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Full Length Research Paper

New molecular diagnosis method combined with nucleic acid sequence-based amplification and probe amplification in rapid detection of Enterovirus 71

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In this study, a new molecular diagnosis assay for rapid detection of virus RNA was established, which combined nucleic acid sequence-based amplification (NASBA) with rapid isothermal detection assay (RIDA). Using this method, we could rapidly identify Human enterovirus 71 RNA with high sensitivity and specificity; the detection limit of the new method was 10^2copy/ml. The whole assay was processed in one tube and the result was determined by naked eyes under ultraviolet radiation without cap-opening, meanwhile, as the products of amplification was RNA which is easily degradative, thus, the aerosol contamination was further decreased. This novel method shows excellent sensitivity, specificity and conveniences, which is easily operated in the elementary medical organizations and resource-limited areas.

Key words: Enterovirus 71, amplification, diagnosis.

INTRODUCTION

In recent years, several large outbreaks of hand-foot-and-mouth disease (HFMD) associated with Enterovirus infection were reported in China (Li et al., 2011). Over 3.4 million cases of hand, foot, and mouth disease (HFMD), including over 1,000 deaths, were reported in China since March 2008 (Wang et al., 2011). Thus, a rapid, specific, and sensitive detection method is in high demand for the early diagnosis and clinical therapy of HFMD. The pathogen of HFMD are several kinds of RNA enterovirus among which *EV71* contributes to severe and fatal cases and is the most popular virus strain in China. Recent years, nucleic acid sequence-based amplification (NASBA) assay was widely used in RNA detection (Asiello et al., 2011; Lau et al., 2006). NASBA is an isothermal amplification method for RNA. This process occurs at one temperature (41°C) without the need of adding intermediate reagents and results in the exponential amplification of RNA and DNA products within 90 min, producing as the major amplification product antisense, and single stranded RNA. In early days, the amplification products of NASBA were usually detected by gel electrophoresis, but in this way, it often caused aerosol contamination which easily led to false positive results (Wacharapluesadee et al., 2005). Recently, some researchers used molecular beacon probes to detect the amplification products, by this way, we could operate the experiment in one-tube without cap-opening. However, the molecular beacon is unstable which is easily influenced by temperature or pH value of the solution (Kouguchi et al., 2010; Lamhoujeb et al., 2008). To resolve these problems and improve the sensitivity and specificity, we developed a new diagnosis

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assay which combined the rapid isothermal detection assay (RIDA) with NASBA. RIDA is a "probe amplification" assay, which uses the single-strand nicking activity of restriction nicking endonucleases to repeatedly cleave synthetic probes hybridizing to the same target sequences (Gao et al., 2008; Shi, 2003). RIDA is achieved through the binding of a reporting probe (RP) to the target ssDNA or RNA, followed by the nicking on the RP with the restriction nicking endonuclease. A number of restriction nicking endonucleases have been obtained from nature or through recombinant engineering which could recognize a specific double-strand binding site and make the cut at only one strand (Too et al., 2004). For example, in a basic RIDA, the RP contains 5′-GAGTC-3′ which can be recognized by N.BstNBI (a kind of restriction nicking endonucleases). Under a certain reaction temperature (Zheleznaya et al., 2007), then, the RP forms stable double strands with its complementary target sequence as the N.BstNBI recognition site. The N.BstNBI enzyme produces a single-strand nick on the RP, which results in the reduction of the melting temperature (Tm) and the subsequent release of the 2 smaller ssDNA probes which were cleaved by the enzyme. When the RP is in excess amount, a new RP will hybridize with the same target and form new double strands, and the new RP will be nicked again. Thus, in RIDA, the same target can be used repeatedly to cleave multiple copies of RPs, which generates large amount of smaller fragments. As a result of RIDA, the existence of the target sequences (DNA or RNA) is indicated through the generation of cleaved RPs. However, this method has an obvious shortage on sensitivity which makes it impossible to be applied in clinical diagnosis. After combined NASBA with RIDA, this shortage will be immediately overcome. In our research, we firstly amplified the RNA target by NASBA; the amplification time in this step could be shortened because the next step of RIDA reaction would amplify the signal of the products by the fluorescence probe. The traditional RIDA method was detected by quantitative PCR instrument which is unavailable in some resource-limited place (Gao et al., 2008). In our research we found that the result can be simply determined with the naked eye by the color difference between positive and negative results under UV irradiation. Thus, the result can be judged more easily without elaborate equipment.

MATERIALS AND METHODS

The culture supernatant samples of *EV71-117, coxsackievirus A16*, *Hepatitis C virus strain FL-J6/JFH* and *Influenza virus A H1N1* were acquired from *Hebei Provincial Center for Disease Control and Prevention* Shijiazhuang, Heibei, China. Among these RNA samples, *EV71* was selected as positive sample; the other three were negative samples. Viral nucleic acids were extracted from 100 µl of culture supernatants with a nucleic acid isolation kit (MagNA Pure LC total nucleic acid isolation kit, Roche Diagnostics GmbH, Mannheim, Germany). The initial concentration of these RNA samples was 10^4copy/ml. The samples were transported in liquid nitrogen and stored at －80°C.

Probe design

The primers of NASBA were designed according to the previously published paper (Shi et al., 2011). VP2 gene sequences of EV71 virus (accession No. AY465356) was chosen as the target and was aligned with available VP2 gene sequences of other strains, including the circulating strains in China responsible for recent epidemics, to identify the conserved regions using DNASIS software. The potential target region of 194 bp corresponding to the genome positions from 1296 to 1489 was selected from the aligned sequences. The sequences of the primers were as follows. P1:(5′-AATTCTAATACGACTCACTATAGGGCACCGGATGGCCAATCCA-3′), P2:(5′-GGTGTGAAGAGCCTATTGAG-3′). The probe of RIDA was designed complementary to the amplification products of NASBA; the probe sequence was: 5′-AGGCGTGTG<u>GAGTC</u>CCTAACCGACTG-3′. Its 5'end was labeled with fluorophore FAM, and the 3'end was labeled with quencher TAMARA. The underlined sequence was the recognize point of endonucleases N.BstNBI. Primers and probes were both synthesized by TAKARA Company.

Preparation of NASBA-RIDA reaction solution and optimization of reaction condition

The reaction system included 15µl NASBA mixture (1 mmol/L dNTPs, 1 mmol/L NTP, 0.5 mmol/L ITP, 0.4 µmol/L primer, 6 U RNasin, 2.5 µL AMV reverse transcriptase Buffer, 2.5 µL T7 RNA polymerase Buffer, 1 µL RNase H Buffer). After adding 1 ul RNA sample into the reaction mix, we heated the tube first at 65°C to destroy the secondary structure of RNA. Then, 0.8 U RNase H, 32 U T7 RNA polymerase,4 U AMV reverse transcriptase, 1 µL DMSO, 7.5 mmol sorbic alcohol,50 pmol/L probe, 2 µL NEB buffer3, and 5 U N.BstNBI endonucleases were added . The final reaction volume for each reaction was 20 µL. The reaction conditions were as follows: first, heating in a water bath at 63°C; different reaction time was screened during this step (20 min, 30 min, 45 min, 60 min, 90 min), adjusting the reaction temperature at 55°C, and heating for 5 min, 10 min and 15 min respectively. Finally, the optimal time was monitored by the visual results. We chose *EV71* RNA as the positive template and *Hepatitis C virus* RNA as the negative template, the concentration of both templates were 10^2 copy/µl, a blank control group was also set up to evaluate the reaction result.

Comparison of the test result of the novel assay

We chose four species of viral RNA samples to identify the specificity of the NASBA-RIDA assay. The samples included *EV71, Cox A 16, Hepatitis C virus* and Influenza virus A *H1N1*. The concentration of those RNA templates was 10^4copy/ml. Distilled water was used as blank control. The whole reaction was processed in one tube as described above.

Comparison of analytical sensitivity of NASBA-RIDA assay and separate RIDA assay

The *EV71* RNA with initial concentration of 10^4 copies/ml was 10×serially diluted, and 10^4, 10^3, 10^2, 10^1, 1 copy/ml RNA were used respectively as the template to carry out the NASBA-RIDA reaction. To evaluate the sensitivity of the separate RIDA reaction, we synthesized a single strand DNA template which was complemented with the fluorescense probe as the template of RIDA reaction. The concentration of the synthesized template was also diluted into 10^4 copies/ml, and 10^4, 10^3, 10^2, 10^1, 1 copy/ml single strand DNA were used respectively as the template to carry out the separate RIDA reaction. RIDA reaction was processed in 55 °C for 60 min.

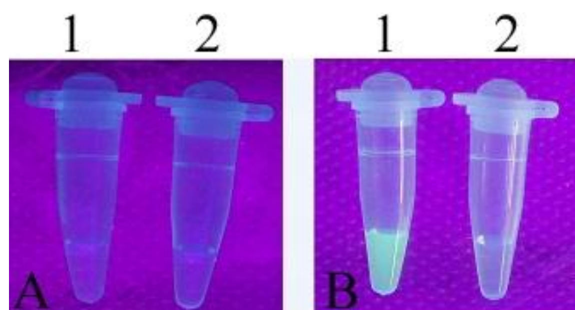

Figure 1. A. Before reaction, the reaction solution for both positive and negative samples are colorless under UV light. Tube 1 is positive sample, tube 2 is negative sample. **B.** After reaction, the positive reaction solution (tube 1) turned to green under UV light, the negative (tube 2) is still colorless.

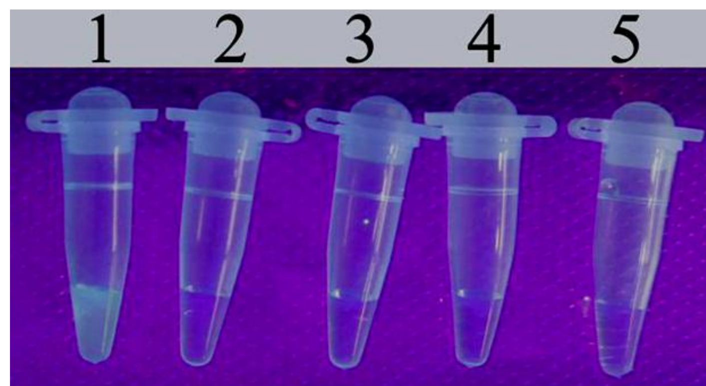

Figure 2. The specificity result of the NASBA-RIDA obtained from the color difference under UV light. The 1 to 5 samples were respectively as follows: *EV71, Cox A 16, Hepatitis C virus,* Influenza virus A *H1N1* and blank control.

Sensitivity of RT-PCR reaction in detection of EV71

The cDNA of EV71 was generated from RNA samples by using 1st-Strand cDNA Synthesis Kit (TIANDZ, China). The primer for PCR reaction was as follow: forward primer: 5'-AAA GGT GGA GCT GTT CAC CTA CAT GCG -3', reverse primer: 5'-AAT CTG GCT TGG GGG CCC CAG GTG -3', The cycling conditions were composed of 5 min at 94°C, followed by 40 cycles with 93°C for 15 s, 55°C for 45 s, and 72°C for 1 min, and a final extension cycle of 72°C for 10 min. The PCR products were examined by 2% agarose gel electrophoresis. The diluted template of RNA sample was set up as described above. 10^4, 10^3, 10^2, 10^1, and 1 copy/ml RNA were used respectively as the template to carry out the RT-PCR reaction.

RESULT

The optimal condition of NASBA-RIDA reaction

After the reaction, we placed the tube under ultraviolet radiation (254nm) to judge the result by color distinction of each tube. The results were judged by naked eyes as follow: the negative sample and blank control were both colorless, the positive sample represented green fluorescence which is obviously distinguished from the color of negative samples (Figure 1). The optimal reaction time for NASBA was 45 min, and 10 min for RIDA reaction.

Observation of the specificity results of different samples under ultraviolet radiation

After reaction, we observed the color intensity under ultraviolet radiation among these samples; the positive sample of *EV71* turned to green color and the rest samples: *cox A virus*, *Hepatitis C virus*, Influenza virus A H1N1 and blank control were all colorless which indicated negative result (Figure 2).

Comparison of sensitivity between NASBA-RIDA and separate RIDA

According to the sensitivity analysis results, NASBA-

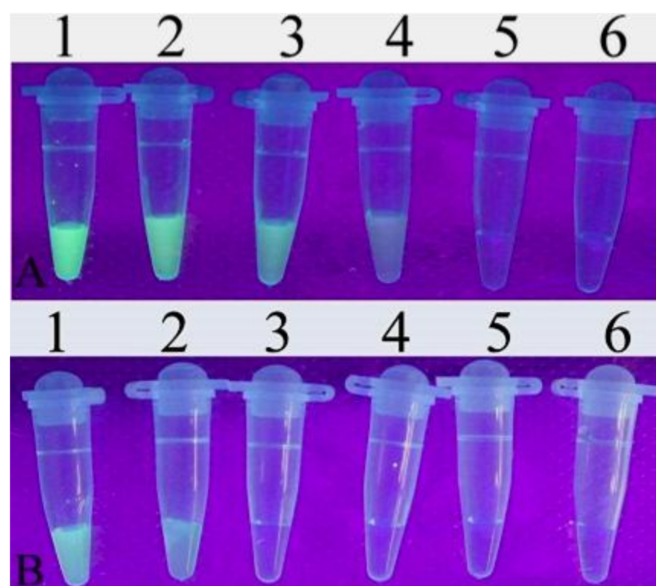

Figure 3. A. The sensitivity result of NASBA-RIDA. The RNA copies amount used in tube 1 to 5 is respectively as follows: 10^4, 10^3, 10^2, 10^1 and 1 copy per ml; tube 6 is the blank control. **B.** The sensitivity result of RIDA reaction. The RNA copies amount used in tube 1 to 5 was respectively as follows: 10^4, 10^3, 10^2, 10^1 and 1copy per mL, tube 6 is blank control.

RIDA method could detect the minimal value of 10 copies/ml RNA; the separate RIDA could detect the minimal value of 10^3 copies/ml cDNA only. Generally, the sensitivity of separate RIDA was 100-times lower than that of the NASBA-RIDA (Figure 3).

Sensitivity results of RT-PCR

The electrophoresis result reveals that the sensitivity of RT-PCR was closed to NASBA-RIDA assay which could detect the minimal value of 10 copies RNA /ml (Figure 4),

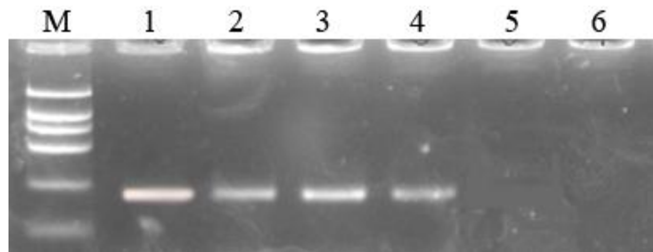

Figure 4. Sensitivity result of RT-PCR. The electrophoresis lanes from left to right are as follows: marker DM2000; the RNA template with content of 10^4, 10^3, 10^2, 10^1, 1 copy per mL and a blank control, respectively.

but the consumption time of RT-PCR which needed 3 hs for the whole reaction was much higher than that of the new method.

DISCUSSION

In this research, we set up a new diagnosis method which does not need temperature cycle but only one change in temperature. The new method we call NASBA-RIDA had many advantages when used in the RNA virus detection. First, the traditional nucleic acid diagnosis assay depends on producing a great deal of amplification products, so it often causes DNA aerosol contamination which would interfere with the subsequent detection, and led to the appearance of false positive results (Higgins et al., 2001; Gibson et al., 2008). However, the new assay was not mainly dependent on amplification nucleic acid. The key point of this assay was amplifying the signal of the fluorescence probe; the amplification of NASBA was limited by cutting short the reaction time (in this assay we set up the NASBA reaction time for only 45 min), so the amount of nucleic acid products were decreased on a certain extent which decreased the risk of contamination. On the other hand, the product of NASBA was RNA which was easily degraded and the RIDA reaction would not produce nucleic acid fragments so the risk of contamination was dramatically decreased by the new assay. The second advantage for this assay was that the combination of these two methods made complementary advantages for each other. As the probe of RIDA was designed according to the specific amplification products of NASBA, so even the reaction of NASBA produced non-specific amplification products; the RIDA would correct the mistake because the probe would not combine with the non-specific products and no fluorescence is released, thus, the final result would still be negative even though there were non-specific products amplified. On the other hand, the single reaction of RIDA has a low sensitivity which makes it impossible to detect the clinical samples with low concentration, so if there were no specificity amplification of NASBA there would be little possibility to see a positive result for NASBA-RIDA in clinical test. In this test, 4 species of viral RNA were used to confirm the specificity of the novel assay and the results indicated that the new assay could distinguish between EV71 and other three virus samples. Besides the improvement of specificity, the novel assay also showed a good sensitivity compared with other assays published before (Scherczinger et al., 1999; Hu et al., 2011; Hi et al., 2011; Lee et al., 2011). Generally speaking, the combination of these two assays improved both the specificity and sensitivity of the detection. The third advantage for this method is the convenience in detecting the procedure. The whole reaction was processed in one tube without cap-opening, and the operator only needed to change the temperature for one time, therefore no expensive equipments and complicated operations are needed for the assay. As a new assay, even though there are so many advantages for this method, it still needs more test on stability and specificity. In the future, we will make further effort on promoting this diagnosis assay and spreading it on RNA virus detection.

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