Loop mediated isothermal amplification biology essay

Science, Biology

ZHANG Shu2, YU Hai-qiong1, LUO Chang-bao1, JIA Kun2, ZHAO Yin1, TIAN Chun-jian1, LIN Zhi-xiong1, LI Shou-jun2Abstract: To establish a molecular biological method for easy and quick detection of equine herpes virus type 1 (EHV-1) and 4 (EHV-4) , a pair of primers were designed according to 6 specific regions of EHV-1 and EHV-4 gB gene, and a loop mediated isothermal amplification(LAMP) assay was established for the first time for the detection of EHV-1 and EHV-4 in China. Amplification temperature was optimized, and the specificity, the sensitivity, and the reproducibility of the LAMP assay were performed. Electrophoresis revealed that ladder-like products were obtained from the EHV-1 DNA by the LAMP at 63℃, while no ladder-like products was found from control samples. The detection limit of EHV-1 DNA LAMP assays was 94*10-6 ng/μL and the EHV-4 DNA LAMP assays was 155. 9*10-6 ng/μL, which was more sensitive than that of traditional PCR method. The LAMP assay established in the present study was quick, simple and original for the detection of EHV-1 and EHV-4, and could be used for the detection of equine herpes virus type 1 and 4 in export and import quarantine. Key words: equine herpes virus type 1 and 4; loop-mediated isothermal amplification(LAMP); Realamp; sensitivityIntroductionEquine herpesvirus types 1 and 4 (EHV-1 and EHV-4) are members of the Alphaherpesvirinae subfamily, genus Varicellovirus. Both virus are endemic in horse populations throughout the word. EHV-1 and EHV-4 are major causative agents of respiratory disease in horses. Equid herpesvirus 1 also causes abortion and neurologic disease(Allen GP, 2004). Respiratory disease among racehorses and abortions in pregnant mares caused by EHV-1 have a great economic impact on the horse industry in the world(Matsumura T,

1992). Because EHV-1 and EHV-4 are antigenically and genetically related(Allen GP, 2004), diagnostic methods are required to distinguish between EHV-1 and EHV-4 infections. Virus isolation (VI), serologic typing, and polymerase chain reaction (PCR) are usually used to diagnose and distinguish between infections with these two related viruses. Virus isolation and PCR are useful for diagnosis in the acute phase of an infection. Because VI is time consuming and laborious, several groups have developed sensitive and rapid PCR tests that detect EHV-1 and EHV-4 DNA in a one-step reaction. (Carvalho R, 2000; Kirisawa R, 1993; Lawrence GL, 1994; Wagner WN, 1992) However, because PCR tests require expensive equipment, these tests are not commonly employed in clinical laboratories. Recently, Loop-mediated isothermal amplification (LAMP) was developed as a novel nucleic acid amplification technique (Notomi, T., 2000). The most significant advantage of the LAMP assay is that it can be carried out under isothermal conditions (60–65°C), and the result can be judged by the naked eye based on the turbidity or fluorescence of the reaction mixture(Mori, Y., 2001; Nagamine, K., 2002; Notomi, T., 2008). The rapidity and simplicity of the LAMP assay make it possible to employ this method in clinical laboratories. We developed the LAMP assay for the detection of EHV-1 and EHV-4. However, it includes an awkward step for extracting DNA from clinical samples, and this prevents the widespread use of the EHV-1 and EHV-4 LAMP assay in clinical laboratories. The sensitivity of the LAMP assay is less affected by the various components of the clinical samples than the PCR assay(Kaneko, H., 2007). It has been reported that herpes simplex virus and human herpesvirus 6 DNA can be directly detected in clinical samples by the LAMP assay without DNA

extraction (Enomoto, Y., 2005; Ihira, M., 2007; Ihira, M., 2010). The rapidity, high specificity, and simplicity of the LAMP method make it possible to introduce this method for use in clinical laboratories in the field. In the current study, LAMP assays could be used for the detection of equine herpes virus type 1 and 4 in export and import quarantine. Materials and methodsViruses and bacteriaThe EHV-1 and EHV-4 was propagated in Equine pulmonary artery endothelial cells(EqPEC). EqPEC currently referred to in the literature as Equine Endothelial cells(EEC) were received 2/15/01 from Brian Moore in Dr. Udeni Balasuryia's lab at UC-Davis. The recommended media was Endothelial Cell Mainteance Media(ECMM). EHV-1(T953) and EHV-4(T445) strains were used in the experimental challenge study. To determine the analytical specificity of the LAMP assays, the following common pathogens were used: EHV-1(T953) strains, EHV-4 (T445) strains, Equine influenza virus1 (H7N7), Equine influenza virus 2(H3N8), EAV(Equine Arteritis Virus), BHV(Bovine Herpes Virus), PRV (porcine pseudorabies virus). Those were bought from Kentucky University or established in the authors' laboratory. DNA and RNA extractionViral DNA and RNA were extracted from 200 ul of culture supernatants with a nucleic acid isolation kit, (TIANGEN, TIANamp Virus DNA/RNA kit DP315 J8802) according to the manufacturer's instructions. Polymerase chain reactionTo confirm the extraction of viral nucleic acids was performed properly and compare the analytical sensitivity of the LAMP assays with conventional PCR assays, the PCR assays were performed with the primer sets□Table 1□specific for the detection of gB of EHV-1 and 4 described previously by using a fast cycling PCR, according to the manufacturer's instructions, in a thermal cycler. The PCR conditions were

as follows: 95 ℃ for 5 min, 35 cycles of denaturation at 95℃ for 30 sec, annealing at 55℃ for 30 sec, and extension at 72℃ for 30 sec, and then a final extension at 72℃ for 10 min. The PCR products were kept at 4℃ until the samples were analyzed by 1. 5 % agarose gel electrophoresis. Loop-mediated isothermal amplificationThe specific primer sets for the gB genes of EHV-1[AY464052 61432-62391(959bp)] and the gB genes of EHV-4 [NF80567 61432-62845(1413bp)] were designed by using PrimerExplorer V4 software. Each primer set includes 6 primers that consist of 2 outer primers (F3 and B3), 2 inner primers (FIP and BIP), and 2 loop primers (loopF and loopB). The sequences and locations of the primers for the LAMP assays used in the current study are shown in Table 1. We try to get the properly LAMP reaction through optimizing the reaction system. In brief, 25ul of reaction mixture that contained as follow: 1ul of Bst DNA polymerase(8U/μL), 2. 5ul of Bst DNA polymerase buffer(10×), 2ul of MgSO4(25mmol/μL), 2. 5ul of Betaine (0. 4mol/ul), 2. 5ul of dNTP (25mmol/μL), 1. 6mM of BIP/FIP, 0. 2mM of B3/F3, 0. 8mM of Loop F/Loop B, 1ul of a fluorescent detection reagent, and 2 ul of extracted samples, with ddH2O up to 25ul. The LAMP reaction was performed at 63 ℃ for 60 min and then was terminated by heating the mixture at 95℃ for 2 min. The LAMP production was analyzed by electro-phoresis on a 1. 5% agarose gel. We examined the properly reaction result with a DNA amplification kit and a fluorescent detection reagent(DASR05-24) according to the manufacturer’s instructions. The LAMP reaction could judged by visual observation. The LAMP reaction was turned green when positive in the presence of calcein, which was contained in the fluorescent detection reagent, whereas the color of the mixture remained orange when

the LAMP assay result was negative. RealAmp MethodThe RealAmp method was performed using the commercially available Loopamp DNA amplification kit(Lucchi NW; 2010) following the manufacturer’s instructions with the exception of the addition of 0. 25 mL per 12. 5 mL reaction volume of a 1: 100 diluted SYBR Green (Invitrogen) or by the use of an in-house reaction buffer. To test the utility of an in-house reaction buffer, pilot experiments were performed in a 12. 5 mL total volume containing a 2X in-house buffer (40 mM Tris-HCl pH 8. 8, 20 mM KCl, 16 mM MgSO4, 20 mM (NH4)SO4, 0. 2% Tween-20, 0. 8M Betaine, 2. 8 mM of dNTPs each), 0. 25 mLofa 1: 100 dilution SYBR green and 8 units of Bst polymerase (New England Biolabs, Ipswich, MA). Genus specific primers were used to amplify the gB of EHV-1 and 4. DNA amplification was carried out at 63℃ for 60 minutes using the eppendorf realplex real-time PCR which was set to collect fluorescence signals at 1 minute intervals. A typical real- time amplification plot obtained using the RealAmp method is shown in Figure 7B and 8B. In the plot, the Y-axis denotes the fluorescence units in milli-volts (mV) and the X-axis shows the time in minutes. ResultsSpecificity of the LAMP assayThe LAMP assays specific primer were designed by PrimerExplorer V4 software. Each primer set includes 6 primers that consist of 2 outer primers (F3 and B3), 2 inner primers (FIP and BIP), and 2 loop primers (loopF and loopB). LAMP assays were performed with EHV-1(T953)strains, EHV-4 (T445)strains, EAV(Equine arteritis virus), PRV, BHV, Equine influenza virus1 (H7N7), Equine influenza virus2 (H3N8), viral in horse to evaluate analytical Specificities. Positive amplifications were observed in EHV-1 (Figure 3). or EHV-4 (Figure 3) strains within a 60-min incubation period. By contrast, other strains were not

amplified after a 60-min incubation period. This result indicates that no false-positive amplifications were observed with these heterologous species in the LAMP assay. Sensitivity of PCR and LAMP assayThe LAMP and PCR methods were used to amplified serial 10-fold dilutions of EHV-1(Figure 5) and EHV-4 DNA(Figure 6). The detection limits of the LAMP assays for EHV-1 and EHV-4 were examined and compared with PCR assays. This result indicates that the LAMP assay is more sensitive than PCR for detecting EHV-1 and EHV-4 DNA. The detection limit of EHV-1 DNA LAMP assays was 94*10-6ng/µL and the EHV-4 DNA LAMP assays was 155. 9*10-6ng/µL. Evaluation of LAMP assay in RealAmpA typical real-time amplification plot obtained using the RealAmp method is showed. Amplification of EHV-1 (Figure 7) and EHV-4 (Figure 8) DNA yielded sigmoid shaped amplification curve while the control tube (no DNA) had no measurable fluorescence indicated by a flat line in the plot. The RealAmp method showed the efficacy of LAMP assays within 10min to 20min. Amplification of EHV-1 and 4 DNA yielded sigmoid shaped amplification curve while the control tube (no DNA) had no measurable fluorescence indicated by a flat line in the plot. DiscussionInfections of EHV-1 and EHV-4 in horses are a major economic concern throughout the world (Allen GP, 2004). The rapid diagnosis of EHV-1 and EHV-4 infections is essential to enable control measures to be implemented and used for in export and import quarantine. Several PCR methods were described for one-step detection and identification of EHV-1 and EHV-4 (Carvalho R, 2000; Kirisawa R, 1993; Lawrence GL, 1994; Wagner WN, 1992). However, PCR methods in clinical laboratories in the field demand expensive equipment and skilled technicians. In contrast, a LAMP reaction can be carried out at a constant

temperature (60–65℃) within 1 hour in a water bath or a heat block. The result of a LAMP assay can be judged by the real-time monitoring of the turbidity of the reaction mixture, visual observation of fluorescence, or gel electrophoresis of the LAMP products. Real-time detection requires specific equipment, such as a real-time turbidimeter to monitor increments in turbidity. Amplified DNA can also be analyzed by gel electrophoresis with which a typical ladder of many DNA bands of different sizes is observed. However, this method could greatly increase the risk of contamination, because the tubes have to be opened, and the reaction mixture, which contains a large amount of amplified DNA, has to be applied onto an agarose gel. In the present study, LAMP assays were developed for the specific detection of EHV-1 and EHV-4,. The analytical sensitivities of the LAMP assays were compared with those of PCR assays. The detection limits of LAMP for EHV-1 gB as well as that of PCR for EHV-1 were 1 94*10-6ng/μL (Fig. 5) and that of LAMP and PCR assays for EHV-4 gB were 155. 9*10-6ng/μL (Fig. 6). It was found that the analytical sensitivities of the LAMP assays for EHV-1 and EHV- 4 developed in the current study were identical to those of PCR assays for EHV-1 and EHV-4, respectively. The LAMP assays developed in the present study provided specificity in the amplification of the targeted DNA. The detection ability of LAMP assays for EHV-1 and EHV-4 in clinical samples was in good overall agreement with that of PCR for EHV- 1 and EHV-4, respectively. LAMP assays were developed for the specific detection of EHV-1 and EHV-4 strains. We builded the LAMP reaction is more suitable for the detection of EHV-1 and EHV-4, it’s Lower the cost of materials and wider scope of application. We have already through the PCR,

DNA LAMP kit and RealAMP to proved it is reliable. The advantages of LAMP assay are that, compared with PCR, a LAMP assay can be undertaken with simple and cost-effective equipment in a short time by using visual detection. The LAMP assays developed in the current study are sensitive and specific, quick, simple and original for the detection of EHV-1 and EHV-4, and could be used for the detection of equine herpes virus type 1 and 4 in export and import quarantine. AcknowledgementsThis work was supported by a grant from the ...Foundation and