th Ed.* : Sandhu T.S. and Shawky S.A. *Duck Virus Enteritis*. Iowa State Press, A Blackwell publishing company.
12. Saif Y.M. 2003. *Disease of Poutry. 11th Ed*.: Swayne, D.E. & Halvorson, D.*A. Influenza*. Iowa State Press, A Blackwell publishing company.
13. Saif Y.M. 2003. *Disease of Poutry. 11th Ed*.:Woolcock P.R. *Duck Hepatitis*. Iowa State Press, A Blackwell publishing company.
14. Veterinary Research Institute : http://www.jphvri.gov.my
15. Yang F.L., Jia W.X., Yue H., Luo W., Chen X., Xie Y., Zen W. and Yang W.Q. 2005. Development of quantitative Real-time Polymerase Chain Reaction for Duck Enteritis Virus DNA. *Avian diseases.* **49**, pp. 397–400.
16. Yuan J.S., Reed A., Chen F. and Jr. C. N. S. 2006. Statistical analysis of real-time PCR data. *BMC Bioinformatics*. **7** (85). pp. 1-12.