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Rapid and visual detection of Group B streptococcus using recombinase polymerase amplification combined with lateral flow strips

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ABSTRACT

Conventional culture method for detecting Group B streptococcus (GBS), a common pathogen of neonatal meningitis and sepsis, is time-consuming and unsensitive. Even though real-time fluorescence PCR-based molecular method is more accurate, it need special instrument and elaborate protocol. Here, we established a novel molecular method combining recombinase polymerase amplification with lateral flow strips for detecting GBS. The cAMP factor (*cfb*) gene is a highly specific and sensitive biomarker to identify GBS and is detectable by using 100 genomic copies as the amplification template. Clinical performance of this assay was evaluated by testing 130 samples, in comparison with culture method and real-time fluorescence PCR, and the results achieved 100% accuracy, which were the same with those of real-time fluorescence PCR, and were better than those of culture method with false-negative detection. This study provides a rapid and visual method, with clinical potential, for the detection of GBS infection of patients.

1. Introduction

Group B streptococcus (GBS), or *Streptococcus agalactiae*, is gram-positive coccus that may result in reproductive tract infection in pregnancy (Oh, 2013). In the 1970s, it was firstly considered as a great risk for the pregnant women and newborns in the United States (Puopolo et al., 2005). GBS infection is the common cause of neonatal meningitis and sepsis, which may bring about severe neurodevelopmental impairment and have significant adverse long-term outcomes (Libster et al., 2012). Up to 40% of all pregnant women were colonized by GBS (de-Paris et al., 2011), the proportion of vertical transmission from mother to the newborns in neonatal GBS colonization is as high as 75%, and 1% to 2% of these infants will develop early-onset GBS infection (Winn, 2007). In 2002, the Centers for Disease Control and Prevention (CDC) published revised guidelines, which recommended prenatal screening of pregnant women at 35-37 weeks' gestation. It ensured that at-risk pregnant women were candidates for intrapartum antibiotic prophylaxis (IAP) (Schrag et al., 2002). This implementation of the guidelines led to the incidence of early-onset neonatal GBS disease reduced from 1.5 to 0.3 per 1000 live births (Daher et al., 2014).

The current gold standard method for the detection of GBS colonization is broth culture in selective medium (de-Paris et al., 2011). The culture is time-consuming which takes 48 h to get the results for fully GBS identification (Philipson et al., 1995). Furthermore, the culture of 35-37 weeks did not always indicate the status of carriers at delivery, as false-negative culture results were observed in some pregnant women who are GBS carriers (Schrag et al., 2002). In the updated CDC guideline in 2012, there was a recommendation to consider using sensitive and rapid nucleic acid amplification tests for diagnosing GBS, such as polymerase chain reaction tests (PCR) (Cagno et al., 2012). PCR test was superior to culture method (Davies et al., 2004), but it needs elaborate protocol and sophisticated thermal cycling equipment (Craw and Balachandran, 2012). Thus, establishing a rapid and convenient molecular method for direct detection of GBS will make prevention program more effective.

Several isothermal nucleic acid amplification technologies have been developed rapidly in recently years (Kim and Easley, 2011), such as loop-mediated isothermal amplification of DNA (LAMP) (Notomi et al., 2000), strand displacement amplification (SDA) (Walker et al.,

Abbreviations: GBS, Group B streptococcus; CDC, Centers for Disease Control and Prevention; IAP, intrapartum antibiotic prophylaxis; PCR, polymerase chain reaction; LAMP, loop-mediated isothermal amplification; SDA, strand displacement amplification; HAD, helicase-dependent amplification; NASBA, nucleic acid sequence-based amplification; RPA, recombinase polymerase amplification; AGE, agarose gel electrophoresis; LF, lateral flow; POCT, point-of-care test; μ-TAS, micro total analysis system.

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1992), helicase-dependent amplification (HDA) (Vincent et al., 2004) and nucleic acid sequence-based amplification (NASBA) (Compton, 1991). For isothermal amplification technologies, nucleic acid sequence amplification was performed at constant temperature without a thermocycler. It is simple, fast and has the equivalent sensitivity and specificity as PCR technology (Craw and Balachandran, 2012). Recombinase polymerase amplification (RPA), one of the isothermal amplification technologies, was a novel approach for rapid and specific DNA amplification developed by Piepenburg et al. (2006). Amplification reaction involves recombinase, single-stranded DNA-binding protein and strand-displacing DNA polymerase. A series of studies published in recent years indicates that RPA technology has been successfully applied to clinical detection. Such as the field of bacteria test, viruses test and food safety and so on (Boyle et al., 2014; Liu et al., 2017; Rohrman and Richards-Kortum, 2012). RPA amplicons could be detected by agarose gel electrophoresis (AGE) and fluorescence monitoring (Xu et al., 2014). Additionally, a simple non-instrumentation 'sandwich assay' called lateral flow strips technology (LF-strips), was also developed to detect amplification product (Jauset-Rubio et al., 2016). Compared to AEG and fluorescence monitoring, the LF-strips-based approach can generate visual results without a specific instrument within 5–10 min.

In this study, we intend to establish a novel molecular method combining RPA with LF-strips for the detection of GBS in pregnancy. This method will be more rapid, simple and cost-efficient than the conventional culture method and PCR technology, which suggests that it has the potential to be an alternative method for diagnosing GBS colonization in the future.

2. Materials and methods

2.1. Reagents

The RPA reaction was performed by using TwistAmp® nfo kit (TwistDx, UK). LF-strips were obtained from HybriDetect (Milenia Biotech GMBH, Germany). Bacterial genomic DNA was extracted using TIANamp Bacteria DNA Kit (Tiangen Biotech, China). Amplification products were purified using QIAquick PCR Purification Kit (Qiagen, Germany). Real-time fluorescence PCR was performed with GBS Nucleic Acid Detection Kit (Fluorescent PCR) (TIB, China), which also provided extraction reagents for extracting genomic DNA from clinical samples.

2.2. Source of microorganisms

GBS ATCC12386 was used in this study. Besides, other 18 microorganisms that are naturally found in vagina or have high homology to GBS were selected to test the specificity of GBS-RPA-LF (Table 1). All of the above microorganisms were obtained from American Type Culture Collection. Extraction of genomic DNA was according to the protocol provided by the TIANamp Bacteria DNA Kit. Concentration and purity of isolated DNA was analyzed by measuring absorbance using NanoDrop 2000 spectrophotometer (Thermo Scientific, US).

Table 2
Primers and probe used in GBS-RPA-LF assay

Primer/Probe[a]	Sequence (5'-3')
G-F	CTCTAGTAAAGCGTGTATTCCAGATTTCCTTATC
G-R-BIO	CTATTGGTAGTCGTGTAGAAGCCTTAACAGATG[b]
G-P	CAACTGAAGCAAATGGATCTAAAATGCGAAT(dSpacer) ACCAGCTTAGTTATCCC[c]

[a] G-F, forward primer; G-R-BIO, reverse primer; G-P, probe
[b] G-R-BIO was labelled at its 5'-end with a biotin
[c] G-P incorporated the carboxyfluorescein (6-FAM) at the 5'-end, an internal abasic nucleotide analogue (dSpacer), and a polymerase extension blocking group (C3-Spacer) at the 3'-end

Table 1
Microorganisms used to test the specificity of GBS-RPA-LF assay.

Microorganisms	Source	Result [c]
Streptococcus agalactiae [a]	ATCC 12386	+
Lactobacillus delbrueckii [a]	ATCC 12315	−
Lactococcus lactis [a]	ATCC 49032	−
Neisseria gonorrhoeae [a]	ATCC 49226	−
Peptostreptococcus anaerobius [a]	ATCC 27337	−
Proteus mirabilis [a]	ATCC 12453	−
Gardnerella vaginalis [a]	ATCC 14018	−
Acinetobacter baumannii [a]	ATCC 19606	−
Bacteroides fragilis [a]	ATCC 25285	−
Bifidobacterium breve [a]	ATCC 15700	−
Mobiluncus curtisii [a]	ATCC 35242	−
Trichomonas vaginalis [a]	ATCC 30001	−
Ureaplasma urealyticum [a]	ATCC 27816	−
Chlamydia trachomatis [a]	ATCC VR-571B	−
Candida albicans [a]	ATCC 10231	−
Streptococcus pyogenes [b]	ATCC 19615	−
Streptococcus pneumoniae [b]	ATCC 12384	−
Staphylococcus aureus [b]	ATCC 6538	−
Enterococcus faecali [b]	ATCC 29212	−

"+", positive result; "−", negative result
[a] Microorganisms common in vagina
[b] Bacteria with high homology to GBS
[c] Specificity of GBS-LF-RPA assay

2.3. Primers and probe design

According to the instruction manual provided by TwistAmp® nfo kits, primers and probe (Table 2) were designed specifically for the cAMP factor (*cfb*) gene (Podbielski et al., 1994). The *cfb* gene sequence can be obtained in GenBank (GenBank accession no. CP021870.1). Primers and probe were synthesized by Sangon Biotech (Shanghai, China) and prepared at a concentration of 10 μM. There are two amplification products in this study. One generated by a pair of primers was 300 bp, and the other generated by the reverse primer and probe was 156 bp.

2.4. GBS-RPA-LF assay

RPA reaction was performed using TwistAmp® nfo kit. Primers and probe as described above. Reaction mixtures containing 29.5 μL 1 × rehydration buffer, 11.2 μL dH2O, 2.1 μL forward primer (10 μM), 2.1 μL reverse primer (10 μM), 0.6 μL probe (10 μM), 2 μL template DNA, and 47.5 μL of the rehydration solution were transferred into a reaction pellet. Next, 2.5 μL magnesium acetate (280 mM) was added into the rehydrated material to initiate the reactions. After mixing well, the tube was immediately inserted into a suitable incubator block for incubation.

The outcome of RPA reactions was analyzed by AGE and LF-strips in this study. The amplification products were purified by QIAquick PCR Purification Kit firstly and resolved by electrophoresis on 1.2% agarose-gel. For LF-strips, removed 5 μL products and mixed with 100 μL buffer, then placed the strips into solution to make mixture infiltrate sample area. Within 5-10 min, a test band and a control band were clearly visible as red lines, indicating that the RPA amplicon was detected (positive). Presence of only a control band (red line) indicates that no RPA amplicon was detected (negative). In any case, the control band must be always visible to confirm that the strips were functioning correctly.

2.5. Application to clinical samples

In this study, 130 clinical samples were collected from women at 35-37 weeks of pregnancy who conducted prenatal screening at Guangzhou Women and Children's Medical Center (Guangzhou, China). Each sample is tested in parallel by three assays, the culture, real-time fluorescence PCR and GBS-RPA-LF. The clinical performance of the GBS-

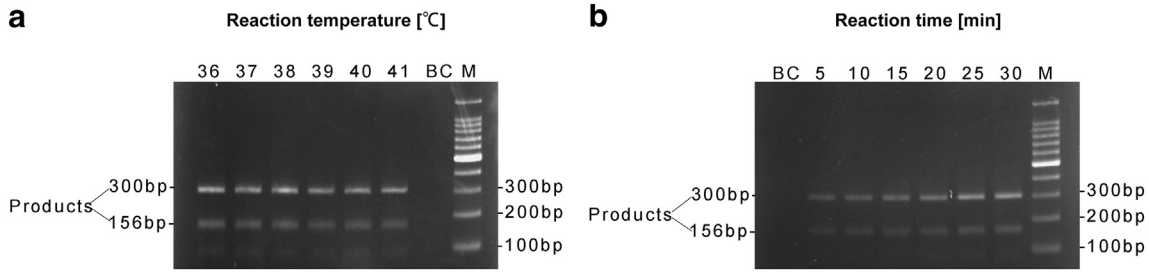

Fig. 1. Optimization of the GBS-LF-RPA reaction temperature (a) and reaction time (b). BC, black control reaction without adding DNA template. M, 100 bp DNA marker.

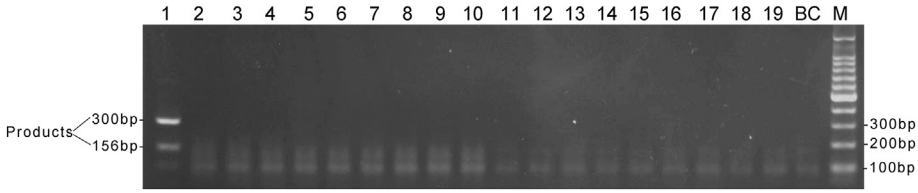

Fig. 2. Specificity of GBS-LF-RPA assay was determined using GBS and other 18 microorganisms. Lane 1, *S.agalactiae*; lane 2, *L.delbrueckii*; lane 3, *L.lactis*; lane 4, *N.gonorrhoeae*; lane 5, *P. anaerobius*; lane 6, *P.mirabilis*; lane 7, *G.vaginalis*; lane 8, *A.baumannii*; lane 9, *B.fragilis*; lane 10, *B.breve*; lane 11, *M.curtisii*; lane 12, *T.vaginalis*; lane 13, *U.urealyticum*; lane 14, *C.trachomatis*; lane 15, *C.albicans*; lane 16, *S.pyogenes*; lane 17, *S.pneumoniae*; lane 18, *S.aureus*; lane 19, *E.faecali*; BC, black control reaction without adding DNA template; M, 100 bp DNA marker.

RPA-LF was evaluated by comparing its detection results with the detection results of culture and real-time fluorescence PCR.

Bacteria culture was carried out in bacteriological laboratory. All clinical samples were cultured on blood agar plates at 37°C in 5% CO_2 for 18-24 h. Bacterial identification was performed on VITEK 2 compact (Merieux, France) and Streptex Rapid Latex Agglutination Test (Oxoid, UK). Before real-time fluorescence PCR and GBS-RPA-LF assay, genomic DNA extraction of clinical samples was performed using extraction reagents provided by the GBS Nucleic Acid Detection Kit. According to the protocol of this kit, real-time fluorescence PCR was carried out on the ABI7500 platforms (Thermo Fisher, US). The GBS-RPA-LF assay was carried out as described previously.

3. Results

3.1. Optimization of the GBS-RPA-LF assay conditions

A series of time and temperature gradients were set to define the optimal reaction conditions of GBS-RPA-LF assay. Firstly, RPA assay was performed within the range of 36–41°C for 40 min, and amplification products were analyzed by AGE (1.2%) subsequently. As shown in Fig. 1(a), there were no significant differences in amplification at 36, 37 and 38°C, but with the temperature increasing from 38 to 41°C, the brightness of target bands decreased. Thus, 37°C was selected as the standard assay temperature. Then, a set of RPA assay was incubated respectively for 5, 10, 15, 20, 25 and 30 min at 37°C. As shown in Fig. 1(b), when incubation time were increased from 5 to 25 min, the corresponding product bands became brighter and brighter. The brightness of the product bands for 30 min incubation was almost the same as that of the product bands that incubation for 25 min, 25 min was considered as the optimum amplification time. Thus, the optimal conditions for RPA assay were incubated at 37°C for 25 min.

3.2. Specificity of the GBS-RPA-LF assay

Under the optimal reaction conditions, the specificity of the GBS-RPA-LF assay was determined by testing GBS and other 18 microorganisms listed in Table 1. As shown in Fig. 2, the target bands were generated only in GBS samples, but not in the other 18 microorganisms or black control samples. No cross-reactions were observed with other microorganisms. The result showed that the primers and probes were specific for GBS detection.

3.3. Detection limit of the GBS-RPA-LF assay

The detection limit of the GBS-RPA-LF assay was determined by using the serially diluted DNA from GBS ATCC12386 samples (corresponding to 10^1 to 10^6 genomic copies per reaction). As shown in Fig. 3(a), target bands were visible on the gel at 100 genomic copies, no products bands were observed in reactions at 10 genomic copies. Fig. 3(b) showed that the test band and the control band were clearly visible at 100 genomic copies (positive), only the control band appeared at 10 genomic copies (negative). The results indicated that the detection limit of the GBS-RPA-LF assay was 100 genomic copies of DNA template per reaction, which was sufficient for clinical detection.

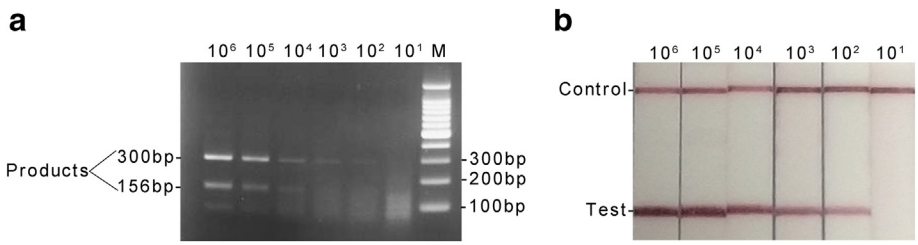

Fig. 3. Detection limit of GBS-LF-RPA assay was analyzed using a series of DNA template ranging from 10^1 to 10^6 genomic copies per reaction. Amplicons were detected by AGE (1.2%) (a) and LF-strips (b). M, 100 bp DNA marker.

Table 3
Clinical performance of the GBS-RPA-LF assay in comparison with bacteria culture and real-time fluorescence PCR.

	Bacterial culture		Real-time fluorescence PCR		GBS-RPA-LF	
Results (n = 130)	Positive 28	Negative 102	Positive 29	Negative 101	Positive 29	Negative 101

3.4. Clinical performance of the GBS-RPA-LF assay

The Clinical performance of the assay to diagnose GBS was evaluated by examining clinical samples (n =130) by culture method and molecular method in parallel. Table 3 shows the performance of the conventional culture, real-time fluorescence PCR and GBS-RPA-LF assay on detecting 130 clinical samples, respectively. Twenty-eight GBS-positive samples and 102 GBS-negative samples were confirmed by culture method. However, 29 GBS-positive samples and 101 GBS-negative samples were identified by molecular method, which is more sensitive and accurate. Medical records of the patient with different diagnosis results between culture and molecular method revealed that the patient had previously been treated with antibiotics, which leading to a false-negative result of culture method. The results of GBS-RPA-LF were consistent with real-time fluorescence PCR, showing a good clinical performance.

4. Discussion

In this study, we developed a novel molecular method for rapid detection of GBS based on RPA in combination with LF-strips. Nucleic acid amplification was performed at 37°C and the results of GBS detection could be available in about 30 min (Fig. 1). The detection of RPA amplicons could be visualized by lateral flow strips, which was rapid, simple and did not require special instrument or well-trained personnel. This assay was specific because only DNA from GBS strain could be amplified. The other microorganisms which are naturally found in vagina or have high homology to GBS were not amplified by the assay (Fig. 2). The detection limit of GBS-RPA-LF was 100 genomic copies per reaction, and its sensitivity is comparable to that of Daher et al. (2014) (Fig. 3). When testing a total of 130 clinical samples, the results of the GBS-RPA-LF method were completely consistent with the existing real-time fluorescence PCR. Therefore, the GBS-RPA-LF assay developed in this study, which was rapid, sensitive and accurate, could feasibly be used as a diagnostic test of GBS and had potential for clinical application.

The conventional method for GBS diagnosis is culturing vaginal/anal swabs in selective medium broth for bacterial identification, which is also the gold standard recommended by CDC. However, it has low sensitivity, poor specificity and the detection results cannot be obtained until 48 hlater. The culture method may also be affected by several factors, resulting in false-negative results. For example, the use of antibiotics before bacterial detection, mild colonization of bacteria in vagina or insufficiency of clinical swab samples. In addition, with regard to certain slow-growing GBS, the GBS-negative result was reported before it had fully cultured on the medium. Among all the clinical samples collected in this study, the results of GBS-RPA-LF were in accordance with the results of real-time fluorescence PCR, while a false-negative result was observed in the culture. Medical records revealed that the patient had previously been treated with antibiotics, which leading to a false-negative result of culture method. Accordingly, the conventional culture method may not be absolutely suitable in the clinical detection of GBS.

Although pregnant women are advised for prenatal screening at 35-37 weeks of gestation according to the guidelines of *Prevention of Perinatal Group B Streptococcal Disease*, some of them conduct screening test for GBS identification at or near the time of delivery, which means that detection results should be available as quickly as possible. Hence, compared to culture which requires 48 h for GBS identification, the advantages of the rapid molecular method for detection of GBS are obvious. Real-time fluorescence PCR can be performed rapidly and accurately which makes up for the shortcomings of culture, but it still takes 100 min to obtain the ultimate result (Ke et al., 2000), sophisticated equipment as well as well-trained personnel are indispensable. Unlike real-time fluorescence PCR assays, isothermal amplification technologies can be performed rapidly at constant temperature without the need for an expensive thermocycler. Several isothermal amplification technologies have been developed in the past few years, such as LAMP, HDA, SDA, and NASBA and so on. The LAMP needs 4 pairs of primers and incubates at 60-65°C for 60 min. Existing protocol of HDA is typically 60–120 min for low copy number targets. For SDA and NASBA, an initial 95°C strand separation step is required. In comparison with the isothermal amplification technology mentioned above, RPA is a novel isothermal amplification technology developed in 2006 which has the following distinct characteristics. In RPA assay, nucleic acid amplification can be completed within 30 min at a lower temperature (37-39°C). The design of primers and probe has no complicated sequence or melting temperature requirement. In addition, this technology is able to tolerate crude samples, and the simplified steps of DNA purification can reduce material and time consumption (Krolov et al., 2014). Thus, RPA assay can achieve rapid portable molecular detection without nucleic acid extraction, which is promised to be an alternative diagnostic tool for point-of-care test (POCT).

RPA amplicons can be analyzed by gel electrophoresis, real-time fluorescence monitoring and lateral flow technology. Daher et al. (2014) established a method for detection of GBS by combining RPA with real-time fluorescence monitoring, which required sophisticated operation and a corresponding fluorescence monitoring system. Here, LF-strips was selected for the analysis of amplified products. It translated result into a visual test in 5-10 min without any special equipment. The LF-strips technology greatly simplified operation process and improved detection efficiency, which made the detection of GBS more rapid and economical.

Owing to its rapidity and simplicity in operation, GBS-RPA-LF is suitable for POCT. However, in the process of this study, we found that the method was extremely susceptible to contamination due to its high sensitivity. It is essential to take precautions accordingly in the whole operation process. On this basis, we suppose that one of the potential applications of the technology combining RPA with LF-strips is to be integrated into the micro total analysis system (μ-TAS) in the future (Mauk et al., 2018), which can achieve fully automated and closed nucleic acid analysis to simplify manual operations, improve efficiency and accuracy of clinical detection.

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Ethical approval

Not required.

Conflict of interest

The authors declare that they have no conflicts of interest.

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