A Rapid, Visible, and Highly Sensitive Method for Recognizing and Distinguishing Invasive Fungal Infections via CCP-FRET Technology

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ABSTRACT: Invasive fungal infection (IFI) is one of the leading causes of death in the intensive care unit (ICU) due to its high morbidity and mortality among immunocompromised patients. Early diagnosis of IFI is typically infeasible because of the lack of clinical signs and symptoms. By virtue of the cationic conjugated polymer-based fluorescence resonance energy transfer (CCP-FRET) technology, we develop a rapid, visible, simple, and sensitive method for simultaneous detection and discrimination of three types of pathogens, including *Candida albicans* (*C. albicans*), *Klebsiella pneumoniae* (*K. pneumoniae*), and *Cryptococcus neoformans* (*C. neoformans*). The CCP-FRET system contains a CCP fluorescent probe and pathogen-specific DNA labeled with fluorescent dyes. These two components spontaneously self-assemble into the complex under electrostatic attraction, resulting in an efficient FRET from CCP to fluorescent dyes when irradiated with a 380 nm ultraviolet (UV) light. The CCP-FRET method can specifically identify the DNA molecules that are extracted from culture pathogen strains or blood samples via PCR and single base extension (SBE) reactions, without any cross-reactions on the DNA of nonspecific strains. In particular, the sensitivity of this method is down to 0.03125 ng, which is ten times higher than that of real-time PCR. We further evaluate its detection efficiency by testing 15 blood samples from neonatal patients who suffer from pathogen infections, in which some of them have undergone antipathogen treatments. Using the CCP-FRET method, 33.3% (5/15) of samples tested positive for *C. albicans* and/or *K. pneumoniae* infections, whereas no pathogen DNAs are recognized with real-time PCR, despite using the same primers. Interestingly, the CCP-FRET method can output unique fluorescent color as well as RGB patterns to different types of pathogen infections, by which the infection type can be conveniently determined. Collectively, the CCP-FRET method is a sensitive and reliable detection platform for rapid identification of fungal and bacterial multiple infections, holding great promise for uses in clinical testing.

KEYWORDS: Candida albicans, Klebsiella pneumoniae pneumoniae, Cryptococcus neoformans, CCP-FRET method, invasive fungal infection, multiple infection detection
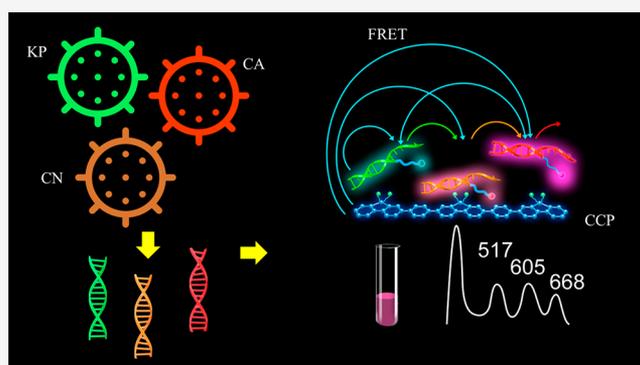
Invasive fungal infection (IFI) remains one of the most frequently occurring and life-threatening diseases in intensive care units (ICUs).[1,2] Most of the patients in ICUs die of IFI rather than primary diseases. The epidemiological studies show that *Candida albicans* (*C. albicans*) accounts for most of the majority of cases.[3−5] *Cryptococcus neoformans* (*C. neoformans*) infection is also rising, which leads to multiple organ damages such as central nervous system and lung damage.[6−8] The diagnosis of IFI is usually delayed due to the lack of typical clinic symptoms and the fact of low sensitivity of blood culture methods as a golden standard of clinical diagnosis. Timely and accurate diagnosis of the IFI is necessary for clinicians to start antifungal drug treatment at the earlier infection stage and also to avoid unnecessary medicine use.[9] Most importantly, as the immunocompromised patients usually suffer more than one pathogen infection, especially

bacterial infection,[10] efficient methods for simultaneous identification of multiple pathogens via one-time sampling test continue to be needed in clinical applications.

Currently, fluorescent probe and PCR technology provide a sensitive tool for identifying specific pathogen strains. A PCR-based fluorescent detection technology, real-time PCR, has been applied in clinical laboratory diagnosis by targeting and amplifying specific rDNA genes or internal transcribed spacer (ITS) regions of pathogen DNA.[11−13] Despite the higher
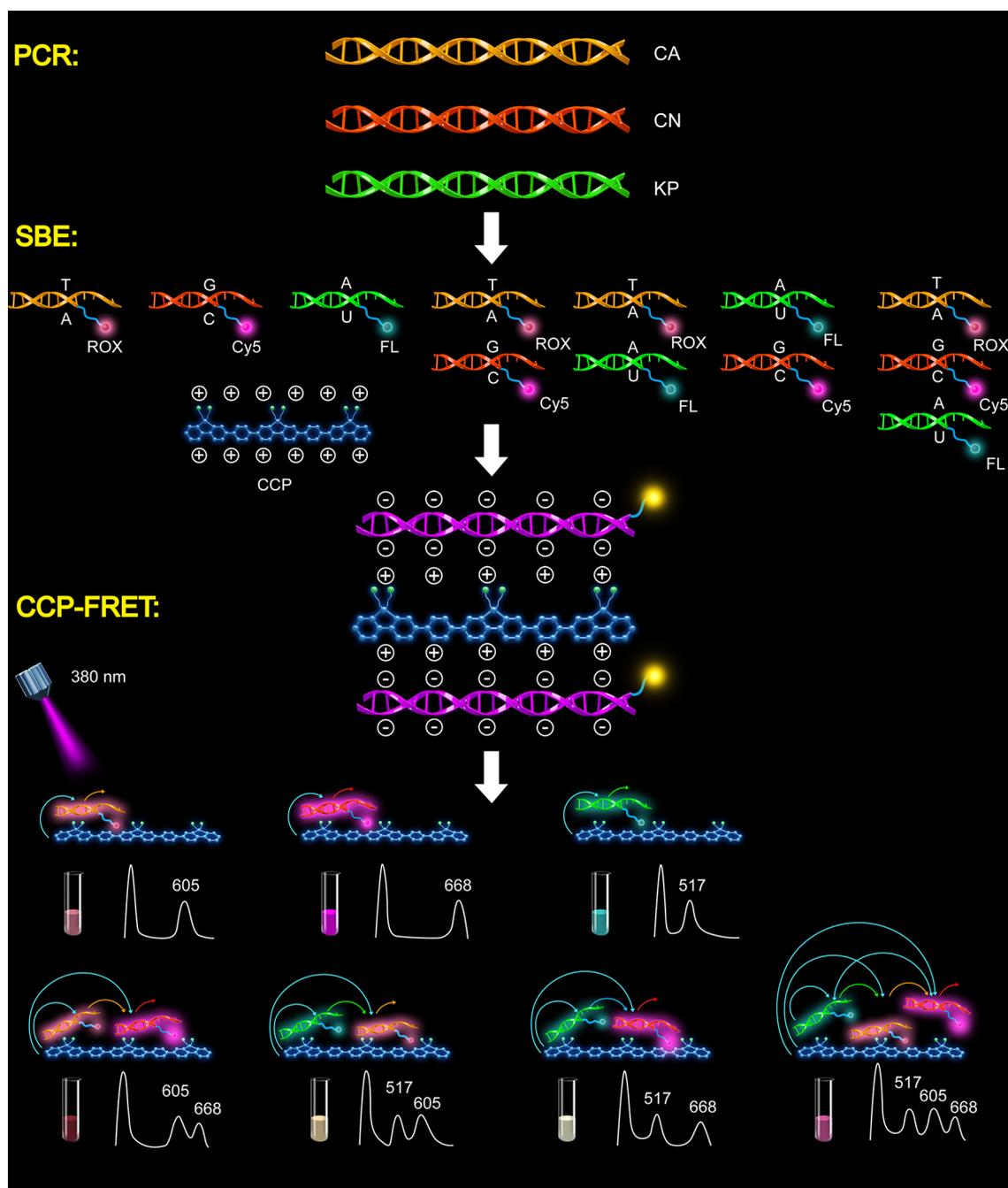
Figure 1. Schematic overview of the cationic conjugated polymer-based fluorescence resonance energy transfer (CCP-FRET) method for identifying and distinguishing multiple pathogen strains. The pathogen DNAs are first amplified by regular PCR reaction (step 1). The PCR system contains three sets of primers targeting three pathogen strains, *Klebsiella pneumoniae* (KP), *Candida albicans* (CA), and *Cryptococcus neoformans* (CN). Then, single base extensions (SBE) are carried out (step 2), in which PCR amplicons are used as SBE templates and ROX-ddATPs, Cy5-ddCTPs, and FL-ddUTPs are incorporated into the CA, CN, or KP primer's 3′-terminus, respectively. Finally, CCP-FRET signals are induced by adding cationic conjugated polymer (CCP) under the excitation of 380 nm ultraviolet light (step 3). In step 3, three pathogen strains and their combinations are distinguished according to the specific FRET emission spectra and unique fluorescent color changes of the reaction system.

sensitivity of the fluorescent assay targeting DNA, it is not suitable for clinical blood and tissue sample detection because of high background noise. On the other hand, the sensitivity of real-time PCR methods does not yet reach the level for the presence of trace pathogen DNA in most real samples. The real-time PCR also requires a professional operation and real-time fluorescent monitoring equipment. In addition, a real-time PCR usually is able to detect the presence of only one pathogen strain in each reaction and is not suitable for multiple

strains, which limits its clinical application. Our group has successfully employed a cationic conjugated polymer-based fluorescence resonance energy transfer (CCP-FRET) technology in detecting known SNP sites,[14] somatic mutations,[15,16] and gene promoter CpG island methylation level[17] of tumor tissue samples. As a core component of the CCP-FRET system, the CCP molecule has the characteristics of fluorescent signal amplification and enrichment in light of its conjugate structure and a large number of absorbing units.[18,19] It
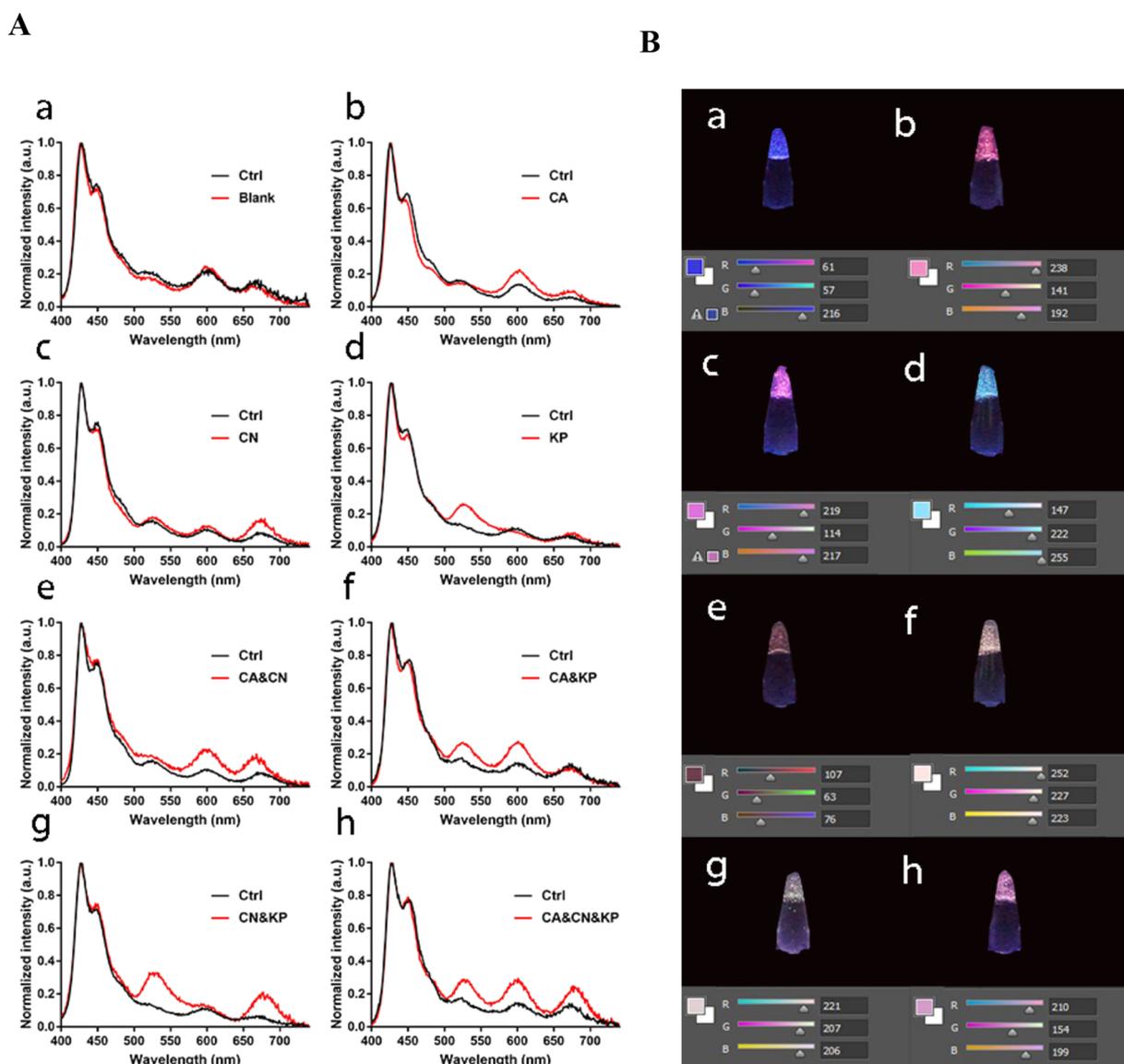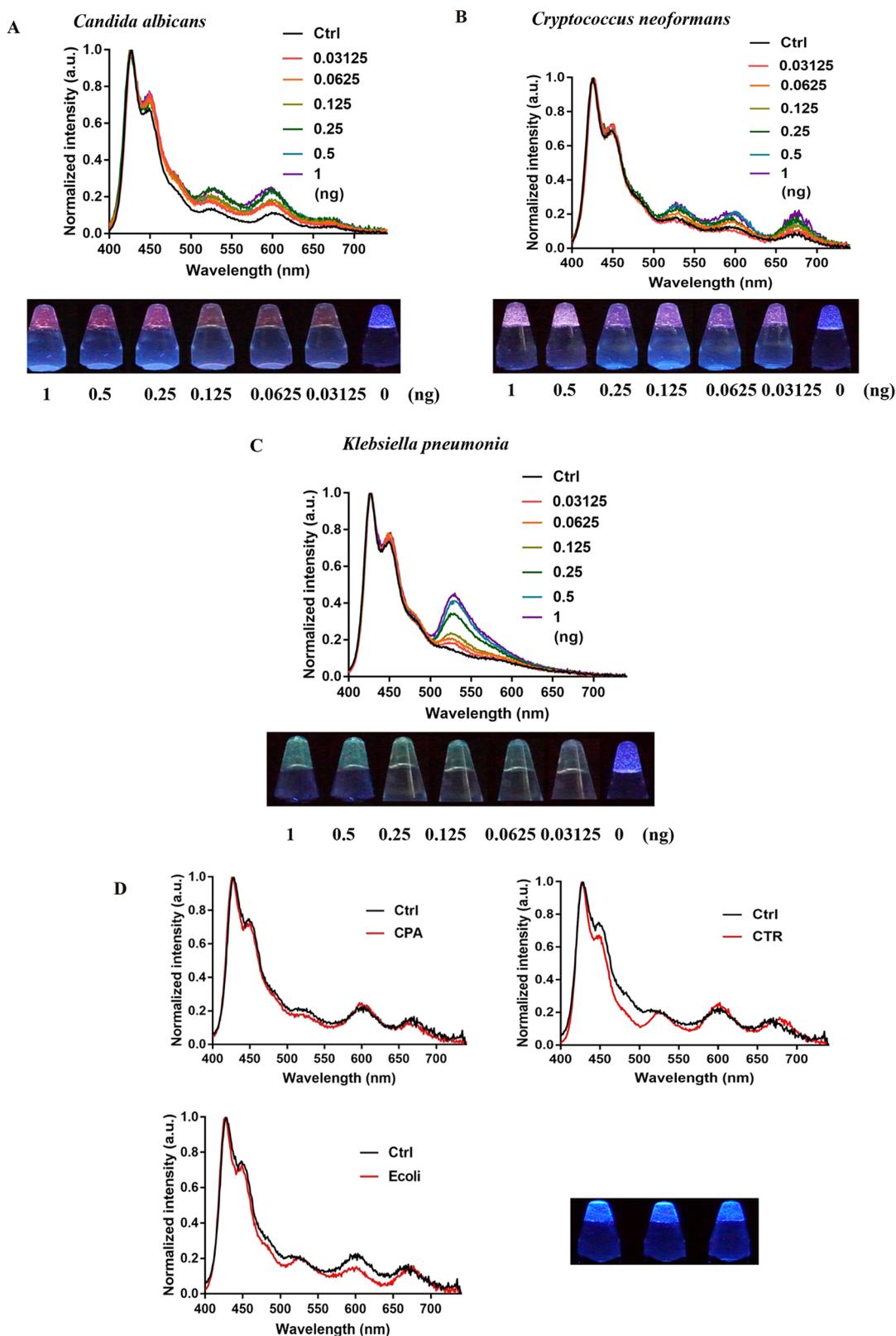
Figure 2. Optimized CCP-FRET method detects multiple pathogen strains. The DNA samples were extracted from *Candida albicans* 10231(CA), *Cryptococcus neoformans* (CN), and *Klebsiella pneumoniae* (KP). Twenty ng of DNA samples were measured by the CCP-FRET method. All the samples were amplified by regular PCR. And then ROX-ddGTPs, Cy5-ddCTPs, and FL-ddUTPs were incorporated into the PCR amplicons by single base extension (SBE). Finally, cationic conjugate polymers (CCPs) were directly added to the reaction system. The FRET signals were excited with 380 nm UV light. (A) Emission spectra of CCP-FRET. (B) The emission fluorescence emission images of CCP-FRET reaction solution. (a) Blank; (b) CA; (c) CN; (d) KP; (e) CA and CN; (f) CA and KP; (g) CN and KP; (h) CA, CN, and KP. The RGB values were analyzed with Adobe Photoshop CS6 software.

transforms the interaction and recognition between biological molecules (nucleic acid) to a remarkably enhanced optical signal,[18] which enables the CCP-FRET system to have higher specificity and sensitivity in recognizing biological molecules and organisms.[19]

Given that *C. albicans*, *C. neoformans*, and *K. pneumoniae* are the main pathogen strains that the immunocompromised neonate patients often suffer from, in this study, we designed a CCP-FRET method that could simultaneously detect three pathogen strains and distinguish seven different types of combination strains just by one reaction, and provide visible specific fluorescent color reactions as well.

Its basic principle and strategy for multiple pathogen detection is illustrated in Figure 1. First, the internal transcribed spacers (ITS), 5.8S or 16S rDNA from *C. albicans* 10231, *C. neoformans*, and *K. pneumoniae*, were respectively

amplified by a regular PCR with three sets of primers in one tube. Second, SBE reaction was initiated with pathogen-specific primers, by which dye-ddNTPs were respectively incorporated into the specific primer's 3′-terminus. The *C. albicans* primer complementarily bound to the PCR-amplified ITS products, and subsequently, ROX-ddATP incorporated into the primer's 3′-terminal OH, similarly, Cy5-ddGTPs into the *C. neoformans* primer's 3′-terminal OH, and FL-ddUTPs into *K. pneumoniae* primer's 3′-terminal OH. Finally, CCP was directly added into the SBE reaction system, initiating the CCP to self-assemble with these fluorescent dye-modified DNA molecules by strong electrostatic interaction. Cationic conjugated polymer, Poly[(9,9-bis(6′-*N*,*N*,*N*-tri-ethylammonium)hexyl)fluorenylene phenylene] (Figure 1), was used as energy donor of the FRET experiments. When the reaction system was excited with 380 nm UV light, the

Figure 3. Evaluation of the sensitivity and specificity of the CCP-FRET method for detecting the pathogen strains. (A−C) To evaluate the sensitivity of the CCP-FRET method, the purified DNA samples extracted from cultured *Candida albicans* 10231 (CA), *Cryptococcus neoformans* (CN), and *Klebsiella pneumoniae* (KP) were 2-fold diluted from 1 ng to 0.03125 ng. As for all the samples, PCR amplification and single base extension (SBE) were carried out and then FRET signals were excited with 380 nm UV light. The upper curves are fluorescence emission spectra in HEPES buffer solution (2.0 mM, pH 8.0). The bottom pictures are the corresponding images of CCP-FRET reactions of CA, CN, and KP in PCR tubes under 380 nm UV light irradiation. (D) To validate the specificity of the CCP-FRET method, nontarget strains, *Candida parapsilosis* (CPA), *Candida tropicalis* (CTR), and *E. coli* (Ecoli) were measured. Twenty ng of extracted DNA samples were detected. FRET fluorescence emission spectra and the images of reaction solution were captured under 380 nm UV light irradiation.
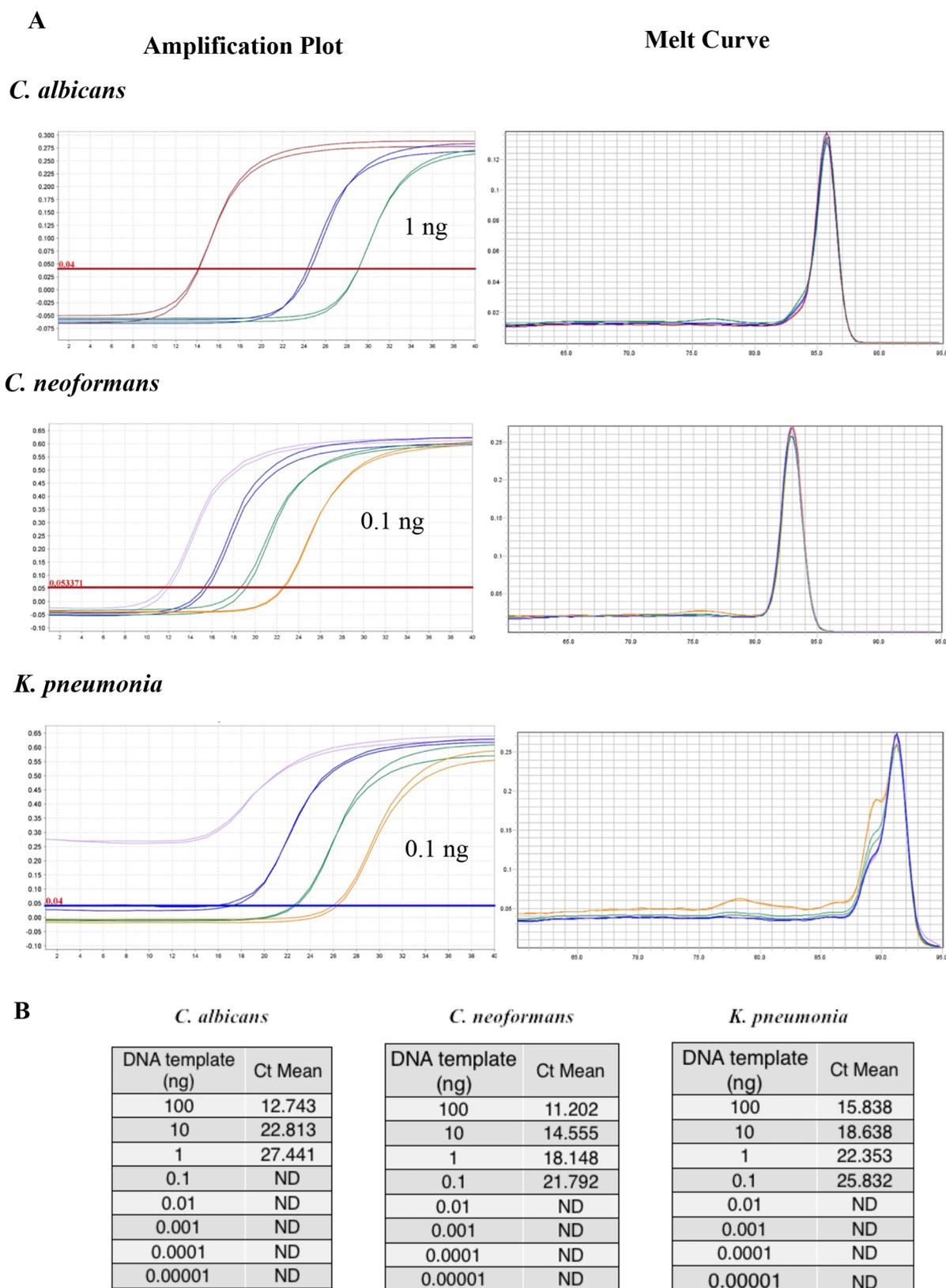
Figure 4. Comparing the sensitivity between the CCP-FRET and real-time PCR. The sensitive assay was determined by using 10-fold serial dilutions of purified DNA sample from *Candida albicans* (CA), *Cryptococcus neoformans* (CN), and *Klebsiella pneumoniae* (KP). (A) Left panel is the amplification plot and right panel is the melt curve. The lowest amount of DNA detected by CCP-FRET method was labeled in amplification plot. (B) Cycle threshold (Ct) values of real-time PCR.

pathogen strain-specific FRET signals were produced between the CCPs and fluorescent dye molecules. According to the

FRET emission spectra, three single pathogen strains and four of their combinations could be distinguished. This method also
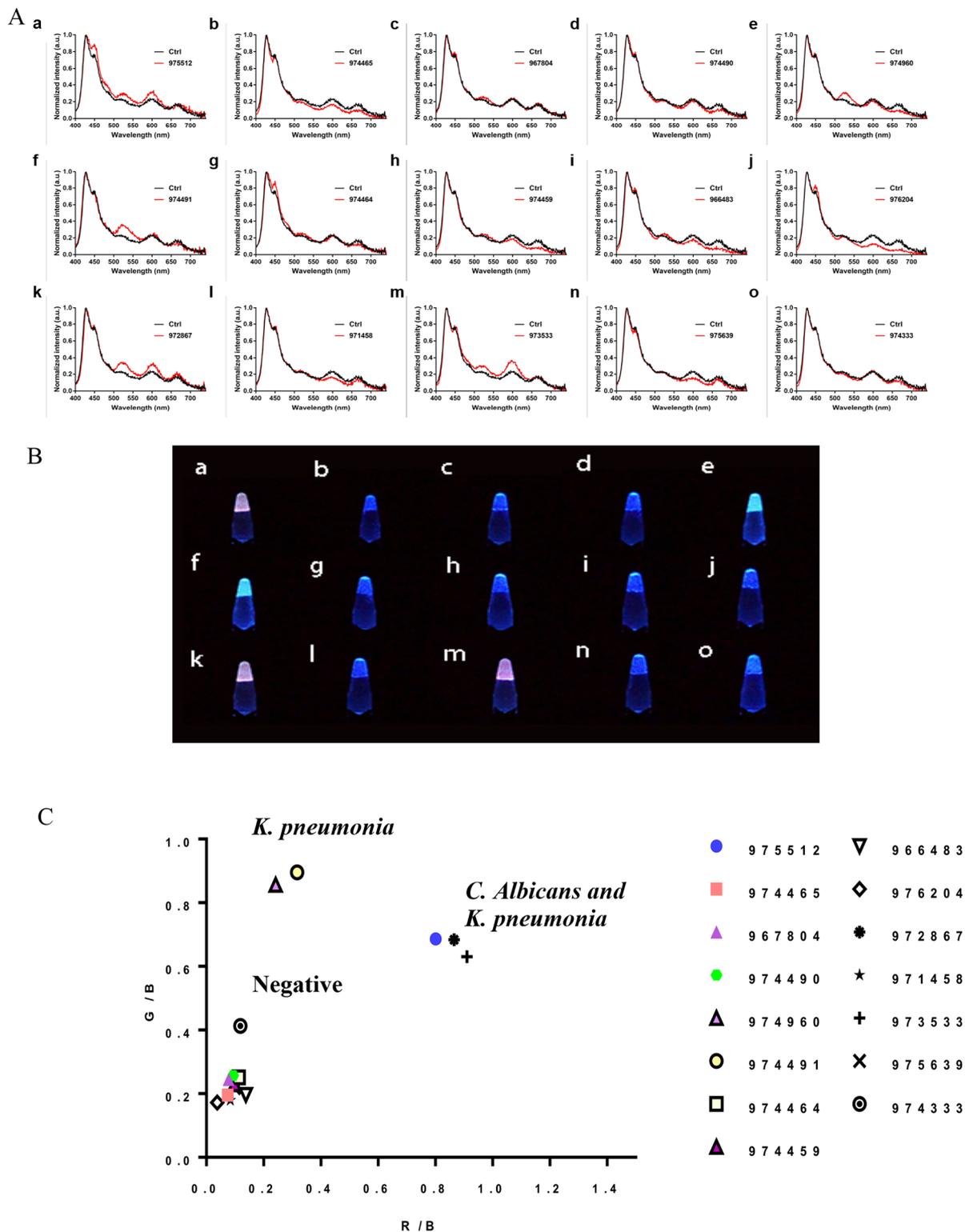
Figure 5. Using the CCP-FRET method to detect the fungal and bacterial pathogen strains in clinical blood specimens. To evaluate the efficiency of the CCP-FRET method for detecting multiple pathogen strains, 18 blood specimens (a−o) from Neonate Intensive Care Unit (NICU) were tested. The DNAs were extracted by QIAamp UCP Pathogen Mini Kit and then measured by the new method. (A) FRET fluorescence emission spectra of clinical specimens. (B) The fluorescence emission images of reaction buffer in PCR tubes under 380 nm UV light irradiation. (C) The plot distribution of the samples by RGB values.

provided a visible and intuitive test result for single or multiple pathogen infection samples.

Using the purified genomic DNAs of *C. albicans*, *C. neoformans*, and *K. pneumoniae* as the template, we successfully established a CCP-FRET method (Figure 2). For the blank control, the emission maximum of CCP itself in HEPES buffer solution appeared at around 425 nm, and weak or no emission of ROX at 605 nm, Cy5 at 668 nm, or FI at 517 nm was

observed, and the solution exhibited royal blue (Figure 2A-a and B-a). For *C. albicans*, an efficient FRET from CCP to ROX led to a little quenching of CCP emission and an increase of ROX emission peak at 605 nm, and the reaction solution exhibited a violet color (Figure 2A-b and B-b). For *C. neoformans*, an evident emission of Cy5 was observed at 668 nm, and the solution emitted an orchid color (Figure 2A-c and B-c). For *K. pneumoniae*, an emission of FI was at 517 nm and emitted light sky-blue color (Figure 2A-d and B-d). For *C. albicans* and *C. neoformans* combination, there is increased ROX and Cy5 emission peaks at both 605 and 668 nm, and the reaction system appeared a dim gray color (Figure 2A-e and B-e). Similarly, for other types of pathogen strain combinations, the corresponding ROX, Cy5, and FI's emission peaks were detected, and the reaction systems gave specific fluorescent color changes (Figure 2A-f−h and B-f−h). Although more than one pathogen strain simultaneously produced signals in one reaction system, the emission pattern of each dye was the same as the single one and nearly not influenced in the mix system because the fluorescence emission of FRET was independently produced in a compact CCP self-assembly system.

To evaluate the sensitivity, we detected a series of 2-fold-diluted pathogen DNA samples ranging from 1 ng to 0.03125 ng by the CCP-FRET method. The results showed that the samples of as little as <0.03125 ng of pathogen DNA were able to be detected (Figure 3A−C). The specific FRET signal and fluorescent color change of reaction buffer were observed, and they were not influenced by DNA content in reaction system.

Furthermore, the specificity was validated by detecting other nontarget strains with this system. As shown in Figure 3D, for *Candida parapsilosis* (*C. parapsilosis*), *Candida tropicalis* (*C. tropicalis*), and *Escherichia coli* (*E. coli*), the CCP-FRET system did not yield any specific FRET signals and fluorescent color change (Figure 3D) compared with blank control. In the system, considering the PCR and SEB primers were the most important factors to obtain higher sensitivity and specificity, we designed, screened, and identified all the primers that did not produce any nonspecific PCR products and dimers (Table S1 and Figure S1). Only one specific peak was detected for each qPCR product in the melting curve analysis, which confirms the specificity of PCR reaction and primers (Figure 4). The primer specificity was also validated by running the PCR products electrophoresis on 2% agarose gel, and there was a single specific band (Figure S1).

Real-time PCR has widely been employed in various clinical laboratory tests due to higher sensitivity. To compare the sensitivity of the CCP-FRET method with that of real-time PCR, the genomic DNAs from *C. albicans*, *C. neoformans*, and *K. pneumoniae* were also serially diluted and then detected by real-time PCR. The same PCR primers and reaction conditions were used in the two methods in order to exclude the potential influence factors for PCR efficiency. The lowest amount of DNA detectable by the CCP-FRET method is down to 0.03125 for *C. albicans*, *C. neoformans*, and *K. pneumoniae* (Figure 3A−C), whereas by the real-time PCR it is 1, 0.1, and 0.1 ng (Figure 4), respectively, indicating the CCP-FRET method has a higher sensitivity than real-time PCR.

To evaluate the efficiency of the CCP-FRET method for detecting fungus in clinical specimens, we performed a double-blind test and employed the new method to identify and distinguish the types of pathogen infections from 15 clinical neonate blood samples from the Neonatal Intensive Care Unit.

We used a highly efficient kit to extract the total DNA from 200 μL of neonate patient blood and obtained a mix DNA sample containing the very small amount of pathogen DNA and mostly blood cell DNA. The spectrophotometric analysis showed the extracted DNA samples have high quality (Table S2). The CCP-FRET method successfully identified five samples with *K. pneumoniae* infection within 3 h with a 100% accurate rate (Figure 5, a, e, f, k, and m), which is consistent with the results of clinical blood culture. Among three *C. albicans* positive samples (Figure 5A-a, k, and m), two samples were *C. albicans* positive by the CCP-FRET method, which is validated by later blood cultures. One was not validated by repeat cultures, and it is speculated that the *C. albicans* number in patient blood was too few to detect by conventional blood culture methods after the patient was treated with antifungal medicine. Notably, these three *C. albicans* positive samples were dual infections of *K. pneumoniae* and *C. albicans*, indicating our new method could identify multiplex pathogen infection only via one reaction test in real patient samples. To assess the detection efficiency for *C. neoformans*, it still required to collect *C. neoformans* positive blood specimen.

Our data demonstrated the imaging effect of the CCP-FRET method. The RGB values are directly obtained and analyzed by Adobe Photoshop CS 6.0 software, by which the samples containing different pathogen DNAs could be distinguished. Compared to the pathogen negative sample, the positive samples show a higher G/B or R/B ratio, which is consistent with the fluorescence emission pattern of FRET reaction system. As expected, the two single *K. pneumoniae* infection samples showed similar fluorescent color (Figure 5B-e and f). The three patient samples of dual infection of *K. pneumoniae* and *C. albicans* also appeared to have identical fluorescent color changes (Figure 5B-a, k, and m) and RGB patterns (Figure 5C) that were obviously distinguished from other noninfection and single *K. pneumoniae* infection ones. These samples were also tested by real time PCR, and the results showed that pathogen DNA were not detected according to the amplification plot and melt curve (Figure S2).

These findings suggested that the CCP-FRET method might be applied in detection of real neonatal blood specimens with higher sensitivity, even suitable for samples from the patients who were treated with antibacterials and antifungals. Meanwhile, it provides a rapid, visible, and intuitional result to help get accurate laboratory diagnoses.

We established a rapid, simple, visible, and highly sensitive CCP-FRET method for detecting and distinguishing fungal and bacterial pathogens in infected patient samples. Our findings demonstrated that the CCP-FRET method could reliably detect and distinguish the bacterial and fungal pathogens with a higher sensitivity of 0.03125 ng DNA. The method is even suitable for the detection of blood specimens, in which the pathogen DNA is very low, contaminated with a large amount of blood cell DNA. In our study, although the neonatal patients received antifungal treatment, this method still could detect the pathogen DNA without requiring additional cultures, purification, or PCR amplification steps.

Compared with the real time PCR, the CCP-FRET method has the features of higher sensitivity and multiple detection. In addition, The CCP-FRET method also provides a visible image with RGB values corresponding to a specific single strain or combinations. Despite a weak *C. albicans* FRET signal in real samples, the reaction buffer appeared with an obvious

fluorescent color change, indicating the color change of reaction buffer was more sensitive. Moreover, we found that the fluorescent color reaction was more stable and kept a longer time than a single conjugate polymer or fluorescent dye. The colors were not bleached even when exposed to UV for more than 10 min (data not shown).

In addition, the CCP-FRET method saves time and money, which makes it more suitable for clinical scale screening. The entire operation can be completed within 3 h and contains three simple steps.

Most importantly, the CCP-FRET system could continue to be developed to distinguish different *Candida* species. As immunocompromised patients in the ICU are often infected by other *Candida app* species, such as *C. glabrata* and *C. krusei*, that show obviously different responses to antifungal medicines, it is necessary to identify these species during the antifungal treatment. By using a *Candida* species-specific primer and fluorescent dye-modified specific ddNTP, the CCP-FRET system might be feasible to differentiate various *Candida* species. Our data showed the CCP-FRET method worked better to detect clinical culture pathogens (Figure S3). There is increasing evidence that some genes have been reported to be associated with *Candida* resistance to drugs, for example, mutations in ERG11.[20] Therefore, it might open a promising avenue to screen and identify pathogen species and their relationship with drug sensitivity.

■ METHODS

The patient blood samples were obtained from Neonatal Intensive Care Unit of Bayi Children's Hospital and had undergone blood culture test. Some of the patients with exposure to polymyxin B or fungizone B were included. The study was approved by the local research ethics committee.

The pathogen strains, *C. albicans* 10231, *K. pneumoniae*, and *C. neoformans* (*Cryptococcus neoformans var. neoformans JEC21*), were from the Institute of Microbiology Chinese Academy of Sciences and Microbiology Department School of Life Sciences of Beijing Normal University.

Pathogen Strain Culture. Reference (*C. albicans* 10231 and *Cryptococcus neoformans var. neoformans JEC21*) and isolated fungal strains were routinely grown on yeast extract peptone dextrose medium, YPD (1% yeast extract, 2% peptone, 2% dextrose, 1.5% agar) at 30 °C for 2−3 days. *K. pneumoniae* was cultured with Luria−Bertani (LB) solid medium (1% tryptone, 0.5% yeast extract, 1% NaCl, and 1.5% agar) at 37 °C overnight.

Pathogen Strain DNA Extraction. The TaKaRa MiniBEST Universal Genomic DNA Extraction Kit (TaKaRa, Japan) was used to extract the total genomic DNA from reference strains and clinical isolates including *C. albicans*, *C. neoformans*, and *K. pneumoniae*.

To get the minute amounts of pathogen DNA from 200 to 400 μL of neonatal blood, pathogen DNAs were extracted using QIAamp UCP Pathogen Mini Kit (QIAGEN, Germany) in accordance with the supplier's instructions. Briefly, fresh neonatal blood was treated with 40 μL of Proteinase K at 56 °C for 10 min and then 200 μL of Buffer APL2 and ethanol were added to the sample to precipitate genomic DNA (containing blood cell DNA and pathogen DNA). The sample mixture was passed through the Qiagen spin mini column to bind and collect the total DNA. Finally, the column with genomic DNA was twice washed with 500 μL of APW1 and APW2 buffer. The bound DNA on the column was eluted with 20 μL of AVE buffer by centrifugation at 8000g for 1 min. The extracted DNA concentration and quality were evaluated by absorbance values at 260 and 280 nm using Nanodrop Spectrophotometers (Thermo Fisher Scientific, ND 2000).

An Optimized CCP-FRET Method for Recognizing and Distinguishing Multiple Pathogens. A new CCP-FRET method was established and optimized to recognize and distinguish fungal and bacterial pathogen strains. First, pathogen-specific DNA fragment from *C. albicans*, *C. neoformans*, and *K. pneumoniae* was amplified by regular PCR. The pathogen primers target the rDNA and internal transcribed spaces that are conservative and specific to the corresponding strain (Table S2). The PCR was performed with 25 μL of the reaction system comprised of a 2 μL series of diluted DNA, 0.4 mM of each dNTP, 1× HS Taq buffer, 0.25 unit HS Taq DNA polymerase (Takara Bio, Japan), and 0.4 μM primers DNA. PCR conditions were as follows: 95 °C for 4 min, followed by 40 cycles at 95 °C for 20 s and 58 °C for 30 s, 72 °C for 20 s, and a final extension at 72 °C for 10 min. Then, 8 μL PCR products were taken and treated with 1 unit Shrimp Alkaline Phosphatase (SAP) (Takara Bio, Japan), 10 units of exonuclease I (Takara Bio, Japan), and 0.05 units of pyrophosphatase (NEB) at 37 °C for 1 h to remove excess primers, dNTPs, and pyrophosphate generated in PCR. SBE was performed in a total volume of 10 μL containing dye labeled ddNTP mix (1 μM ROX-ddATP, 2 μM Fl-ddUTP, 2 μM Cy3-ddCTP) (Perkin Elmer, USA), 1 μM of each extension primer (Table S1), 1 unit of thermosequenase DNA polymerase (GE, USA), and 3.4 μL exonuclease/SAP/pyrophosphatase-treated multiplex PCR products. Extension reactions were performed in a thermal cycler (Bio-RAD, T100) and the conditions were 94 °C for 2 min, followed by 60 cycles of 94 °C for 30 s and 60 °C for 30 s.

Finally, to initiate the self-assembly and FRET reaction, 6 μL of SBE products were diluted with 586 μL of 25 mM HEPES, pH 8.0, and then 8 μL of 15 mM CCP was added to the solution. The FRET reaction fluorescence signals were acquired at an excitation wavelength of 380 nm with FS5 fluorescence spectrometer (Edinburgh Instruments, UK) with an excitation wavelength of 380 nm.

The Image of Reaction System of CCP-FRET. The images of CCP-FRET reaction buffer were captured under the UV excitation by Nikon digital photograph. The RGB signals were analyzed with PS software (Adobe Photoshop CS6).

Real-Time PCR Method for Pathogen Detection. Real-time PCR was carried out to compare the sensitivity with that of CCP-FRET method. The TB Green Premix Ex Taq (TaKaRa, Japan) was used to detect 2-fold serial dilutions of pathogen DNAs. The sequences of the primers were the same as the CCP-FRET system (Table S1). The primers were diluted in the master mix buffer to 0.4 μM (each primer). The reactions were carried out in a fluorescence quantitation PCR machine (ABI Quant Studio 6 Flex). The pathogen DNA was first pre-denatured at 95 °C for 30 s before DNA amplification. The second step conditions consisted of denaturation at 94 °C for 5 s, annealing and extension at 60 °C for 20 s for 40 cycles. The third step was 94 °C for 15 s, 60 °C for 60 s, and 94 °C for 15 s for collecting melt curve data.

■ ASSOCIATED CONTENT

ⓢ Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsinfecdis.1c00393.

The primer sequences for PCR and single base extension (SBE) of CCP-FRET method (Table S1); The quality and quantity of the extracted DNA from neonate patient blood (Table S2); Validation of the specificity of primers in the multiple pathogen-detecting CCP-FRET system (Figure S1); Real-time PCR testing the pathogen DNA molecules extracted from the patient specimens (Figure S2); Using the CCP-FRET method to test pathogen strains isolated from the culture plates of neonate patient blood (Figure S3) (PDF)

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Author Contributions

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

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