Development of a reliable method to diagnose *Streptococcus agalactiae* infection by droplet digital PCR

CURRENT STATUS: UNDER REVIEW

BMC Microbiology  □ BMC Series

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DOI:
10.21203/rs.2.16922/v2

SUBJECT AREAS
Applied & Industrial Microbiology  General Microbiology

KEYWORDS
Droplet Digital PCR (ddPCR), Streptococcus agalactiae, real-time quantitative PCR (qPCR)
Abstract

**Background:** *Streptococcus agalactiae* (GBS) is the causative pathogen of puerperal sepsis in pregnant women and meningitis in infants. Infection of GBS is responsible for the increased morbidity in pregnant women and the elderly, and bring challenges to clinical diagnosis and treatment. However, culture-based approaches to detect *S.agalactiae* is time-consuming with limited sensitivity. Besides, real-time quantitative PCR demand for expensive instruments with tedious steps. Thus, we aim to establish a new detection method for more accurate and rapid detection of *S.agalactiae*.

**Results:** The ddPCR primer targeted the CspE gene showed a better amplified efficiency in the reaction. The limitation of ddPCR for identifying GBS DNA was able to reach 5 pg/µL. Moreover, no positive amplified signals could be detected in the reactions which served 11 non-GBS strains DNA as templates. Furthermore, the coefficient of variation of this method is 4.5%, indicating an excellent repeatability of ddPCR assay.

**Conclusions:** In our study, ddPCR was performed as a rapid detection of *S.agalactiae* with high sensitivity and specificity. This technique can promote the accuracy of the diagnosis of GBS infection and provide a scientific basis for clinical treatment.

**Background**

*Streptococcus agalactiae* (Group B Streptococcus, GBS) is a facultative anaerobic gram-positive opportunistic pathogen, which colonizes the gastrointestinal and genitourinary tract of approximately 30% of the healthy adults\[^1\]. Moreover, infection of GBS is the main cause of pneumonia, septicemia and meningitis in neonates, especially for the high morbidity rate of pregnant women\[^2-4\]. So far, detection of *Streptococcus agalactiae* varies from culture-based methods to novel molecular tools\[^5,6\]. Traditional culture is laborious and time-consuming with limited sensitivity. Although real-time qPCR and other rapid technique, such as MALDI-TOF-MS, are now commercialized, the cost and expertise limit their use in most laboratories\[^7\]. Therefore, early diagnosis of infection requires a novel method with rapid and specificity in the detection of GBS.

Recently, droplet digital PCR (ddPCR) has been utilized in quantifying nucleic acid and detecting pathogen\[^8-10\]. This method dilutes and divides the mixtures into many microdroplets with oil. Each
microdroplet is amplified as an independent reaction system with or without target genes. The amplified condition of ddPCR is similar to that of real-time PCR with the probe for signal detection. Eventually, absolute concentration will be calculated precisely according to the Poisson distribution without a standard curve\[^{11-14}\].

Droplet digital PCR is an ultraprecise, reliable and economical method in the diagnosis of infectious disease. However, detection of GBS based on ddPCR has not been reported yet. Thus, we aim to evaluate the ddPCR for the detection of GBS and test whether ddPCR can be an alternative assay for the rapid diagnosis of GBS infection.

**Results**

**Primer screening test**

Two sets of primes showed different amplification to GBS ATCC13813 monitored by the SLAN-96P real-time system(Figure 1). The amplified signal was firstly detected 15 cycles after the reaction and the peak emerged at approximately 45 cycles with $CspE$ primer. No other amplification was seen in the $Sip$ primer and negative control. Therefore, the $CspE$ primer was selected for the subsequent tests.

**Sensitivity and specificity of ddPCR for GBS**

The limit of ddPCR for detecting GBS DNA was able to reach 5 pg/µL. As shown in Figure 2, the horizontal axis represented the event number of four concentrations templates, and the vertical represented the sample amplitude. The positive and negative microdroplets were shown in blue and gray, respectively. The number of events was 0 in the concentration of 0.5 pg/µL, suggesting no amplification in this reaction. (Figure 3). No positive microdroplets could be detected in the reactions using non-GBS strains DNA as templates(data not shown). Therefore, the ddPCR with $CspE$ primer has a satisfactory sensitivity and specificity for GBS detection.

**Repeatability test of ddPCR**

GBS reference strain was run in triplicate (Figure 4). The positive events number was 1661, 1560 and 1704, respectively, with a CV of 4.5%, indicating that ddPCR has an excellent repeatability.

**Discussion**
Streptococcus agalactiae is the leading cause of neonatal pneumonia, infantile septicemia, bacterial meningitis, as well as perinatal infection of pregnant women and infants\textsuperscript{[15,16]}. Therefore, a novel detection based on ddPCR was developed to detect GBS by targeting the CpsE gene in the current study.

Nowadays, a bacterial culture is still a gold standard method to identify GBS infection, but it is time-consuming with limited sensitivity and vulnerable to interference\textsuperscript{[17]}. Also, real-time qPCR requires expensive equipment and only quantifies nucleic acid relatively. Recently, a novel technique, ddPCR, was for DNA quantifications absolutely without depending on the standard curve\textsuperscript{[18]}. ddPCR is the third generation PCR with higher diagnostic efficiency compared to conventional methods. In our study, we showed that ddPCR could detect S.agalactiae precisely as low as 5 pg/µL.

Moreover, amplification was observed in GBS but not in non-GBS strains, which indicated the high specificity of ddPCR primers. Furthermore, ddPCR had an excellent repeatability with a CV of 4.5%. Given these advantages, it can be used to determine the expression and copy number variation analysis of the target gene\textsuperscript{[19,20]}.

In the present study, the fluorescent dye EvaGreen was used to monitor GBS. However, EvaGreen binds to dsDNA and could cause false-positive results if there existed dimer formations. Thus, the melting curve was analyzed and no double-peak was found, indicating no primer dimer formation. Furthermore, we discovered that the CspE primer amplifies the target gene of GBS more effectively than the Sip gene.

Some limitations of our study should not be ignored. Firstly, ddPCR is an assay mainly with fluorescent probes. Application with EvaGreen dye may significantly interfere with the experimental results when the primer dimers were forming. Therefore, the demand for primers specificity is very high. Secondly, bubbles in the process will produce less than 12 000 microdroplets. Thus the experiment does not meet the Poisson distribution, leading to inaccurate results. Thirdly, it was reported that the fbs-B gene was targeted with LAMP to identify GBS \textsuperscript{[5]}. Which gene is more effective in detection needs to be further studied. Moreover, we just established a ddPCR method to identify
GBS, and clinical validation should be considered with clinical samples in the next. Meanwhile, since the purity of DNA templates are easily affected and varied from different samples, DNA extraction should be optimized to extend the ddPCR testing from the laboratory to further clinical detection.

Conclusions
In conclusion, our study suggested that ddPCR is a specific, economical and reliable method. Further experiments should be performed to prove that it is an alternative tool in further clinical detection of GBS.

Methods

Bacterial strains
GBS standard strain ATCC13813 was purchased from Shanghai cell bank of the Chinese Academy of Sciences. Eleven non-GBS strains were isolated from the Clinical Laboratory of Third Affiliated Hospital of Guangzhou Medical University and initially identified by Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry. Non-GBS strains stored at -70°C, were used for specificity experiments, including Candida tropicalis, Candida albicans, Klebsiella pneumoniae, Streptococcus pyogenes, Acinetobacter baumannii, Escherichia coli, Staphylococcus haemolyticus, Candida parapsilosis, Streptococcus anginosus, Enterobacter aerogenes, Pseudomonas aeruginosa.

DNA extraction
The ATCC13813 and other non-GBS strains were inoculated onto sheep blood agar and cultured in 37°C constant thermostat incubator for 18-24h. The bacterial colonies picked on the agar plates were inoculated into sterile physiological saline to prepare 1ml bacterial suspensions\[^{21,22}\]. Then the bacterial DNA was extracted and purified according to the instruction manuals of the TIANGEN DNA kit (TIANGEN BIOTECT, Beijing) and was stored at -20°C.

Primer design and synthesis
The sequence of Streptococcus agalactiaeCpsE and Sip gene were obtained from GenBank\[^{23,24}\]. Special primers were designed by Primer Premier 5.0(Premier Laboratories, Canada) and synthesized by Thermo Scientific of Shanghai Trade Co. Ltd. (Table 1). The EvaGreen® dsDNA fluorescent dye (EvaGreen®) was used in this detection.
Droplet Digital PCR reaction

The ddPCR reaction was performed in a QX200 Droplet Digital PCR System (Bio-Rad Laboratories, CA) according to the manufacturer's instruction\textsuperscript{[11]}. Each test was prepared in a 20ul volume of the reaction mixture, which comprised 10 μL of 2× QX200™ ddPCR™ EvaGreen® Supermix (no dUTP; Bio-Rad), forward and reverse primers and 4 μL of DNA templates. For microdroplets generation, 20ul mixture and 70 ul droplet generation oil were added to the DG8™ cartridge (Bio-Rad), then loaded into a QX200 Droplet Generator (Bio-Rad). Next, microdroplets were transferred into 96-well PCR plate and heat-sealed with foil to prevent aerosol pollution. Then the PCR was performed on a Bulk PCR Thermal Cycler using the following conditions: Pre-denature for 1 cycle at 95°C for 10min; denature for 45 cycles at 95°C for 15s; anneal and extend for 45 cycles at 60°C for 1 min. Finally, the fluorescence signal in each plate was analyzed by a QX200 Droplet Reader and QuantaSoft\textsuperscript{TM} Version 1.7.4\textsuperscript{[10,25]}. Each reaction adopted negative control and performed in duplicate.

Primer screening analysis

Two sets of primers were dissolved into a working solution, and the DNA of the ATCC13813 strain was served as the template. Then the qPCR assay was performed to amplify $CpsE$ or $Sip$ gene in a SLAN-96P real-time system (HONGSHI. Shanghai). Briefly prepared a total volume of 25μL mixture according to the kit instructions, which contained DNA template, primers, SYBR GREEN dye and PCR Master Mix etc. The reaction procedure was set up identically as the ddPPCR amplification condition mentioned above. Finally, the amplification efficiency was compared to select a better primer for the subsequent assay.

Sensitivity of ddPCR reaction

DNA obtained from GBS reference strain ATCC13813 was used to determine the limitation of ddPCR assay towards the selected gene. The initial concentration of DNA was adjusted to 5ng/μL and then diluted four times with sterile saline, that is, 5ng/μL, 0.5ng/μL, 50pg/μL, 5pg/μL and 0.5pg/μL. The ddPCR reaction was performed as described above with four concentrations of the DNA template to determine the sensitivity of ddPCR in the GBS test.
Specificity and repeatability of ddPCR reaction

The DNA of the GBS ATCC13813 strain and other 11 non-GBS strains were used to assess the specificity of the primers under identical ddPCR conditions. For further repeatability analysis of ddPCR, the reaction was carried out by testing one positive strain and one negative strain three times under identical conditions. Finally, the fluorescence signal was evaluated.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Competing interests

The authors declare that there are no competing interests associated with the manuscript.

Funding

This study was supported by grants as follow:

1. "Climbing Plan" Guangdong University Student Science and Technology Innovation Cultivation Special Fund Projects (grant number pdjh2017b0421);
2. Science and Technology Innovation Project of Guangzhou Medical University (grant number 2016A046);
3. Natural Science Foundation of Guangdong Province(2015A030313684);
4. National Natural Science Foundation of China(81803884).

Authors' contributions

YF Zeng, JJ Chen, XY Li and XG Guo conceived and designed the study. YF Zeng, JJ Chen and YG Wang cultured bacteria and carried out the ddPCR. CM Chen, S Ouyang, and TX Ji extracted DNA and performed the qPCR. YF Zeng, JJ Chen, XY Li and CM Chen conducted data analysis to make figures and tables. YX Xia and XG Guo participated and gave guidance throughout the process. All members
participated in the writing, review, discussion and revision of the manuscript and adopted the final version unanimously.

**Acknowledgments**

We thank all members of our research team for their contributions to this work.

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### Tables

| Target           | Sequences (5’-3’)          |
|------------------|----------------------------|
| *Sip* upstream   | CTGCCAACACCTATGACC          |
| *Sip* downstream | CTGCTACAGTTCTTACC          |
| *CpsE* upstream  | GCAAAAGAACAGATGGAACAAAGTG |
| *CpsE* downstream| CGCCGTAAGTAGCAACAGAT       |

### Figures

**Figure 1**

The primer of the qPCR screening experiment in the present study.
Figure 2

Sensitivity assay for ddPCR using continuous 10-fold dilutions of DNA templates from GBS ATCC13813.
Figure 3

The number of events in each amplification concentration.
Figure 4

The repeatability test of ddPCR using DNA from the GBS ATCC13813 strain.