Direct Detection of Equine Herpesvirus Type 1 DNA in Nasal Swabs by Loop-Mediated Isothermal Amplification (LAMP)

Manabu NEMOTO1)*, Minoru OHTA2), Koji TSUJIMURA2), Hiroshi BANNAI2), Takashi YAMANAKA2), Takashi KONDO1), Hiroshi BANNAI1), Takashi YAMANAKA1), Koji TSUJIMURA2), Hiroshi BANNAI2), Takashi KONDO1), Tomio MATSUMURA1)

1) Epizootic Research Center, Equine Research Institute, Japan Racing Association, 1400–4 Shiba, Shimotsuke, Tochigi 329–0412 and 2) Racehorse Clinic, Ritto Training Center, Japan Racing Association, 1028 Misono, Ritto, Shiga, 520–3085, Japan

(Received 14 February 2011/Accepted 23 April 2011/Published online in J-STAGE 6 May 2011)

ABSTRACT. We evaluated loop-mediated isothermal amplification (LAMP) as a means of detecting equine herpesvirus type 1 (EHV-1) DNA directly from nasal swabs. To increase the sensitivity, we added a step in which the samples were heat-treated to the original LAMP procedure. The detection limit of the LAMP assay with heat treatment was 10 times more sensitive than the original LAMP assay even when the DNA extraction step was omitted. In addition, the LAMP assay with heat treatment was more sensitive than the original LAMP assay and the polymerase chain reaction using clinical samples. The LAMP assay with heat treatment is easy to perform and so should be applicable to the diagnosis of EHV-1 infections in clinical laboratories.

KEY WORDS: equine herpesvirus type 1 (EHV-1), heat treatment, loop-mediated isothermal amplification (LAMP).

Equine herpesvirus type 1 (EHV-1) is a major causative agent of respiratory disease in horses and can also lead to miscarriage and neurological disease [1, 2]. Respiratory disease among racehorses caused by EHV-1 has a great economic impact on the horse industry in Japan [9]. Virus isolation, serological tests, and a polymerase chain reaction (PCR) are usually employed to diagnose EHV-1 infection. Because virus isolation and serological tests are time-consuming and laborious, several groups have developed sensitive and rapid PCR tests to detect EHV-1 DNA [1, 2]. However, because PCR tests require expensive equipment, these tests are not commonly employed in clinical laboratories.

A technique called loop-mediated isothermal amplification (LAMP) was developed as a novel nucleic acid amplification technique [14]. 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 [10–12, 17]. 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 [13]. However, it includes an awkward step for extracting DNA from clinical samples, and this prevents the widespread use of the EHV-1 LAMP assay in clinical laboratories. Kaneko et al. [6] have reported that the sensitivity of the LAMP assay is less affected by the various components of the clinical samples than the PCR assay. It has also 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 [3–5].

The omission of the DNA extraction step should promote the use of the EHV-1 LAMP assay in clinical laboratories. Several groups have reported that an additional heat-treatment step increases the sensitivity of the LAMP assay [4, 15, 16]. Therefore, to increase the sensitivity, we added a heat-treatment step to the LAMP assay in this study. Furthermore, we applied a LAMP assay without DNA extraction to the rapid diagnosis of EHV-1 infection using nasal swabs. In addition, we examined the effect of mediums immersing a nasal swab on the LAMP assay.

The EHV-1 89c25p strain [8] (2.5 × 10⁶ plaque-forming units/200 µl) was used to determine the analytical sensitivity of the LAMP assay. To evaluate the effect of mediums on the LAMP assay, the EHV-1 89c25p strain was serially diluted 10-fold with saline, phosphate buffered saline (PBS, pH:7.4) or transport medium [PBS supplemented with 0.6% (w/v) tryptose phosphate broth (Sigma-Aldrich Corporation, St. Louis, MO, USA.), 500 unit/ml penicillin, 500 µg/ml streptomycin and 1.25 µg/ml amphotericin B (Gibco®, Invitrogen Corporation, Carlsbad, CA, U.S.A.)]. Nasal swabs were collected from horses using 1.0 × 1.5 cm absorbent cotton swab (JMS Co., Ltd., Hiroshima, Japan). To mimic clinical samples, a nasal swab collected from a healthy horse was added to 2.5 ml of each medium. The 100 clinical samples used in this study were collected from Thoroughbred racehorses with a fever (≥38.5°C) in winter in December 2008 - April 2010. The nasal swabs were immediately immersed in 2.5 ml of transport medium and were transported from a clinical site to our laboratory. The nasal swabs were centrifuged at 5,000 rpm for 5 min to precipitate debris, and the resultant supernatants were used in the LAMP assay. Viral DNA was extracted from 100 µl of a sample using a nucleic acid isolation kit (MagNA Pure LC Total Nucleic Acid Isolation Kit, Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer’s
instructions. For the direct detection of viral DNA, the DNA extraction step was omitted and the supernatants were used for the LAMP assay.

We used the specific primer set for the glycoprotein E (gE) gene of EHV-1 [13]. The LAMP assay was carried out with a DNA amplification kit (Loopamp®, Eiken Chemical Co., Ltd.) and a fluorescent detection reagent (Loopamp®, Eiken Chemical Co., Ltd.). The reaction mixture (25 µl) contained 12.5 µl of 2 × reaction mix buffer (40 mM Tris–HCl [pH 8.8], 20 mM KCl, 16 mM MgSO₄, 20 mM [NH₄]₂SO₄, 0.2% Tween-20, 1.6 M betaine, and 2.8 mM each of 4 deoxyribonucleotide triphosphates), 0.2 mM each of 2 outer primers, 1 µl of a fluorescent detection reagent (Loopamp®, Eiken Chemical Co., Ltd.), and 4.5 µl of samples prepared with or without DNA extraction. The mixture was incubated at 63°C for 60 min. The result of LAMP reaction was judged by visual observation with the naked eye. The mixture turned green when the LAMP reaction was positive in the presence of calcein, which was contained in the fluorescent detection reagent, whereas the mixture remained orange when the LAMP assay result was negative.

To increase the sensitivity of the LAMP assay, we incorporated a heat-treatment step (heat-treatment LAMP assay). The reaction mixture (23 µl) containing 12.5 µl of 2 × reaction mix buffer, 0.2 mM each of 2 outer primers, 1.6 mM each of 2 inner primers, 0.8 mM each of 2 loop primers, 1 µl of Bst DNA polymerase, and 4.5 µl of samples prepared with or without DNA extraction was heated at 96°C for 5 min and the result of the LAMP reaction was judged by visual observation.

The PCR assay was performed with the primer set specific for EHV-1 glycoprotein C gene [7] by using a fast cycling PCR (Qiagen GmbH, Hilden, Germany), according to the manufacturer’s instructions. The PCR conditions were as follows: 95°C for 5 min, 35 cycles of denaturation at 95°C for 5 sec, annealing at 60°C for 5 sec, and an extension at 72°C for 20 sec, and then a final extension at 72°C for 1 min. The PCR products were kept at 4°C until the samples were analyzed by 1.2% agarose gel electrophoresis (Flash-Gel® System for DNA, Lonza Rockland Inc., Rockland, ME., U.S.A.). The detection limits of the LAMP assays are shown in Table 1. The heat-treatment LAMP assay without DNA extraction could detect the DNA from a 10⁴ virus dilution, which was 100 and 10 times more sensitive than results obtained with the non-heat-treatment LAMP assay without DNA extraction and the original LAMP assay with DNA extraction, respectively. As previously reported [4, 15, 16], the addition of a heat-treatment step increased the sensitivity of the LAMP assay. The detection limits of the LAMP assays without DNA extraction were the same as in saline, PBS and a transport medium containing a nasal swab (Table 1). These results indicate that saline, PBS, antibiotics and a small amount of tryptose phosphate broth have very little effect on the EHV-1 LAMP reaction.

Clinical samples (n=100) were used to evaluate the reliability of the heat-treatment LAMP assay without DNA extraction. As a result, 39 out of 100 clinical samples were found to be positive. Among these positive samples, 11 and 10 samples were found to be negative by the original LAMP assay and the PCR assay, respectively (Table 2). These LAMP products exhibited a ladder pattern on the agarose gel and were specifically digested by a restriction enzyme, Ban II (data not shown). Therefore, these LAMP products were confirmed to be the expected amplicons of EHV-1 DNA. These results indicate that the heat-treatment LAMP assay without DNA extraction is also more sensitive than the original LAMP assay and the PCR assay when clinical samples were used to detect EHV-1 DNA.

Table 1. Analytical sensitivity of the non heat-treatment LAMP assay without DNA extraction, the heat-treatment LAMP assay without DNA extraction and the original LAMP assay with DNA extraction

<table>
<thead>
<tr>
<th>Medium containing nasal swabb)</th>
<th>Non heat-treatment LAMP without DNA extraction</th>
<th>Heat-treatment LAMP without DNA extraction</th>
<th>Original LAMP with DNA extraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>10⁻⁴</td>
<td>10⁻⁶</td>
<td>10⁻³</td>
</tr>
<tr>
<td>PBS</td>
<td>10⁻⁴</td>
<td>10⁻⁶</td>
<td>10⁻³</td>
</tr>
<tr>
<td>Transport medium</td>
<td>10⁻⁴</td>
<td>10⁻⁶</td>
<td>10⁻³</td>
</tr>
</tbody>
</table>

a) The LAMP reactions were carried out with 10-fold serial dilutions of EHV-1 89c25p strain diluted with saline, PBS or a transport medium containing a nasal swab obtained from a healthy horse.

Table 2. Comparison of the heat-treatment LAMP assay without DNA extraction with the original LAMP assay and the PCR assay for detecting EHV-1 DNA from clinical samples

<table>
<thead>
<tr>
<th></th>
<th>Original LAMP</th>
<th>PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>Heat-treatment LAMP</td>
<td>28</td>
<td>11</td>
</tr>
<tr>
<td>without DNA extraction</td>
<td>0</td>
<td>61</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>28</td>
</tr>
</tbody>
</table>
samples were examined. The omission of the DNA extraction step could save the cost, the time and labor needed to prepare the LAMP assay samples. The addition of the heat-treatment step made the LAMP assay more sensitive than the original LAMP assay even though the DNA extraction step was omitted. The present study shows that the heat-treatment LAMP assay is useful for the direct detection of EHV-1 DNA in nasal swabs. The fact that it is easy to use should make the heat-treatment LAMP assay without DNA extraction applicable to the diagnosis of EHV-1 infection in clinical laboratories.

ACKNOWLEDGMENTS. We are grateful to Mr. Akira Kokubun, Ms. Kazue Arakawa, Ms. Junko Igarashi and Ms. Kaoru Makabe for invaluable technical assistance.

REFERENCES


