Journal of Virological Methods 270 (2019) 126–130

Contents lists available at ScienceDirect

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet

A reverse transcription-insulated isothermal PCR assay for the detection of duck hepatitis A virus type 3 based on the POCKIT™ system

Yupeng Ren[a,b,1], Hua Yue[b,1], Lin Zhu[a,*,1], Ruici Kan[b,1], Cheng Tang[b,1], Bin Zhang[b,1]

[a] College of Veterinary Medicine, Sichuan Agricultural University, Wenjiang, Chengdu City, Sichuan Province, 611130, PR China
[b] College of Life Science and Technology, Southwest University for Nationalities, Chengdu City, Sichuan Province, 610041, PR China

ARTICLE INFO

Keywords:
Duck hepatitis A virus
Insulated isothermal RT-PCR
Detection

ABSTRACT

Duck hepatitis A virus type 3 (DHAV-3) infection is characterized by severe hepatitis. In recent years, DHAV-3 has become widespread in Asia and has led to economic losses. Conventional methods for DHAV-3 detection usually depend on the use of larger equipment that is not portable and is not fit for on-site diagnoses. In this study, a rapid reverse transcription insulated isothermal (RT-iiPCR) technique was developed for the on-site detection of DHAV-3 based on the POCKIT™ system in a convenient device. The concentration of primer pairs and probes were optimized for amplification of the DHAV-3 VP3 gene of DHAV-3, with no amplification of 12 other duck pathogens. The detection limit of viral RNA was 3.85×10^1 copies/μL, and the analytical sensitivity and specificity levels were both 100% in the detection of 40 liver samples. Furthermore, 97.5% of the RT-iiPCR results were in agreement with those of rRT-PCR, with a kappa value of 0.93. This method is time-saving and better suited to field diagnoses because of its portable device.

1. Introduction

Duck hepatitis A virus (DHAV) is the causative agent of duck viral hepatitis which is characterized by an increased number of liver vacuoles, liver necrosis and hemorrhage, and a high mortality rate, especially in young ducklings (Yugo et al., 2016). Persistent DHAV infection is considered to be an age-dependent and dose-dependent predisposition in young ducks (Fu et al., 2009; Jilbert et al., 1998), and the highest DHAV viral loads are found in the liver ($10^{11.15}$ copies·g^{-1}), spleen ($10^{10.37}$ copies·g^{-1}), and kidney ($10^{10.30}$ copies·g^{-1}) (Zhu et al., 2018).

DHAV was categorized as a member of the Picornaviridae by the Virus Taxonomy Ninth Report of the International Committee on Taxonomy of Viruses (ICTV). According to phylogenetic analyses and neutralization tests, DHAV is genetically divided into three genotypes: DHAV-1, DHAV-2, and DHAV-3(Doan et al., 2016). However, DHAV-3 is widely distributed throughout Asia (Wen et al., 2018), and epidemiological investigations revealed that a significantly higher proportion of DHAV infections were caused by DHAV-3 (66.2%) between 2013 and 2015 in South Korea compared with DHAV-1 (Soliman et al., 2015). In China, the highest number of outbreaks caused by DHAV-1 occurred in 2010–2012, but this declined continuously in subsequent years. From 2013–2015, the most frequently detected genotype in China was DHAV-3, representing 57.1% of 14 isolates identified in five provinces. Phylogenetic analyses showed that all DHAV-3 strains isolated in this study belonged to a monophyletic group, which has become the predominant viral type since 2013, particularly in Shandong and Sichuan provinces (Wen et al., 2018). Such changes of the predominant DHAV type and its epidemic features show the importance of developing new detection methods and vaccination strategies.

Many duck pathogens causing hepatitis and their similar clinical symptoms make them difficult to identify. Therefore, immunological and molecular methods have been used for rapid diagnostics. An indirect enzyme-linked immunosorbent assay (ELISA) based on recombinant VP3 protein of DHAV-3 has been developed to efficiently monitor antibody levels in sera from duck flocks with suspected DHAV infection (Shen et al., 2015). However, this assay does not distinguish between DHAV-1 and DHAV-3 because of cross-reactivity between the two genotypes. PCR-based assays are rapid and sensitive compared with classical diagnostic assays such as virus isolation, or when specific pathogens are difficult to isolate or culture *in vitro* (Espy et al., 2006). Thus, techniques such as RT-PCR (Chen et al., 2013), real-time RT-PCR

* Corresponding author.
E-mail addresses: renyupeng1986@163.com (Y. Ren), yhua900@163.com (H. Yue), 434766626@qq.com (L. Zhu), 1204020524@qq.com (R. Kan), tangcheng101@163.com (C. Tang), binovy@sina.com (B. Zhang).
[1] Current address: College of Life Science and Technology, Southwest University for Nationalities, South Section 4, First Ring Rd, Wuhou District, Chengdu City, Sichuan Province, PR China.

https://doi.org/10.1016/j.jviromet.2019.05.006
Received 8 April 2018; Received in revised form 7 May 2019; Accepted 13 May 2019
Available online 14 May 2019
0166-0934/

based on SYBR-Green I fluorescence (Huang et al., 2012), and real-time TaqMan RT-PCR (Hu et al., 2016) have been developed to detect DHAV. Although these detection methods are rapid and very sensitive, they rely on laboratory-based instruments.

Insulated isothermal PCR (iiPCR) is a type of gene amplification technique based on the principle of Rayleigh–Bénard convection. It involves the amplification of target cDNA by cycling reaction components through different temperature zones to achieve denaturation, annealing, and extension steps of PCR (Chang et al., 2012; Tsai et al., 2012b). Since it was first reported in 2002 (Krishnan et al., 2002), iiPCR assays have been used for the detection of many animal pathogens, such as the H3N8 subtype of equine influenza virus (Balasuriya et al., 2014), foot and mouth disease virus (Ambagala et al., 2017), and providing high sensitivity comparable with an OIE-certified commercial nested PCR kit for white spot syndrome virus (Tsai et al., 2012a). This approach has also been used as a qualitative method for rapid on-site malaria detection, which achieved 96.9% efficiency and a detection limit of ≥100 copies of plasmodial DNA (Chua et al., 2016). All iiPCR reactions are completed using the POCKIT™ system (GeneReach USA, Lexington, MA), which is a small device which is convenient for on-site rapid detection. Besides, the process steps of RNA extraction from livers sample in the field involves the centrifugation of crushed tissues. It could be completed by a cubee™ Mini-Centrifuge with portable power source manufactured by Genereach Biotechnology corporation.

In this study, an on-site rapid RT-iiPCR assay was developed targeting the *VP3* gene of DHAV-3. A one-step duplex rRT-PCR assay for DHAV (Hu et al., 2016) was evaluated in parallel with RT-iiPCR for analytical sensitivity and specificity, and the accuracy of the two assays for detecting DHAV-3 in liver samples was compared. In order to be more suitable to on-site detection, the PetNAD™ nucleic acid rapid extraction kit was used in liver samples. And It was compared with the TRIzol method.

2. Materials and methods

2.1. Viruses, bacteria, and clinical samples

Seven clinical isolates of DHAV-3, eight virus species, and four bacterial species that potentially infect ducklings were used to evaluate the specificity of the RT-iiPCR assay (Table 1). Forty archived sequential DHAV-3 liver samples (containing 31 positive samples and 9 negative samples), which were collected from sick ducklings less than 3 weeks old in May 2016 in China. These samples had been confirmed by the reported real-time quantitative PCR (Huang et al., 2012) before they were used in this study. The amplifications were sequenced and analyzed at the Laboratory for Preventive Veterinary Science of Southwest University for Nationalities. All animal procedures used in the present study were conducted in accordance with good animal practices as defined by the Laboratory Animal Use License (Certificate No. SYXK (CHUAN) 2014 – 187).

2.2. Virus nucleic acid extraction and cDNA synthesis

The performance of two different nucleic acid extraction methods using the manufacturer's instructions was evaluated in this study. Initially, DHAV-3 nucleic acid was extracted from 15 clinical samples (10 of which were identified as positive) using the PetNAD™ nucleic acid rapid extraction kit (GENERADAR Biotechnology Corp., Xiamen, China). Clinical samples were centrifuged at 13,000 × *g* for 2 min prior to nucleic acid extraction. The centrifugation of clinical samples was completed by a cubee™ Mini-Centrifuge with portable power source manufactured by Genereach Biotechnology corporation. Then, 200 μL of supernatant was mixed with 600 μL of buffer PB1, and shaken for 1 min. Next, 600 μL of buffer PB2 was added, and the mixture was transferred to a spin column. Wash steps were performed as described in the manufacturer's manual. Total RNA was eluted in 50 μL of elution buffer PB5. All nucleic acid preparations were stored at –80 °C prior to testing. Subsequently, these samples were compared with those obtained using TRIzol reagent (Applied Biosystems Inc., Carlsbad, CA). Total RNA was then reverse-transcribed into cDNA using a PrimeScript™ reverse transcription kit (TaKaRa Biotechnology, Dalian, China). These cDNA was in preparation for the rRT-PCR assay.

Table 1
Reference strains and isolates used in this study for RT-iiPCR specificity testing.

Pathogens	Source	RT-iiPCR detection	rRT-PCR detection
DHAV-3 strain SWUN3501	a	Pos	31.51
DHAV-3 strain SWUN3502	a	Pos	27.81
DHAV-3 strain SWUN3503	a	Pos	26.34
DHAV-3 strain SWUN3504	a	Pos	34.19
DHAV-3 strain SWUN3505	a	Pos	29.11
DHAV-3 strain SWUN3506	a	Pos	24.37
DHAV-3 strain SWUN3507	a	Pos	27.77
DHAV-1 strain SWUN3523	a	Neg	Neg
NDV SWUN 2690	a	Neg	Neg
GPV SWUN 5301	a	Neg	Neg
MPV SCAU 251	b	Neg	Neg
AIV CAHEC 343	c	Neg	Neg
FAV-ISWUN 7002	a	Neg	Neg
DAstV-1 CAU121	d	Neg	Neg
DAstV-2 CAU122	d	Neg	Neg
P. multocida SWUN 0300	a	Neg	Neg
E. coli (O46) SWUN 0314	a	Neg	Neg
S. enteritidis SWUN 5223	a	Neg	Neg
R. anatipestifer SWUN 0233	a	Neg	Neg

a, Southwest University for Nationalities, China; b, Sichuan Agricultural University, China; c, China Animal Health and Epidemiology Center, China; d, China Agricultural University, China.
Pos, positive; Neg, negative. NDV, Newcastle disease virus; GPV, gosling parvovirus; MPV, muscovy parvovirus; AIV, avian influenza virus; FAV-I, fowl adenovirus-I; DAstV: duck astrovirus.

2.3. Bacterial nucleic acid extraction

DNA was extracted from plate cultures of *Pasteurella multocida* SWUN 0300, *Escherichia coli* (O46) SWUN 0314, *Salmonella enteritidis* SWUN 5223, and *Riemerella anatipestifer* SWUN 0233 using a DNA extraction kit (TaKaRa Biotechnology) according to the manufacturer's instructions. Subsequently, these DNA samples were used to determine the specificity of the RT-iiPCR assays.

2.4. Development of RT-iiPCR for DHAV-3

Primers and probes were designed according to the complete genome sequence (GenBank database) from 22 different DHAV-3 strains (accession nos. DQ256132.1, DQ256133.1, DQ812093.1, KC993890.1, GQ485311.1, GQ485310.1, KU860090.1, KP233203.1, JF835025.1, GQ122332.1, EU755009.1, EU877916.1, KP995438.1, JF914944.1, JX312194.1, GU066821.1, GU066823.1, GU066824.1, EU352805.2, GU066822.1, EU747874.1, and DQ256134.1), eight different DHAV-1 strains (accession nos. FJ971623.1, GU944671.1, FJ496342.1, FJ496339.1, FJ496344.1, J496341.1, FJ496340.1, and KU923754.1), and two DHAV-2 strains (accession nos. EF067923.1 and EF067924.1). The target was a 161 bp nucleotide sequence specific to the *VP3* gene of DHAV-3 (accession no. EU352805.2), which was chosen using Primer Express software, version 1.0, for TaqMan technology. Primer sequences were: VP3F, 5′-GATATAGTCATGGTGCT TAG-3′ and VP3R, 5′-AGGGACCCTCTCCAATATTG-3′; and the TaqMan probe was VP3P, FAM-GGCTCACACAGTGACCCCTGGCAACA-BHQ1.

The RT-iiPCR assay was optimized by testing DHAV-3 strain SWUN3501. Based on Uni-ii PCR Starter Kit instructions (GENERADAR Biotechnology Corp.), various concentrations of components were tested and screened in a total volume of 50 μL, including forward and reverse primers at 10 μmol μL^{-1} (0.5–4 μL), the 10 μmol μL^{-1} probe

(0.05–0.4 μL), 5U·μL^{-1} Taq DNA polymerase (1–5 μL), and 20U·μL^{-1} reverse transcriptase (0.5–2 μL). Optimum conditions were determined by the absorption ratios at A520/B550.

The RT-iiPCR reaction was carried out using the POCKIT™ device as RT at 42 °C for 10 min, then iiPCR at 95 °C for 30 min. Reaction signals were processed by an optical detection module and shown automatically on the display screen. Results were converted automatically to "+" (positive), "–" (negative), or "?" (inconclusive), according to the default S/N thresholds of POCKIT™ (Miszczak et al., 2011.).

2.5. The detection limits of RT-iiPCR

DHAV-3 strain SWUN3501 RNA was extracted by PetNAD™ nucleic acid rapid extraction. Copy numbers were detected by rRT-PCR as previously described by Hu et al. (2016). The primer and probes sequences of rRT-PCR were: F3, 5′- GTGCTTAGACGCTGGCAGAT-3′ and R3, 5′-TTCGATTGAAAACTATCTGAAACCTATC-3′; Taqman probe was P-VP0, FAM-TCAGTGGGCTAACACAGTAGCCCCTG-BHQ. The concentrations of relevant reagents and the thermocycling conditions for the detection of DHAV-A were as described previously (Hu et al., 2016). Ten-fold serially diluted standard RNA templates with RNase-free water were simultaneously assayed by RT-iiPCR and rRT-PCR to compare their sensitivity. All samples were amplified using optimum conditions.

2.6. RT-iiPCR specificity

All of pathogens, used in the test of specificity, was confirmed by rRT-PCR (Hu et al., 2016). A total of 12 non-DHAV-3 pathogens were used to determine the specificity of the RT-iiPCR (Table 1). Nucleic acid templates were extracted from DHAV-1 attenuated strain SWUN3523, duck astrovirus 1 (DAstV-1), DAstV-2, gosling parvovirus, muscovy parvovirus, avian influenza virus (H5N1), fowl adenovirus-I, *P. multocida*, *E. coli* (O46), *S. enteritidis*, and *R. anatipestifer*.

2.7. RT-iiPCR repeatability and reproducibility

Six different RNA dilutions from positive samples (1×10^{-2}–1×10^{-7}) were used to evaluate the reproducibility of RT-iiPCR. Each sample was amplified in triplicate.

2.8. Analysis of RT-iiPCR and comparison with rRT-PCR

The one-step duplex rRT-PCR assay for the detection of DHAV-1 and DHAV-3, which was used as a comparison with RT-iiPCR, was established as previously described (Hu et al., 2016). A total of 40 clinical liver samples collected from sick ducklings were analyzed simultaneously by rRT-PCR and RT-iiPCR. Analytical Sensitivity and specificity were determined by 2 × 2 contingency tables. The degree of agreement between the two assays was assessed by calculating Cohen's kappa (k) values.

3. Results

3.1. Protocol optimization

Optimization testing showed that the combination of 3.5 μL (10 μmol μL^{-1}) primers, 0.25 μL (10 μmol μL^{-1}) probes, 1 μL (5 U·μL^{-1}) Taq DNA polymerase, and 1.25 μL (20 U·μL^{-1}) reverse transcriptase, Premix Ex Taq 25 μL in a total volume of 50 μL achieved the maximum A520/B550 value of 4.65. These reagent volumes were used for all subsequent experiments.

3.2. Comparison of detection limits of rRT-PCR and RT-iiPCR

The rRT-PCR assay detected a DHAV-3 RNA concentration of 3.85×10^8 copies/μL, which was serially diluted and tested in duplicate. The rRT-PCR assay detection limit was a dilution of 10^{-6} to 10^{-7}, and regression analysis indicated that good linearity was achieved (slope = –3.3968, R^2 = 0.998; Fig. 1). The rRT-PCR amplification efficiency (%), calculated by the formula $E = [10^{-1/slope} - 1] \times 100$, was 96.97%. By comparison, the RT-iiPCR detection limit was 10^{-6} to 10^{-7}dilutions (Table 2), indicating that the sensitivities of the two methods were approximately identical.

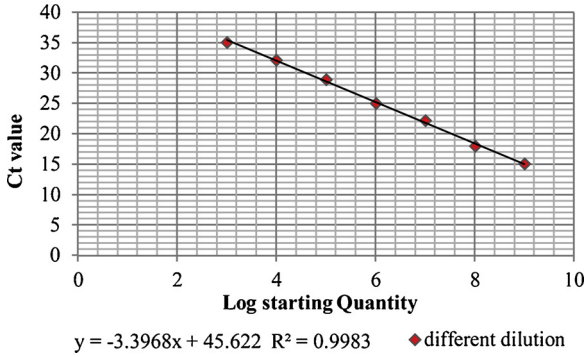

Fig. 1. The linear relationship of every dilution detected by rRT-PCR.

Table 2
Evaluation of rRT-PCR and RT-iiPCR sensitivity for DHAV-3 amplification.

Dilution	DHAV-3 SWUN3501 virus strain	
	RT-iiPCR	rRT-PCR (Ct value)
10^{-2}	Pos	15.11 ± 0.039
10^{-3}	Pos	18.03 ± 0.057
10^{-4}	Pos	22.21 ± 0.018
10^{-5}	Pos	25.07 ± 0.024
10^{-6}	Pos	28.97 ± 0.031
10^{-7}	**Pos**	**32.16 ± 0.082**
10^{-8}	Neg	Neg
10^{-9}	Neg	Neg

Limits of detection (LOD) are bold. Neg, negative; Pos, positive.

3.3. RT-iiPCR specificity

DHAV-3 RNA could be detected from all seven DHAV-3 strains both by RT-iiPCR and by rRT-PCR, but nucleic acids extracted from 12 other duck pathogens could not be amplified (Table 1). These data indicate the high level of RT-iiPCR specificity for DHAV-3.

3.4. Comparison of the two nucleic acid extraction methods

Samples extracted with TRIzol and PetNAD™ nucleic acid rapid extraction were simultaneously compared by rRT-PCR and RT-iiPCR. Both methods of RNA extraction were able to achieve the same positive detection rate for 15 DHAV-3 samples of 66.7% (10/15) (Table 3), which agreed with prior sequencing results. PetNAD™ nucleic acid rapid extraction therefore achieves the similar results as TRIzol but is more rapid and does not need a high-speed centrifuge, which can be inconvenient for on-site pathogen detection.

3.5. Comparison between rRT-PCR and RT-iiPCR in the analysis of liver samples

The rRT-PCR and RT-iiPCR were compared in the detection of 40 archived liver samples (containing 31 positive samples and 9 negative samples), which had been confirmed by the reported Real-Time Quantitative PCR (Huang et al., 2012) to evaluate their sensitivity in detecting DHAV-3. For this purpose, RNA was extracted by the PetNAD™ nucleic acid rapid extraction kit. The rRT-PCR assay

Table 3
Performance evaluation of PetNAD™ and TRizol RNA extraction methods for DHAV-3 detection by rRT-PCR and RT-iiPCR.

Sample ID	Sequencing result	petNAD™		Trizol	
		rRT-PCR (Ct value)	RT-iiPCR	rRT-PCR (Ct value)	RT-iiPCR
SCSl11	Pos	30.16	Pos	28.77	Pos
SCSl12	Pos	28.93	Pos	25.64	Pos
SCSl13	Pos	25.76	Pos	24.16	Pos
SCWJ21	Pos	31.31	Pos	32.79	Pos
SCWJ3	Pos	27.52	Pos	23.41	Pos
SCWJ4	Pos	28.43	Pos	26.93	Pos
SCWJ5	Pos	30.34	Pos	27.14	Pos
SCWJ6	Pos	29.53	Pos	25.72	Pos
SCWJ7	Pos	26.78	Pos	24.15	Pos
SCWJ8	Pos	21.31	Pos	24.22	Pos
SCSl41	Neg	Neg	Neg	Neg	Neg
SCSl50	Neg	Neg	Neg	Neg	Neg
SCSl61	Neg	Neg	Neg	Neg	Neg
SWUN05	Neg	Neg	Neg	Neg	Neg
SWUN07	Neg	Neg	Neg	Neg	Neg

Neg, negative; Pos, positive.

Table 4
The level of agreement between rRT-PCR and RT-iiPCR assays for the detection of DHAV-3 in liver samples.

		The known samples		
		Positive	Negative	Total
rRT-PCR	Positive	30	0	30
	Negative	1	9	10
	Total	31	9	40
		The known samples		
		Positive	Negative	Total
RT-iiPCR	Positive	31	0	31
	Negative	0	9	9
	Total	31	9	40
		rRT-PCR		
		Positive	Negative	Total
RT-iiPCR	Positive	30	1	31
	Negative	0	9	9
	Total	30	10	40

successfully detected DHAV-3 nucleic acid in 30/31 established positive liver samples (Table 4). However, the RT-iiPCR assay detected 31/31 positive samples. The analytical sensitivity and specificity of the rRT-PCR of DHAV-3 and RT-iiPCR were determined from 2 × 2 contingency tables as compared to sequencing analysis. The analytical sensitivity and specificity were respectively 96.77% and 100% for the rRT-PCR while the RT-iiPCR showed 100.00% both. Therefore, rRT-PCR and RT-iiPCR were in good agreement with the real consequences in liver samples. RT-iiPCR was 97.5% in agreement with rRT-PCR (Table 4) with a kappa value of 0.93.

3.6. Evaluation of RT-iiPCR reproducibility

RT-iiPCR were used to analyze six samples in triplicate which were obtained from serial dilutions (from 10^{-2} to 10^{-7}) of DHAV-3 strain SWUN3501. The consistency of this analysis was indicative of the high reproducibility of RT-iiPCR.

4. Discussion

DHAV-3 is one of the major pathogens of duck hepatitis and is widespread throughout the world, causing major economic losses for commercial duck farms. Liver injury is characteristic for DHAV-3, but this can also be caused by many other duck pathogens such as DHAV-1, DHAV-2, Newcastle disease virus, DAstv-1, and bacteria (Fu et al., 2009), so diagnosis can be difficult. Therefore, there is a need to develop a rapid, effective, and reliable detection method. Although virus isolation and purification are currently the recognized classical method for virus detection, its performance depends on the type of culture medium, susceptibility of the host cell, coinfection of multiple pathogens, and many other uncertain conditions (Kumar et al., 2016).

In recent years, RT-iiPCR as a new fast molecular diagnostic method were more and more used in the detection of pathogens (Ambagala et al., 2017; Carossino et al., 2016). The reagents and reaction systems are contained in a small device (POCKIT™), and results are obtained after automatic data processing with a default algorithm, without the need for manual data analysis and interpretation. Thus, this method facilitates the rapid diagnosis of disease and can be applied to many situations.

In the present study, a new RT-iiPCR assay for DHAV-3 detection based on the POCKIT™ system was developed. Similar to quantitative RT-PCR, this assay is also based on TaqMan® probe hydrolysis to generate a fluorescent signal. The optimum reaction system included 35 μmol primers, 2.5 μmol probes, 5 U Taq DNA polymerase, and 25 U reverse transcriptase, which were contained in one tube for a single reaction and processed using vacuum freeze-drying technology. The premixed reagents simply require dissolving in sterile deionized water before use. This technique also reduces the operating time, which prevents sample contamination (Wilkes et al., 2015). Moreover, seven DHAV-3 strains collected from different area were correctly diagnosed by our RT-iiPCR assay, which demonstrated high specificity for DHAV-3. The low limit of detection for RT-iiPCR was 3.85×10^{1} copies/μL, which was similar to rRT-PCR (from 10^{0} to 10^{-7} dilutions) (Hu et al., 2016). The results of rRT-PCR and RT-iiPCR were in excellent agreement with the actual findings in liver samples, while RT-iiPCR was 97.5% in agreement with rRT-PCR with a k value of 0.93. Although RT-iiPCR had no significant improvement in sensitivity or accuracy compared with rRT-PCR, its advantages of rapid nucleic acid extraction, short processing time, and portable device mean that it has potential for field-deployable diagnoses. The RT-iiPCR also would be run as part of a suite of PCR tests for differential diagnosis or for more targeted detection.

5. Conclusions

This study established an RT-iiPCR method based on the POCKIT™ system that can be processed in a mini-type device weighing less than 0.83 pounds which is convenient to transport. The entire detection process from RNA extraction to obtaining the results takes only about 1 h. Moreover, the technique is both sensitive and specific. It was demonstrated that the RT-iiPCR/POCKIT™ system can be used as a reliable and effective tool for the diagnosis and detection of DHAV-3, and that it could facilitate molecular epidemiological investigations of DHAV-3 and prevent and control DHAV breeding reservoirs.

Competing interests

The authors declare that there is no conflict of interest.

Funding

This present study was supported simultaneously by The National Key Research and Development Program of China (2017YFD0501101) and the Fundamental Research Funds for the Central Universities

(2017NZYQN01).

Acknowledgements

We would like to thank Prof. Hua Yue for insightful comments regarding study design and the manuscript, and Dr. Bin Zhang for providing the nucleic acids from various duck bacterial pathogens used in this study.

References

Ambagala, A., Fisher, M., Goolia, M., Nfon, C., Furukawa, T., Ortega, P.R., Lung, O., 2017. Field-deployable reverse transcription-insulated isothermal PCR (RT-iiPCR) assay for rapid and sensitive detection of foot-and-Mouth disease virus. Transbound. Emerg. Dis. 64 (5), 1610–1623.

Balasuriya, U.B., Lee, P.Y., Tiwari, A., Skillman, A., Nam, B., Chambers, T.M., Tsai, Y.L., Ma, L.J., Yang, P.C., Chang, H.F., Wang, H.T., 2014. Rapid detection of equine influenza virus H3N8 subtype by insulated isothermal RT-PCR (iiRT-PCR) assay using the POCKIT™ Nucleic Acid Analyzer. J. Virol. Methods 207, 66–72.

Carossino, M., Lee, P.Y., Nam, B., Skillman, A., Shuck, K.M., Timoney, P.J., Tsai, Y.L., Ma, L.J., Chang, H.F., Wang, H.T., Balasuriya, U.B., 2016. Development and evaluation of a reverse transcription-insulated isothermal polymerase chain reaction (RT-iiPCR) assay for detection of equine arteritis virus in equine semen and tissue samples using the POCKIT system. J. Virol. Methods 234, 7–15.

Chang, H.F., Tsai, Y.L., Tsai, C.F., Lin, C.K., Lee, P.Y., Teng, P.H., Su, C., Jeng, C.C., 2012. A thermally baffled device for highly stabilized convective PCR. Biotechnol. J. 7 (5), 662–666.

Chen, L.L., Xu, Q., Zhang, R.H., Yang, L., Li, J.X., Xie, Z.J., Zhu, Y.L., Jiang, S.J., Si, X.K., 2013. Improved duplex RT-PCR assay for differential diagnosis of mixed infection of duck hepatitis A virus type 1 and type 3 in ducklings. J. Virol. Methods 192 (1-2), 12–17.

Chua, K.H., Lee, P.C., Chai, H.C., 2016. Development of insulated isothermal PCR for rapid on-site malaria detection. Malar. J. 15, 134.

Doan, H.T., Le, X.T., Do, R.T., Hoang, C.T., Nguyen, K.T., Le, T.H., 2016. Molecular genotyping of duck hepatitis A viruses (DHAV) in Vietnam. J. Infect. Dev. 10 (9), 988–995.

Espy, M.J., Uhl, J.R., Sloan, L.M., Buckwalter, S.P., Jones, M.F., Vetter, E.A., Yao, J.D.C., Wengenack, N.L., Rosenblatt, J.E., Cockerill, F.R., Smith, T.F., 2006. Real-time PCR in clinical microbiology: applications for routine laboratory testing. Clin. Microbiol. Rev. 19 (1), 165–256.

Fu, Y., Pan, M., Wang, X., Xu, Y., Xie, X., Knowles, N.J., Yang, H., Zhang, D., 2009. Complete sequence of a duck astrovirus associated with fatal hepatitis in ducklings. J. Gen. Virol. 90 (Pt 5), 1104–1108.

Hu, Q., Zhu, D., Ma, G., Cheng, A., Wang, M., Chen, S., Jia, R., Liu, M., Sun, K., Yang, Q., Wu, Y., Chen, X., 2016. A one-step duplex rRT-PCR assay for the simultaneous detection of duck hepatitis A virus genotypes 1 and 3. J. Virol. Methods 236, 207–214.

Huang, Q.X., Yue, H., Zhang, B., Nie, P.T., Tang, C., 2012. Development of a real-time quantitative PCR for detecting duck hepatitis a virus genotype C. J. Clin. Microbiol. 50 (10), 3318–3323.

Jilbert, A.R., Botten, J.A., Miller, D.S., Bertram, E.M., Hall, P.M., Kotlarski, J., Burrell, C.J., 1998. Characterization of age- and dose-related outcomes of duck hepatitis B virus infection. Virology 244 (2), 273–282.

Krishnan, M., Ugaz, V.M., Burna, M.A., 2002. PCR in a Rayleigh-Bénard convection cell. Science 298 (5594), 793.

Kumar, N., Barua, S., Riyesh, T., Chaubey, K.K., Rawat, K.D., Khandelwal, N., Mishra, A.K., Sharma, N., Chandel, S.S., Sharma, S., Singh, M.K., Sharma, D.K., Singh, S.V., Tripathi, B.N., 2016. Complexities in isolation and purification of multiple viruses from mixed viral infections: viral interference, persistence and exclusion. PLoS One 11 (5), e0156110.

Miszczak, F., Shuck, K.M., Lu, Z., Go, Y.Y., Zhang, J., Sells, S., Vabret, A., Pronost, S., Fortier, G., Timoney, P.J., Balasuriya, U.B., 2011. Evaluation of two magnetic-bead-based viral nucleic acid purification kits and three real-time reverse transcription-PCR reagent systems in two TaqMan assays for equine arteritis virus detection in semen. J. Clin. Microbiol. 49, 3694–3696.

Shen, Y., Cheng, A., Wang, M., Chen, S., Jia, R., Zhu, D., Liu, M., Sun, K., Yang, Q., Chen, X., 2015. Development of an indirect ELISA method based on the VP3 protein of duck hepatitis A virus type 1 (DHAV-1) for dual detection of DHAV-1 and DHAV-3 antibodies. J. Virol. Methods 225, 30–34.

Soliman, M., Alfajaro, M.M., Lee, M.H., Jeong, Y.J., Kim, D.S., Son, K.Y., Kwon, J., Choi, J.S., Lim, J.S., Choi, J.S., Lee, T.U., Cho, K.O., Kang, M.I., 2015. The prevalence of duck hepatitis A virus types 1 and 3 on Korean duck farms. Arch. Virol. 160 (2), 493–498.

Tsai, Y.L., Lin, Y.C., Chou, P.H., Teng, P.H., Lee, P.Y., 2012a. Detection of white spot syndrome virus by polymerase chain reaction performed under insulated isothermal conditions. J. Virol. Methods 181 (1), 134–137.

Tsai, Y.L., Wang, H.T., Chang, H.G., Tsai, C., Lin, C., Teng, P., Su, C., Jeng, C., Lee, P., 2012b. Development of TaqMan probe-based insulated isothermal PCR (iiPCR) for sensitive and specific on-site pathogen detection. PLoS One 7 (9), e45278.

Wen, X., Zhu, D., Cheng, A., Wang, M., Chen, S., Jia, R., Liu, M., Sun, K., Zhao, X., Yang, Q., Wu, Y., Chen, X., 2018. Molecular epidemiology of duck hepatitis a virus types 1 and 3 in China, 2010-2015. Transbound. Emerg. Dis. 65 (1), 10–15.

Wilkes, R.P., Lee, P.Y., Tsai, Y.L., Tsai, C.F., Chang, H.H., Chang, H.F., Wang, H.T., 2015. An insulated isothermal PCR method on a field-deployable device for rapid and sensitive detection of canine parvovirus type 2 at points of need. J. Virol. Methods 220, 35–38.

Yugo, D.M., Hauck, R., Shivaprasad, H.L., Meng, X.J., 2016. Hepatitis virus infections in poultry. Avian Dis. 60 (3), 576–588.

Zhu, Y., Wang, M., Cheng, A., Zhu, D., Jia, R., Liu, M., Chen, S., Zhao, X., Yang, Q., Wu, Y., Chen, X., 2018. Distribution of duck hepatitis a virus type 3 in artificial infected ducklings and analysis of the relationship between histopathological changes and expression of cytokines. pDf. Acta Veterinaria et Zootechnica Sinica 49 (1), 164–172.