A probe-directed recombinase amplification assay for detection of *MTHFR* A1298C polymorphism associated with congenital heart disease

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Single nucleotide polymorphisms (SNPs) play an important role in susceptibility to complex diseases, treatment efficacy and adverse drug responses. Conventional methods to detect SNPs are usually based on PCR or DNA sequencing, which are typically time-consuming and require sophisticated equipment. In this proof-of-concept study, a probe-directed recombinase amplification (PDRA) assay was developed to detect the A1298C polymorphism of 5,10-methylenetetrahydrofolate reductase (*MTHFR*). The PDRA assay included two real-time reactions to detect the A and C nucleotides of A1298C polymorphism. Each reaction contained only one primer and one probe and was finished at 39°C within 35 min. The results of genotyping of 150 clinical samples using PDRA were completely consistent with those by direct sequencing. Additionally, when the 1000 Genomes Project HCB frequencies were used as the control group, *MTHFR* A1298C was found to be associated with congenital heart disease. In conclusion, the proposed novel PDRA assay is a valuable tool for the detection of SNPs and demonstrates significant potential to be widely applicable in both research and clinical settings.

With the completion of the human genome project and the international haplotype map project, single nucleotide polymorphisms (SNPs) have become very useful markers to examine variability between individuals in susceptibility to complex diseases, treatment efficacy and adverse drug responses [1–3]. This in turn enables clinicians and healthcare workers to take appropriate actions. A variety of methods have been adopted for the genotyping of SNPs. Conventional methods include real-time PCR, DNA sequencing, restriction fragment length polymorphism (RFLP) analysis and amplification refractory mutation system PCR (ARMS-PCR) [4–7]. However, these methods are time-consuming, laborious and require sophisticated equipment.

Recently, isothermal DNA amplification technologies that run at a constant temperature have gained interest due to their excellent compatibility with point-of-care (POC) assays [8–10]. Among them, loop-mediated isothermal amplification (LAMP) was most commonly used in SNP detection [11–13]; however, the complex LAMP primer design and the requirement for high temperature (65ºC) have limited its application. A few studies have reported that the recombinase polymerase amplification (RPA) was able to detect point mutations of a tumor gene by the use of intercalating dye, internal control and peptide nucleic acid [14,15], but have shown limited specificity and sensitivity. Recombinase-aided amplification (RAA) is a novel isothermal amplification and detection assay, utilizing specific enzymes and protein for rapid detection of nucleic acids at 39ºC in less than 30 min.The RAA assay depends on three major proteins: single-strand DNA-binding protein (SSB),

METHOD SUMMARY

We describe a novel PDRA assay for rapid isothermal detection of *MTHFR* A1298C polymorphism with two reactions. Each reaction contained only one primer and one probe. The results could be obtained at 39°C within 35 min by measuring fluorescence without opening the sample tubes, minimizing concerns on contamination of amplified products. The results of genotyping of 150 clinical samples using PDRA were completely consistent with those by direct sequencing.

recombinase UvsX extracted from *Escherichia coli* and DNA polymerase. RAA uses UvsX to pair the specific primers with their homologous sequence in the template DNA and SSB to form a D-loop structure to prevent immediate formation of double-stranded DNA, while DNA polymerase is for amplification and extension. RAA can also use a fluorescent probe system for real-time detection of DNA or RNA amplicons [16,17]. It has been reported to detect viral and bacterial pathogens with its high specificity and sensitivity [16–18], but no study has reported on the detection of SNPs.

MTHFR is a critical enzyme in the folate metabolism pathway, which converts 5,10-methylenetetrahydrofolate acid into 5-methyltetrahydrofolate, and is also of key importance for homocysteine metabolism, supplying methyl groups for methylation reactions catalyzed by methionine synthase (MTR) [19]. Several polymorphisms in the *MTHFR* gene have been identified. Among them, A1298C (rs1801131) has been extensively studied [20–23]. The A1298C polymorphism is located in exon 7 of the *MTHFR* gene, with an A to C transition at base pair 1298 leading to a glutamate to alanine substitution. A1298C substitution results in a thermos-labile form of *MTHFR*, affecting DNA synthesis, genome stability and maintenance of appropriate homocysteine level in the blood [22,24]. In addition, many studies suggest this polymorphism plays an important role in the etiology of various diseases, such as ischemic stroke, neural tube defects and congenital heart disease (CHD) [20,21,25,26].

In this proof-of-concept study using *MTHFR* as an example, we developed a novel probe-directed recombinase amplification (PDRA) assay for rapid and specific real-time detection of A1298C polymorphism.

Materials & methods

Clinical sample collection

A total of 150 children with CHD hospitalized in the Department of Cardiac Surgery, Children's Hospital of Hebei, China, were recruited from January 2017 to October 2017. A total volume of 2 mL venous blood samples were collected and stored at -20°C. This study was approved by the Institutional Review Boards of National Institute for Viral Disease Control and Prevention, Center for Disease Control and Prevention of China, and all aspects of the study were performed in accordance with national ethics regulations. The written informed consent form was obtained from children's parents or caregivers before participating in this study.

DNA extraction

Genomic DNA was extracted from 200-µl venous blood samples by use of the TIANamp Blood DNA kit (Tiangen, Beijing, China) according to the manufacturer's instructions. The DNA was eluted in 150 µl of nuclease-free water and stored at -20°C until used. Extracted DNA samples had a final concentration ranging from 10–75 ng/µl in general.

Direct sequencing of clinical samples

Detection of *MTHFR* A1298C polymorphism of 150 clinical samples was performed by PCR amplification with a ExTaq Hot Start Version kit (TaKaRa, Dalian, China), followed by direct DNA sequencing. The primers for amplification of the *MTHFR* gene region are listed in Table 1. The 25-µl PCR mixtures contained 2 µl of genomic DNA, 1 µl of each primer (10 µM), 2.5 µl of buffer, 2 µl of dNTP, 0.125 µl of Ex Taq and 16.375 µl of ddH_2O. All samples were amplified under the same thermal cycling conditions: the initial denaturation step at 95°C for 3 min, followed by 50 cycles at 95°C for 15 s, 60°C for 45 s, and finally kept at 4°C. The PCR products were then sent to Sangon Biotech (Shanghai, China) for sequencing.

Design of primer & probes for the PDRA assay

The PDRA assay included two real-time RAA reactions (A and C) to detect the A and C nucleotides of the A1298C polymorphism, respectively. Each PDRA reaction simply contained one primer and one probe. The forward primers of the two reactions were the same, and the probes of two reactions had only one nucleotide difference in the site of polymorphism. The flanking sequence of the *MTHFR* A1298C polymorphism was obtained from the dbSNP database of the National Center for Biotechnology Information (NCBI). One forward primer and two specific reverse probes for the A and C nucleotides of the A1298C polymorphism were designed with the aid of Oligo7 software. The primer is generally 30–35bp. The probe is typically 46–52 nucleotides (nt) long, at least 30 nt of which are placed at the 5' end next to an internal abasic site mimic (tetrahydrofuran [THF]) site flanked by a dT-fluorophore and a corresponding dT-quencher group, and at least 15 nt are located at the 3' end. Meanwhile, the mutation site was designed to be located just before the THF site of each probe (Figure 1). The primer and the probe sequences of the PDRA assay are listed in Table 2. All of the primer and probes were synthesized and purified by high-performance liquid chromatography (HPLC) by Sangon Biotech (Shanghai, China).

PDRA assay of clinical samples

PDRA assay was performed using RAA kits with Exo (Qitian, Jiangsu, China) with some modifications. Both A and C reactions were carried out in a 50 µl volume of a 0.2 mL freeze-dried reaction tube containing a dried enzyme pellet (SSB, UvsX, DNA polymerase, Exonuclease III), 25 µl of rehydration buffer, 17 µl of ddH_2O, 2.1 µl of forward primer (10 µM), 1.4 µl of A or C nucleotide-specific PDRA probe (10 µM), 2 µl of DNA template, and 2.5 µl magnesium acetate (280 mM). The tubes were then transferred to a tube holder on a RAA fluorescence detection device QT-RAA-F7200 (Qitian, Jiangsu, China) and incubated at 39ºC for 35 min. A total of 150 clinical specimens were tested using the PDRA assay (both A and C reactions). A negative control (nuclease-free water) was included in each run.

Interpretation of the PDRA results

For either the A or C reaction, a fluorescence unit of 500 within 35 min was defined as the cut-off value for a positive result. Thus, when both the A and C reactions are positive, the genotype of the specimen is designated as heterozygosity (AC). Alternatively, if only one reaction is positive, the genotype is judged as AA or CC homozygotes accordingly.

Results & discussion

Unlike the basic RAA assay and real-time RAA assay, the proposed PDRA assay for A1298C polymorphism of *MTHFR* contains two individual reactions (A and C). Each reaction has only one probe and one primer. The probe not only serves as a probe, but also a reverse primer; the mutation base (SNP) is designed to be located just before the THF site of the probe. Combined with a forward primer and the use of a real-time RAA kit, the PDRA assay works only when

Table 1. The primers of the *MTHFR* gene for PCR and sequencing.

Primer	Sequence (5′ to 3′)	Size (bp)
Forward primer	CCTTGTCTCAATTCTCTGTCCC	729
Reverse primer	AGCCTGTCTTTGCCTCCCT	

the target SNP of the template is exactly matched to the corresponding base in the designed probe. The fluorophore is released and the fluorescence signal is accumulated via Exonuclease III cleaving at the THF site.

Real-time RAA requires the use of two primers (forward and reverse) and one probe (in the middle) and allows real-time detection. In our preliminary study, we have undertaken real-time RAA by introducing mismatches into the 3′ end of the forward or reverse primer according to the principle of allele-specific PCR. However, this strategy is not suitable for SNP detection because Exonuclease III, one of the components in real-time RAA kits, is able to cut from the 3′ end of double DNA not only the probe that binds to the template, but also the primers binding to the template; thus, the mismatches designed at the forward or reverse primer can also be cut off, resulting in failure to distinguish SNP. In the case of the PDRA assay, as Exonuclease III stops its function at the THF site, when the mutation base (SNP) is designed to be located just before the THF site of the probe, the THF site can protect mutation bases from being cut off, while the remaining >30 bases of the probe can act as a primer for extension. The results from the clinical samples in this study prove the feasibility of the principle of the PDRA assay.

Several requirements have to be met to perform a successful PDRA assay, as seen in Figure 1. First, secondary structures causing probes to fold back should be avoided. Second, template DNA should have a certain region where two T residues are replaced by a dT-fluorophore residue and a dT-quencher residue of the probe, respectively, and one base between two T residues is replaced by the THF residue. Furthermore, the distance between fluorophore and quencher is required to be no more than five bases. In addition, the PDRA assay also requires the mutation base located before the THF site. This requires the presence of a T base before and after the mutation site. However, when the A base is present in the template DNA before and after the mutation site, the probe can also be designed as a reverse primer, as was done in this experiment.

A total of 150 samples were typed with both PDRA assay and direct sequencing to assess the accuracy and efficiency of the assay. The genotypes scored from PDRA assay were completely consistent with direct sequencing. No cross-reaction was observed in both the A and C reactions except for detection failure of both the A and C reactions in two specimens with very low concentrations (<10 ng/μl). The accuracy of the PDRA assay for three samples with different genotypes was confirmed by direct sequencing (Figure 2).

Statistical analyses were performed using IBM SPSS Statistics, version 21 (IBM Corporation, NY, USA), and significance levels were set at 0.05. In patients with CHD, 78(52%) were female and 72 (48%) were male. Ages ranged from 1 month to 14 years old. The genotype frequencies of the *MTHFR* A1298C polymorphism were as follows: AA, n = 119 (79.33%); AC, n = 29 (19.33%); CC, n = 2 (1.34%). In addition, the genotype frequencies of *MTHFR* A1298C were in Hardy–Weinberg Equilibrium (χ^2 = 0.02; p = 0.877), suggesting the genetic allele and genotype frequencies are constant in the population. Taking 1000 Genomes Project HCB frequencies as the control group in this study, the resulting odds ratio (OR) for patients carrying allele C compared with the controls was 0.43 (95% CI: 0.27–0.68; p < 0.001), suggesting the allele C of *MTHFR* A1298C might be a protective factor for CHD, which is consistent with a study of the central and southern populations in the USA conducted by Hobbs *et al.* [27]. Crude genetic analysis of AC and CC genotypes compared with AA genotype in CHD-affected children vs controls revealed an OR of 0.39 (95% CI: 0.22–0.69; p = 0.001) and 0.26 (95%

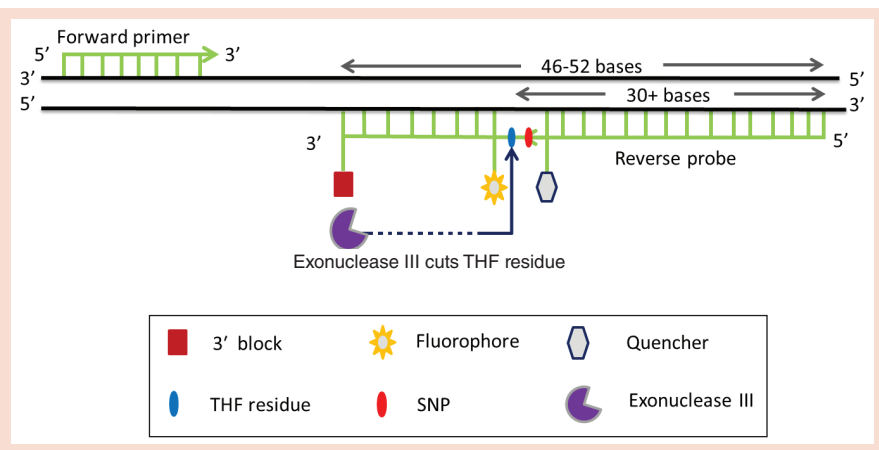

Figure 1. Design of the PDRA assay probe.
SNP: Single nucleotide polymorphism; THF: Tetrahydrofuran.

Table 2. Primer and probes used for the PDRA assay.

Primer	Sequence (5′ to 3′)
A1298C-F†	TTGGGAGCGGGAGGGCAGAAGAAGTTTGCAT
A1298C-A-P‡,¶	GTAAAGAACGAAGACTTCAAAGACACT[FAM-dT]**T**(THF)[BHQ-dT]TCACTGGTCAGCTCC[C_3-spacer]
A1298C-C-P§,¶	GTAAAGAACGAAGACTTCAAAGACACT[FAM-dT]**G**(THF)[BHQ-dT]TCACTGGTCAGCTCC[C_3-spacer]

†Forward primer for both A and C reactions of the A1298C polymorphism.
‡A nucleotide-specific reverse probe for A reaction.
§C nucleotide-specific reverse probe for C reaction.
¶For probe modifications: BHQ: Black hole quencher; C_3-spacer: 3′ phosphate blocker; FAM: 6-carboxyfluorescein; THF: Tetrahydrofuran.

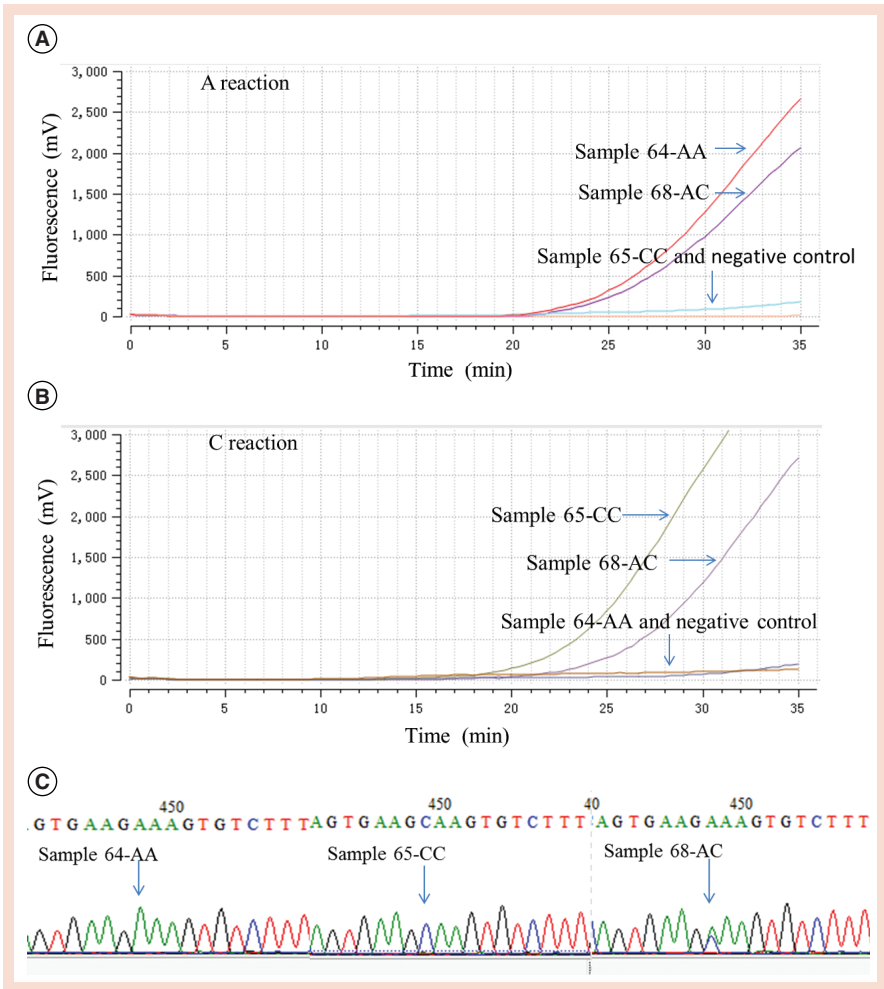

Figure 2. Comparsion of the PDRA assay and direct sequencing results of three samples with different genotypes. (A) A reaction of the PDRA assay for samples 64-AA, 65-CC and 68-AC; among them samples 64 and 68 were positive. **(B)** C reaction of the PDRA assay for samples 64-AA, 65-CC and 68-AC, among them samples 65 and 68 were positive. **(C)** Direct sequencing of samples 64-AA, 65-CC and 68-AC.

CI: 0.05–1.44; p = 0.222), respectively (Table 3). Compared with the wild AA genotype, heterozygosity AC shows a lower risk of CHD. However, there is no difference in the distribution of CC-type individuals in the two groups, due to the limitation of sample size.

To further investigate the influence of template concentration on the specificity of the PDRA assay, two recombinant plasmids containing AA and CC homozygotes of the *MTHFR* gene fragment were prepared and quantified. The specificity of the C reaction was tested by type AA recombinant plasmid, while the specificity of the A reaction was examined by type CC recombinant plasmid. No crossreaction of the PDRA assay (in triplicates) was observed using recombinant plasmids up to 10^5 copies per reaction as input templates (data not shown).

In addition, in order to make the PDRA assay even simpler, we made an initial attempt to merge the two reactions into one tube. However, the experiment failed. We suspect that the failure may be due to the competitive relationship of two probes in one tube or the strict requirements for multiple reactions. In spite of this limitation, the PDRA assay is still very convenient and simple to perform. Compared with the conventional methods capable of identifying *MTHFR* A1298C, such as QPCR, the PDRA assay has the advantages of lower cost ($3 vs $8 per reaction), simpler design (one primer and one probe vs two primers and one probe), and shorter turnaround time (35 vs 90 min). In the era of personalized medicine, with further development, we believe the PDRA assay will be an alternative in SNP detection and point mutation diagnostics of cancer biomarkers and drug-resistant mutants.

In conclusion, we have developed a novel PDRA assay for rapid isothermal detection of SNPs and provided an alternative for the detection of point mutations when appropriate. To our knowledge, the proposed method is the first report to detect SNPs using one probe and one primer. This method is intended to fill the gaps of isothermal detection for point mutations. Future research is needed to overcome the limitations of PDRA.

Author Contributions

S.D. and X.M. wrote the paper. G.L. and L.W. conceived and designed the experiments; S.D., X.L., C.C. and T.Y. performed the experiments; F.Q., L.Z., M.Z. and Z.F. elaborated the plasma samples and contributed analysis tools.

Table 3. Genotype and allele frequencies of the *MTHFR* A1298C polymorphism in CHD patients.

Genotype/Allele	n	Frequency (%)	1000 Genomes Project HCB frequency (%)	OR	95% CI	OR (p-value)
AA	119	79.33	59.23	reference		
AC	29	19.33	36.89	0.39	0.22–0.69	0.001
CC	2	1.34	3.88	0.26	0.05–1.44	0.222
A	267	89.00	77.67	reference		
C	33	11.00	22.33	0.43	0.27–0.68	<0.001

Financial & competing interests disclosure

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No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

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