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Full Length Research Paper

A new molecular diagnosis method combined single primer isothermal amplification with rapid isothermal detection assay in detection of group B *Streptococcus*

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Herein, a new molecular diagnosis assay for rapid detection of bacterial DNA was established, which combined single primer isothermal amplification (SPIA) with rapid isothermal detection assay (RIDA). Using this method, we could rapidly identify Group B *Streptococcus* DNA 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 a linear amplification system, the products cannot be used as the amplification target, 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: SPIA combined with RIDA; Molecular diagnosis; Group B *Streptococcus.*

INTRODUCTION

Group B *Streptococcus* (GBS) is an important cause of mortality in infants. The incidence of neonatal GBS infection ranges from 0.5 to 2 per 1000 live births in different countries. GBS neonatal disease is classified as either early-onset disease (<7 days) or late-onset disease (>7 to 90 days) (Bergseng et al., 2007). GBS colonization in pregnant women is the single most important risk factor for early-onset new-born disease due to vertical transmission and colonization of the infant during delivery. Antibiotic treatment in intrapartum could lower the incidence of early onset disease (Schraget al., 2002). While generally associated with maternal cervicovaginal colonization and neonatal disease, GBS also frequently causes serious infections including acute urinary tract infections in older persons and those with chronic medical illness. Thus, a rapid, specific, and sensitive detection method is in high demand for the early diagnosis and clinical therapy of GBS. Recent years, isothermal nucleic acid amplification assay was widely used in clinical diagnosis for bacterial DNA (Asiello et al., 2011; Lau et al., 2006).

Single primer isothermal amplification (SPIA) is a new isothermal amplification method for DNA detection. This process occurs at one temperature (50°C) without the need of adding intermediate reagents and results in the linear amplification of DNA products within 90 min, producing the amplification products of single stranded DNA. The SPIA reaction is initiated by the addition of a reaction mixture containing a chimeric DNA/RNA primer, a DNA polymerase with strong strand-displacement

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Abbreviations: GBS, Group B *Streptococcus*; **SPIA,** Single primer isothermal amplification; **PCR,** polymerase chain reaction; **RIDA,** rapid isothermal detection assay; **RP,** reporting probe; **Tm,**melting temperature; **NASBA,**nucleic acid sequence-based amplification.

activity and RNase H (NurithKurna et al.,2005). The RNase H cleaves the RNA portion of the heteroduplex at one end of the double-stranded cDNA, thus generating a unique partial duplex cDNA with a single-stranded DNA tail at the 3′ end of the second-strand cDNA. This tail is the priming site for the SPIA amplification step. The sequence of the SPIA amplification primer, a chimeric DNA/RNA primer, is complementary to the sequence of the single-stranded 3′ end of the second-strand cDNA in the partial duplex (NurithKurna et al., 2005). This DNA/RNA chimeric primer is composed of a DNA sequence at the 3′ end and an RNA sequence at the 5′ end. DNA amplification is carried out by extension of this primer, when hybridized to target DNA, by a DNA polymerase with strand-displacement activity and by cleavage of the RNA portion of the primer in the RNA/DNA heteroduplex created by primer hybridization to the target by RNase H. Cleavage of the 5′ RNA portion of the primer annealed at the priming site clears this site for hybridization of a new primer molecule, which is extended along the template DNA by DNA polymerase (NurithKurna et al.,2005). Strand-displacement DNA synthesis leads to displacement of the previous primer extension product away from the template DNA. This cycle of primer binding, extension, displacement, and cleavage causes efficient generation of multiple copies of the amplification product. The amplification step is carried out at a constant temperature (between 47 and 50°C). Of note, the initiation of primer extension to generate single-stranded DNA amplification product is not dependent on the completion of a previous primer extension step. Thus, multiple primer extension products are generated by multiple DNA polymerase molecules moving along the same template DNA (NurithKurna et al., 2005). This process is rapid, efficient, isothermal, and linear. In addition, cleavage of the 5′ portion of the primer extension product (the amplification product) by RNase H renders it nonamplifiable in the given amplification system, thus eliminating containment requirements, and reduces potential deleterious effects of amplification product contaminating naïve samples, a common cause of false-positive results in tests conducted with polymerase chain reaction (PCR) amplification systems (NurithKurna et al., 2005). In early days, the amplification products of SPIA were usually detected by gel electrophoresis, but this method was inconvenience for clinical diagnosis. 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., 2009). To resolve these problems and improve the sensitivity and specificity, we developed a new diagnosis assay which combined the rapid isothermal detection assay (RIDA) with SPIA. 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 et al., 2011). 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., 2011). 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., 2009), 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 apply in clinical diagnosis. After combined SPIA with RIDA, this shortage will be immediately overcome. In our research we firstly amplified the DNA target by SPIA, the amplification time in this step could be shorten 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

Samples

The culture supernatant samples of GBS-strain ATTC 12386, *Staphylococcus haemolyticus*-strain ATCC 29970, *Enterococcus faecalis*-strain ATCC 19434, *Streptococccuspyogenes*-strain DSM 2071 were acquired from Centers for Disease Control of Zhejiang Province. Among these samples, GBS-strain ATTC 12386 was selected as positive sample, the other three were negative bacterial samples which could be most frequently confused with GBS in clinical diagnosis. Bacterial 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 DNA

samples were 104 copy/mL. The samples were transported in Liquid nitrogen and stored at -80°C.

Probe design

The chimeric DNA/RNA primer of SPIA was designed according to the principle in previously published paper (NurithKurna et al., 2005). cAMP gene sequences of GBS-strain ATTC 12386 (accession No. AY465356) was chosen as the target and was aligned with available cAMP gene sequences of other GSB strains, including the circulating strains in China responsible for recent epidemics, to identify the conserved regions using DNASIS software. The sequences of the primers are as follows. P1:(5′-UCGAUCAUGTTAGCCGC -3′), the underlined sequence in 5′ end were RNA and the other bases in 3′ end were DNA sequence. The probe of RIDA is designed complementary to the amplification products of nucleic acid sequence-based amplification (NASBA), the probe sequence is: 5′-AGGCGTGTGGAGTCCCTAACCGACTG-3′. Its 5'end was labeled with fluorophore FAM, 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 SPIA-RIDA reaction solution and optimization of reaction condition

The reaction system includes 15μL SIPA mixture(1 mmol/L dNTPs, 1 mmol/L NTP, 0.5 mmol/L ITP, 0.4 μmol/L primer, 6 U Bst DNA polymerase, 0.8 U RNase H, 2.5 μLBst DNA polymerase Buffer, 1 μLRNase H Buffer). Before adding 1ul DNA sample into the reaction mix, we should first heat the DNA sample at 99°C for 10s to destroy the double strand structure. Then the denatured single strand DNA was added to the mixture. The reaction conditions were as follows: First, heating in a water bath at 50°C, different reaction time was screened during this step (20, 30, 45, 60 and 90min), then adjusting the reaction temperature to 55°C, RIDA mixture(1 μL DMSO, 7.5 mmolsorbic alcohol, 50 pmol/L probe, 2 μL NEB buffer3, 5U N.BstNBI endonucleases)n was added, heating for 5, 10 and 15min, respectively. Finally the optimal time was monitored by the visual results. We chose GBS-strain ATTC 12386 DNA as the positive template and *E.faecalis*-strain ATCC 19434 DNA as the negative template, the concentration of both templates were 10^2copy/μL; a blank control group was also set up to evaluate the reaction result.

Comparison the test result of the novel assay

We chose 4 species of bacterial DNA samples to identify the specificity of the SPIA-RIDA assay. The samples included GBS-strain ATTC 12386, *S.haemolyticus*-strain ATCC 29970, *E.faecalis*-strain ATCC 19434 and *S.pyogenes*-strain DSM 2071. The concentration of those DNA templates was 104copy/mL. Distilled water was used as blank control. The whole reaction was processed in one tube as described above.

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

The GBS-strain ATTC 12386 DNA with initial concentration of 10^4 copies/mL was 10×serially diluted, and 10^4, 10^3, 10^2, 10^1, 1 copy/mL DNA were used respectively as the template to carry out the SPIA-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 104 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 60min.

Sensitivity of PCR reaction in detection of GBS-strain ATTC 12386

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 DNA sample was set up as described above. 10^4, 10^3, 10^2, 10^1, 1 copy/mL DNA were used respectively as the template to carry out the PCR reaction.

RESULT

The optimal condition of SPIA-RIDA reaction

After the reaction, we placed the tube under ultraviolet radiation (254 nm) 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 SPIA was 45min, and 10min 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 GBS-strain ATTC 12386 turned to green color and the rest samples: *S.haemolyticus*-strain ATCC 29970,*E.faecalis*-strain ATCC 19434, *S.pyogenes*-strain DSM 2071 and blank control are all colorless which indicted negative result(Figure 2).

Comparison of sensitivity between SPIA-RIDA and separate RIDA

According to the sensitivity analysis results SPIA-RIDA method could detect the minimal value of 10 copies /mL DNA, 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 SPIA-RIDA (Figure 3).

Sensitivity results of PCR

The electrophoresis result revealed that the sensitivity of

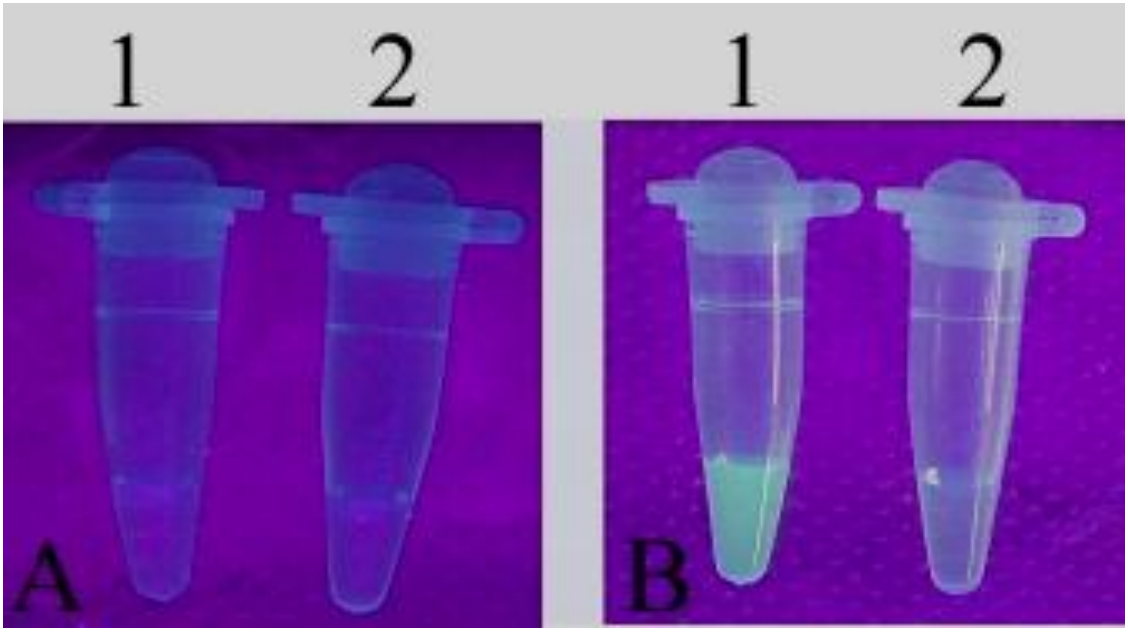

Figure 1. A,Before reaction, the reaction solution for both positive and negative samples are colorless under UV light. Tube 1 is positive sample, tube 2 is negative sample. B,After reaction, the positive reaction solution (tube 1) turned to green under UV light, the negative (tube 2) is still colorless.

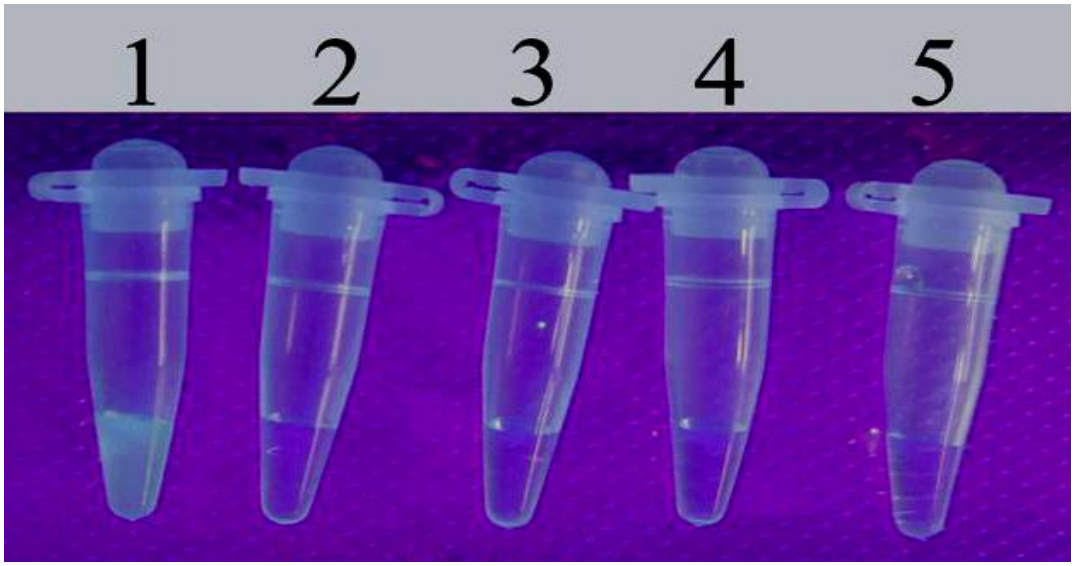

Figure 2. The specificity result of the SPIA-RIDA obtained from the color difference under UV light. The 1 to 5 samples are respectively as follows: 1, GBS-strain ATTC 12386;2, *Staphylococcus haemolyticus*-strain ATCC 29970;3, *Enterococcus faecalis*-strain ATCC 19434, 4, *Streptococccuspyogenes*-strain DSM 2071; 5, blank control.

PCR was closed to SPIA-RIDA assay which could detect the minimal value of 10 copies DNA/mL (Figure 4), but the consumption time of PCR which needed 3 h for the whole reaction is much higher than that of the new method.

DISCUSSION

In this research, we have set up a new diagnostic method which does not need temperature cycle but only one change in temperature. The new method we presently

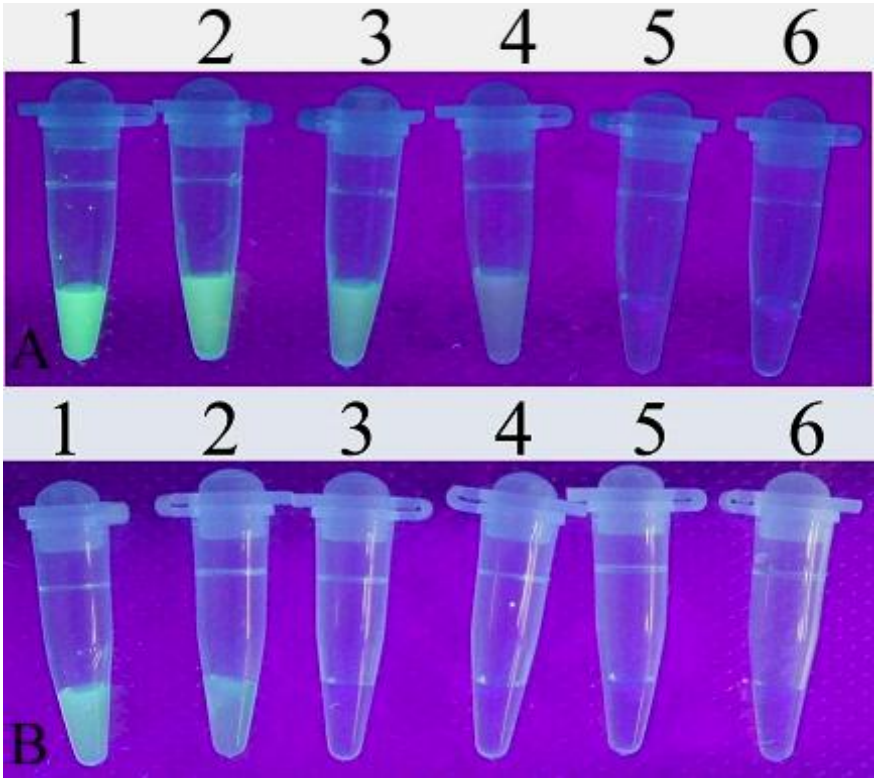

Figure 3. A. The sensitivity result of SPIA-RIDA. B, Sensitivity result of RIDA reaction. The DNA copies amount used in tube are 1, 10^4; 2, 10^3; 3, 10^2; 4, 10^1; 5, 1 copy per mL, repectively. Tube 6 is blank control.

call SPIA-RIDA, has many advantages when used in the bacterial DNA 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 lead to the appearance of false positive results (Higgins et al., 2001; Gibson et al., 2008). However, the new assay does not mainly depend on amplification of nucleic acid.

The key point of this assay was amplifying the signal of the fluorescence probe, the amplification of SPIA was limited by cutting short the reaction time (in this assay we set up the SPIA 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, as a linear amplification system, the products of SPIA cannot be used as the amplification target 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 SPIA 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 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 SPIA there would be little possibility to see a positive result for SPIA-RIDA in clinical test. In this test, 4 species of bacterial DNA were used to confirm the specificity of the novel assay, the results indicted that the new assay could distinguish between GBS and other three bacterial samples.

Besides the improvement of specificity, the novel assay also showed a good sensitivity compared with other assays published (Scherczingeret 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 of thedetecting procedure. The whole reaction only needs to change the temperature once; therefore no expensive equipments and complicated operations are needed for the assay.

As a new assay, there are so many advantages for this method though 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 bacterial DNA detection.

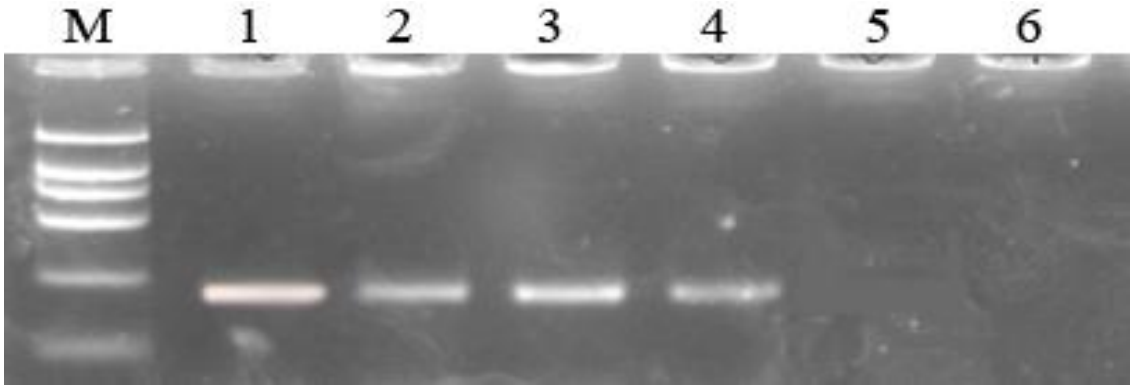

Figure 4. Sensitivity result of PCR. The electrophoresis lanes from left to right were as follows: marker DM2000; the DNA template with content of 10^4, 10^3, 10^2, 10^1, 1 copy per mL and a blank control.

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