Virus Research 183 (2014) 50–55

Contents lists available at ScienceDirect

Virus Research

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

Real-time fluorescence loop-mediated isothermal amplification for the diagnosis of hemorrhagic enteritis virus

Xuemei Liu [1], Yuhao Li [1], Chenggang Xu, Jianru Qin, Jianyong Hao, Min Feng, Liqiang Tan, Weixin Jia, Ming Liao, Weisheng Cao *

Key Laboratory of Veterinary Vaccine Innovation of the Ministry of Agriculture, College of Veterinary Medicine, South China Agricultural University, Guangzhou 510642, The People's Republic of China

ARTICLE INFO

Article history:
Received 23 September 2013
Received in revised form 21 January 2014
Accepted 22 January 2014
Available online 31 January 2014

Keywords:
Hemorrhagic enteritis virus
RealAmp
Nested PCR

ABSTRACT

Suspected cases of hemorrhagic enteritis associated with hemorrhagic enteritis virus (HEV) are becoming more frequent among yellow chickens in the Guangdong Province of China. In this study, we have developed a one-step, ecumenical, real-time fluorescence loop-mediated isothermal amplification (RealAmp) assay for the rapid diagnosis of HEV. The RealAmp assay was performed at 63 °C and reduced the assay time to 15 min, using a simple and portable device, the ESE-Quant Tube Scanner. The detection limit of DNA was 1 fg/μl, and the detection was specific only to HEV. We also used nested PCR to evaluate the application of the RealAmp assay. The coincidence rate of the two methods was 100%. Our data indicated that the RealAmp assay provides a sensitive, specific, and user-friendly diagnostic tool for the identification and quantification of HEV for field diagnosis and in laboratory research.

1. Introduction

Hemorrhagic enteritis virus (HEV) is an officially recognized member of the viral avian Adenoviridae family's group II turkey adenovirus type 3 (TAdV-3). It is related to the frog adenovirus (FrAdV-1) of the *Siadenoviruses* (Davison et al., 2000). HEV is prevalent in poultry flocks worldwide and causes serious economic losses due to splenomegaly, bloody diarrhea, severe intestinal hemorrhages, immunosuppression and mortality. HEV is ubiquitous, birds between 6 and 12 weeks of age are affected most frequently. Great losses due to HE were frequently reported in USA prior to the development of vaccine. Field outbreak of turkey hemorrhagic enteritis in Hungary and Czech Republic remains a big problem (Palya et al., 2007). And it is still regarded as a major important infection in commercial turkey production especially when secondary bacterial infections may exacerbate and extend the course of the disease for several weeks.

Turkeys, chickens and pheasants are the only known natural hosts for HEV. HEV is spread by contaminated manure or by direct contact with infected birds (Fadly and Nazerian, 1984). The transfer of the disease between turkeys and chickens has not been investigated, but chickens may play an important role in the epidemiology of this disease (Rautenschlein et al., 1998). Chickens are susceptible to HEV, which can induce the activation of immune reactions (Rautenschlein et al., 1998) and splenomegaly (Nazerian and Fadly, 1982; Silim et al., 1978). A HEV antibody has also been reported in commercial chicken flocks (Domermuth et al., 1982; Yamaguchi et al., 1982). However, little is known about the disease in chicken. Recently, some local yellow chicken flocks in Guangdong Province were reported to have developed intestinal hemorrhagic disease. Most of the affected chickens had similar symptoms: splenomegaly, bloody diarrhea and lethargy. We performed HEV antibody-ELISAs to survey the seroepidemiology in the field. The results showed that the group positive rate was 68.9% (20/29) and the individual positive rate was 35.9% (90/251) (data not shown). These indicated that HEV may be endangering the local chicken flocks in China.

Accurate diagnosis is important to prevent and control HE. The existing tools for the detection of HEV infection include the agar-gel precipitation (AGP) test (Domermuth et al., 1972), ELISA (van den Hurk, 1986), immunohistochemistry (Fitzgerald et al., 1992), *in situ* hybridization (Saunders et al., 1993) and diagnostic PCR/nested PCR (Beach et al., 2009; Hess et al., 1999). However, these methods require complicated laboratory infrastructure and are time-consuming. The restricted growth of the virus limits the isolation of HEV because it is usually performed on a specific

Abbreviations: HEV, hemorrhagic enteritis virus; RealAmp, real-time fluorescence loop-mediated isothermal amplification; LAMP, loop-mediated isothermal amplification; HE, hemorrhagic enteritis; VAS, the Virginia avirulent strain; SPF, Specific Pathogen Free.

* Corresponding author at: College of Veterinary Medicine, South China Agricultural University, 483 Wushan Road, Tianhe District, Guangzhou 510642, People's Republic of China. Tel.: +86 020 85282536; fax: +86 020 85280234.
E-mail addresses: weishengcao@yahoo.com, caoweish@scau.edu.cn (W. Cao).
[1] These authors contributed equally to this work.

0168-1702/$ – see front matter © 2014 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.virusres.2014.01.023

lymphoblastoid B-cell line of turkeys (MDTC-RP19) (Nazerian and Fadly, 1982) or in turkey peripheral blood leukocyte cultures (Nazerian et al., 1982; Nazerian and Fadly, 1982; van den Hurk, 1990). Therefore, developing a sensitive, specific, simple and cost-effective diagnostic tool for HE field diagnosis and laboratory research is necessary.

The recently developed loop-mediated isothermal amplification (LAMP) (Notomi et al., 2000) method, which has been applied to the detection of several pathogens, is relatively simple and can be improved for better use in the field (Pham et al., 2005; Wei et al., 2012; Xu et al., 2009; Zhang et al., 2010). In recent years, various isothermal amplification methods for the LAMP assay have been developed, such as the ESE-Quant Tube Scanner device, which offers the possibility of developing even simpler point-of-care systems. In the present study, we used the simple portable ESE-Quant Tube Scanner device (Qiagen, MD, Hilden, Germany) that contains both the amplification platform (heating block) and the fluorescent detection unit for end point use (acquire real-time data) into a single LAMP assay unit (Lucchi et al., 2010). We demonstrate the utility of this method with specific primers and compare it to a nested PCR method (Beach et al., 2009). Additionally, we attempted to develop a fast and valuable diagnostic system for the detection of HEV infections among farm-breed chickens.

2. Materials and methods

2.1. Field samples selection

Between 2010 and 2012, several flocks of local yellow chickens in Guangdong Province, China were reported to have intestinal hemorrhagic disease. Most of the affected chickens had similar symptoms: splenomegaly, bloody diarrhea and lethargy. We selected 22 cases of suspected hemorrhagic enteritis (HE) infection from different farms. Spleen and sera samples were aseptically collected and frozen until used.

2.2. Experimental infection

A total of 24 twenty-four days old White Leghorn Specific Pathogen Free (SPF) chickens and 31 twelve days old turkeys were randomly divided into 2 groups, respectively. The experimental group of 20 SPF chickens and 21 turkeys, in two separate isolators, was orally inoculated with 200 μl (100 doses) of the Virginia avirulent strain (VAS) of HEV vaccine (ORALVAX HE®, Schering-Plough Animal Health Corporation). In two additional isolators, the control group of four SPF chickens and ten turkeys was each orally inoculated with 200 μl of phosphate-buffered saline (PBS). After 7 days post-infection, all of the vaccinated and control birds were euthanized. Spleens and sera samples were collected and frozen for further study. All chickens and turkeys were handled according to the provisions of the SCAU's Institutional Animal Care and Use Committee guidelines.

2.3. HEV antibody ELISA test

All sera samples were maintained frozen at −20 °C before being tested. The sera samples were tested for HEV antibody by ProFLOCK® HE-T enzyme-linked immunosorbent assay (ELISA) kit (SynBiotics, USA) according to the manufacturer's instructions. Sera from uninfected chickens were used as negative controls. Three independent HEV-Ab ELISA tests were performed to avoid false positives.

2.4. DNA extraction

Tissue samples were aseptically collected and stored at −70 °C until DNA was extracted. A tissue homogenate was prepared with cold PBS in a sterile environment and centrifuged at 6000 × *g* for 5 min at 4 °C. Tissue pellets were used to extract genomic DNAs using an EZNA SQ tissue DNA Kit (Omega Bio-Tek, GA, USA). Viral DNAs of the VAS of HEV were extracted using a Viral DNA Kit (Omega Bio-Tek) according to the manufacturer's instructions. The DNA was aliquoted and stored at −20 °C. All DNA samples were tested by both RealAmp and nested PCR methods.

2.5. Nested PCR assay

Nested PCR was performed with primers and cycling conditions as described (Beach et al., 2009) with slight modifications. Reactions were performed in 15 μl total volume containing template DNA, 25 pM each primer, 7.5 μl Go Taq Green Master Mix (Promega Corporation, USA), and ddH_2O added to a final volume of 15 μl. After completion of the thermocycler program, PCR products were analyzed on 2% agarose-TAE gels, stained with ethidium bromide, and photographed using a gel documentation system (UVITEC, Cambridge, UK).

2.6. Primer design and RealAmp method

Primers specific for HEV (shown in Table 1) were designed to amplify the gene encoding hexon protein, using Primer Explorer V4 software (http://primerexplorer.jp/elamp4.0.0/index.html) (Eiken Chemical Co., Ltd., Tokyo, Japan). RealAmp was performed in a 25 μl volume using the commercially available Loopamp DNA Amplification Kit (DEAOU Biotechnology Co., Ltd., Guangzhou, China) following the manufacturer's instructions. The DNA amplification was performed at 63 °C for 60 min using the ESE-Quant Tube Scanner, which was set to collect fluorescence, signals at 30 s intervals. The ESE-Quant Tube Scanner device contains a sophisticated fluorescence sensor, which slides back and forth under a set of eight tubes, collecting fluorescence signals over time and allowing real-time documentation of increasing fluorescence signals. A signal slope and combined analysis are used for signal interpretation confirmed by second-derivative analysis (Euler et al., 2012). In the plot, the *X*-axis denotes the time in minutes, and the *Y*-axis shows the fluorescence units in milli-volts (mV). Amplification of the positive DNA yielded a sigmoidal amplification curve, while the negative control tube had no measurable fluorescence as indicated by a flat line in the plot.

2.7. RealAmp assay sensitivity and specificity

The standard viral DNA for sensitivity and specificity was extracted from aliquots of vaccines (HE, VAS). And the viral DNA was diluted to 1 ng/μl firstly, and then 10-fold serial dilutions were prepared to a final sensitivity experiments. The sensitivity experiment repeated 3 times. The analytical specificity of the RealAmp assay was evaluated by testing egg drop syndrome virus (EDSV), infectious bursal disease virus (IBDV), subgroup J avian leukosis virus (ALV-J), avian influenza virus (AIV) H6N1, and the avian reovirus (ARV). All of these virus isolates were obtained from MOA Key Laboratory for Animal Vaccine Development in Guangdong, China.

2.8. Statistics

The sensitivity and specificity of the RealAmp method was calculated using the nested PCR assay as a reference test. The