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Non-invasive prenatal testing for fetal Ss, Kidd, and CTL2 blood group prediction by multiplex digital droplet PCR

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Yufeng Wang*, Xiaoyue Chu*, Xihui Chen, Anan Yin, Yan Yao, Li Wang, Hua Xu, Fangfang Liu, Kun Chen and Yuanming Wu

Correspondence to:
Fangfang Liu
Department of Neurobiology, School of Basic Medicine, Air Force Medical University, Xi'an 710032, Shaanxi, P.R. China.
lianpipi@fmmu.edu.cn

Kun Chen
Department of Anatomy, Histology and Embryology and K.K. Leung Brain Research Centre, School of Basic Medicine, Air Force Medical University, Xi'an 710032, Shaanxi, P.R. China.
chenkun@fmmu.edu.cn

Yuanming Wu
Department of Biochemistry and Molecular Biology, School of Basic Medicine, Air Force Medical University, Xi'an 710032, Shaanxi, P.R. China.
wuym@fmmu.edu.cn

Yufeng Wang
Medical Research and Experiment Center, Medical College of Yan'an University, Yan'an University, Yan'an, P.R. China

Xiaoyue Chu
Hua Xu
Blood Group Reference Laboratory, Shaanxi Institute of Blood Transfusion, Shaanxi Blood Center, Xi'an, P.R. China

Xihui Chen
Yan Yao
Department of Biochemistry and Molecular Biology, School of Basic Medicine, Air Force Medical University, Xi'an, P.R. China

Anan Yin
Department of Neurosurgery, Xijing Institute of Clinical Neuroscience and Department of Plastic surgery, Xijing Hospital, Air Force Medical University, Xi'an, P.R. China

Abstract

Background: Some blood groups, such as S and s blood groups in the MNS blood group system, and Kidd and CTL2 blood group systems, can cause severe fetal and newborn alloimmune disorders. Non-invasive prenatal testing (NIPT) to predict fetal blood groups and knowledge of local blood group gene frequency are both important for pregnancy management decisions. Droplet digital PCR (ddPCR) has high specificity and sensitivity in detecting fetal single nucleotide variation.

Objectives: The objective is to predict fetal Ss, Kidd, and CTL2 blood groups using multiplex ddPCR. The gene frequencies of three blood groups were detected by ddPCR in northwest China.

Design: This is a prospective study.

Methods: Cell-free fetal DNA isolated from 26 healthy single pregnant women at different gestational stages was tested with QX200 Droplet Digital PCR. Results were compared with fetal genotypes. DNA samples purified from 20 blood pools containing a total of 1000 donors in northwest China were subjected to ddPCR to detect the gene frequency of three blood groups.

Results: Ss, Kidd, and CTL2 blood groups of 26 pregnant fetuses were accurately detected by multiplex ddPCR. The multiplex ddPCR results were consistent with the Sanger sequencing results of 26 fetal blood samples after birth. The gene frequencies of the three blood groups detected by ddPCR were 9.30% for S, 90.70% for s, 48.43% for Jk^a, 51.57% for Jk^b, 66.57% for HNA-3A, and 33.43% for HNA-3B.

Conclusions: It is reliable to predict fetal Ss, Kidd, and CTL2 blood groups by multiplex ddPCR. Meanwhile, we designed a simple and efficient method for inferring the gene frequency of three blood groups based on ddPCR.

Keywords: blood group, blood type incompatibility, digital droplet PCR, fetal blood testing, neonatal alloimmune disease

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Background

As a nucleic acid quantification method, digital droplet polymerase chain reaction (ddPCR) allows for absolute target quantification and has high sensitivity.[1] ddPCR directly calculates the fluorescence signal in each reaction unit and calculates it according to the Poisson distribution function. Therefore, we do not need standard curves and internal parameters to obtain accurate initial copy number concentrations of samples. In addition, ddPCR has been widely accepted in the detection of extremely small nucleic acid samples, rare mutation detection, and identification of small differences in copy number.[2,3]

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Li Wang
Department of Biochemistry and Molecular Biology, School of Basic Medicine, Air Force Medical University, Xi'an, P.R. China

School of Aerospace Medicine, Air Force Medical University, Xi'an, P.R. China

*These authors contributed equally to this work.

Moreover, ddPCR can realize multiplexing. Using probe based on EvaGreen analysis, multiple analyses can be designed to quantify multiple objectives in a single well.[4] This will result in cost savings when running a pure assay.

In recent years, ddPCR technology has been applied to non-invasive prenatal testing (NIPT), and it is particularly noteworthy that in the first trimester (4.5 weeks), all maternal plasma samples were correctly used for *SRY* gene detection with an unprecedented accuracy of 100%.[5] Moreover, non-invasive prenatal detection of ABO and Rh blood groups by the ddPCR method has been established.[6] However, there have been no studies on multiplex ddPCR testing for NIPT for blood group point mutations. It is urgent to establish a method for NIPT of blood groups based on multiplex ddPCR.

A person's blood group is determined by the types of antigens on the surface of blood cells.[7] Blood group incompatibility between the fetus and the mother can lead to fetal and neonatal alloimmune disease.[8] The cause of fetal and neonatal alloimmune disease is usually an ABO or Rh incompatibility between the maternal and fetal blood group.[9,10] However, some other blood groups, such as the S and s blood groups in the MNS blood group system, Kidd (Jk) blood group system, and CTL2 blood group system, can also cause alloimmune disorders of fetuses and neonates. These blood groups are divided according to different blood group antigens occurring due to point mutations. Some antibodies including anti-S and anti-s may cause hemolytic disease of the fetus and newborn (HDFN).[11,12] Anti-Jk^a and anti-Jk^b are also responsible for HDFN. Among the blood antigen–induced HDFN in Chinese women, anti-Jk^a and anti-Jk^b are responsible for 0.51% of cases.[13] The antigens of the CTL2 blood group system, HNA-3A and HNA-3B, are located on the surface of neutrophils. Blood group incompatibility between the maternal and fetal blood by the CTL2 blood group system accounts for neonatal alloimmune neutropenia.[14] Moreover, these three blood groups can cause serious fetal and neonatal alloimmune disease.[15–17] Therefore, we need to establish an early NIPT of fetal Ss, Kidd, and CTL2 blood groups for pregnancy management.

In addition, because ddPCR can accurately quantify characteristics, we established a more convenient and efficient method to detect the gene frequency of three blood groups in 1000 people in Northwest China. On the one hand, fetal or neonatal alloimmune disorders caused by anti-Jk^a, anti-Jk^b, and anti-S can result in fetal or neonatal death, and the only treatment is systemic blood exchange.[18,19] Therefore, it is important to know the frequency distribution of local blood groups, which will guide the preparation of blood in the event of alloimmune disorders of fetuses and neonates. On the other hand, the number of samples in the blood bank is huge. Mastering a simple, rapid, and efficient method to detect the distribution frequency of blood group genes is beneficial to the storage and management of blood samples and to provide better medical services for blood banks.

In conclusion, we attempted to establish a multiplex ddPCR method to predict early fetal S and s blood groups in the MNS blood group system, and Kidd, and CTL2 blood group systems. In addition, a convenient and efficient method for gene frequency calculation was established with the characteristic of quantitative ddPCR.

Methods

Donor population recruitment

The study recruited 26 pregnant women with healthy singleton pregnancies at various stages of pregnancy and 1000 blood donors mainly from northwest China. Tubes containing the anticoagulant ethylenediaminetetraacetic acid were used to collect 10 ml of venous blood from pregnant women and 2 ml of venous blood from donors. After birth, 200 µl of blood was collected from the newborn.

Extraction of blood samples

Genomic DNA was extracted from each blood sample using the Ezup Column Blood Genomic DNA Purification Kit (Bioengineering) according to the manufacturer's instructions.

Digital droplet PCR workflow and assay composition

Primers and probes used for blood group detection by ddPCR are shown in Table 1. The reaction mix was as follows: 10 µl of ddPCR supermix for probes (Bio Rad, USA), 0.6 µl of primers (10

Table 1. Primers and probes used for blood group genotyping.

Antigen	Primers and probs	Sequences
S/s	Ss FP	5′-TGCACATGTCTTTCTTATTTGGACTTACA-3′
	Ss RP	5′-CCTGGTACAGTGAAACGATGG-3′
	S	5′6-FAM-TTGTCCCATTTCTC-3′MGB
	s	5′6-VIC-TTGTCCCGTTTCTC -3′MGB
JKa/JKb	JK FP	5′-GAGTTCTGACCCCTCCTGTCTTA-3′
	JK RP	5′-TGAGCGCCATGAACATTCC-3′
	JKa	5′6-FAM-CATTTGAGGACATCTACT-3′MGB
	JKb	5′6-VIC-CCATTTGAGAACATCTACT -3′MGB
HNA-3A/HNA-3B	HNA-3 FP	5′-TCCCTCCATCTCTCTTGGCAG-3′
	HNA-3RP	5′-ATCCCCTGACTCACAGGGTTTG-3′
	HNA-3A	5′6-FAM-TTCGAGATGGTGACTGCCCTGCT-3′MGB
	HNA-3B	5′6-VIC-TTCAAGATGGTGACTGCCCTGCT-3′MGB
Control genes		
SRY	*SRY* FP	5′-CTGCTATGTTAAGCGTATTC-3′
	SRY RP	5′-CACACTGATACTTAGAGTTAC-3′
	SRY	5′6-VIC-ACAGCGATGATTACAGTCCAGC-3′MGB
CCR5	*CCR5* FP	5′-TACCTGCTCAACCTGGCCAT-3′
	CCR5 RP	5′-TTCCAA AGTCCCACTGGGC-3′
	CCR5	5′6-FAM-TTTCCTTCTTACTGTCCCCTTCTGGGCTC-3′MGB
RASSF1	*RASSF1* FP	5′-AGCCTGAGCTCATTGAGCTG-3′
	RASSF1 RP	5′-ACCAGCTGCCGTGTGG-3′
	RASSF1	5′6-FAM-CCAACGCGCTGCGCAT-3′MGB
ACTB	*ACTB* FP	5′-GCGCCGTTCCGAAAGTT-3′
	ACTB RP	5′-CGGCGGATCGGCAAA-3′
	ACTB	5′6-VIC-ACCGCCGAGACCGCGTC-3′′MGB

μM), 0.2 μl of probes (10 μM) (Sangon Biotech), 8.2 μl of ddH_2O, and 1 μl of DNA (10 ng/μL), reaching a final reaction volume of 20 μL.

Different primer and probe combinations were used to develop the simplex, duplex, triplex probe mix, and quadruplex assays.

Cell-free DNA extraction from pregnant women blood plasma

First, 10 ml of fresh blood was extracted from pregnant women and mixed gently upside down, and then immediately centrifuged at 1600*g* for 10 min at 4°C. The supernatant was separated and centrifuged again at 16,000*g* for 10 min at

Table 2. Primers for blood group sequencing.

Blood group	Primers	Sequences
Ss blood group	Ss ID FP	5′-CTGGGAGGGATGTGGGAGAA-3′
	Ss ID RP	5′-ACAAAGGTTAATTGGGGCTTGC-3′
Kidd blood group	JK ID FP	5′-ATTAACCCTCACTAAAGGGA-3′
	JK ID RP	5′-ATACGACTCACTATAGGG-3′
CTL2 blood group	CTL2 ID FP	5′-CACGTACCTGAATGCTCGC-3′
	CTL2 ID RP	5′-GAGCAGAGGATGGCACCAGT-3′

4°C. After the precipitate was removed, the supernatant was stored at –20°C for a short time and –80°C for a long time until required for extraction. Cell-free DNA was extracted from plasma (4 ml) using a QIAamp Circulating Nucleic acid kit according to the manufacturer''s protocol (Qiagen, USA). The final elution sample volume was 30 µl.

Testing circulating free DNA by ddPCR

The sample volume in the reaction system was increased to 8 µl and the corresponding water content was reduced. The total reaction volume was 20 µl. The procedure of ddPCR amplification was that a thermal cycle with a warming rate of 2°C/s per step is set to run at 95°C for 10 min; then denatured at 94°C for 30 s, annealed and extended at 60°C for 1 minute, 40 cycles; enzyme inactivation at 98°C for 10 min; and finally hold step at 4°C.

Fetal blood group identification

Genomic DNA was extracted from each blood sample using the Ezup Column Blood Genomic DNA Purification Kit (Bioengineering) according to the manufacturer's instructions. Primers used to identify blood groups are shown in Table 2.

Blood pool organization for detecting population gene frequency

The blood samples of the 1000 donors were mixed into 20 pools of 50 samples and DNA was extracted according to the above methods and detected by ddPCR. Then, we directly tested the unknown blood group samples and calculated the gene frequency. In a blood pool of 50 donors, the ratio of alleles was calculated using two-dimensional (2D) ddPCR assay. The heredity of three blood groups conforms to Hardweinberg's law of equilibrium[20–22] We used a population gene frequency to caculate the population genotype frequency: $(p + q)^2 = p^2 + 2pq + q^2 = 1$, where p is the gene frequency of A, q is the gene frequency of a, p^2 is the genotype frequency of genotype AA, q^2 is the genotype frequency of genotype aa, and 2pq is the genotype frequency of genotype Aa. We pooled the genotype frequencies in each blood pool. Finally, the genotype frequencies of 1000 donors in 20 blood pools were calculated.

Data analysis

According to the previous studies, the cut-off point for all ddPCR assay was ⩾10,000 drops. If the droplet number was low, the experimental results were discarded or repeated.[4]

All ddPCR data were generated using Bio Rad's Quanta Soft™ software 1.7.4.0197. The single and double analysis experiments were analyzed using the same software. However, for the three-set probe mix and four-set analysis, the analysis was performed using Quanta Soft™ Analysis Pro software version 1.0.596. The reporting of this study conforms to the STARD statement.[23]

The 95% confidence level was set and the confidence interval was calculated using SPSS 26.0 software.

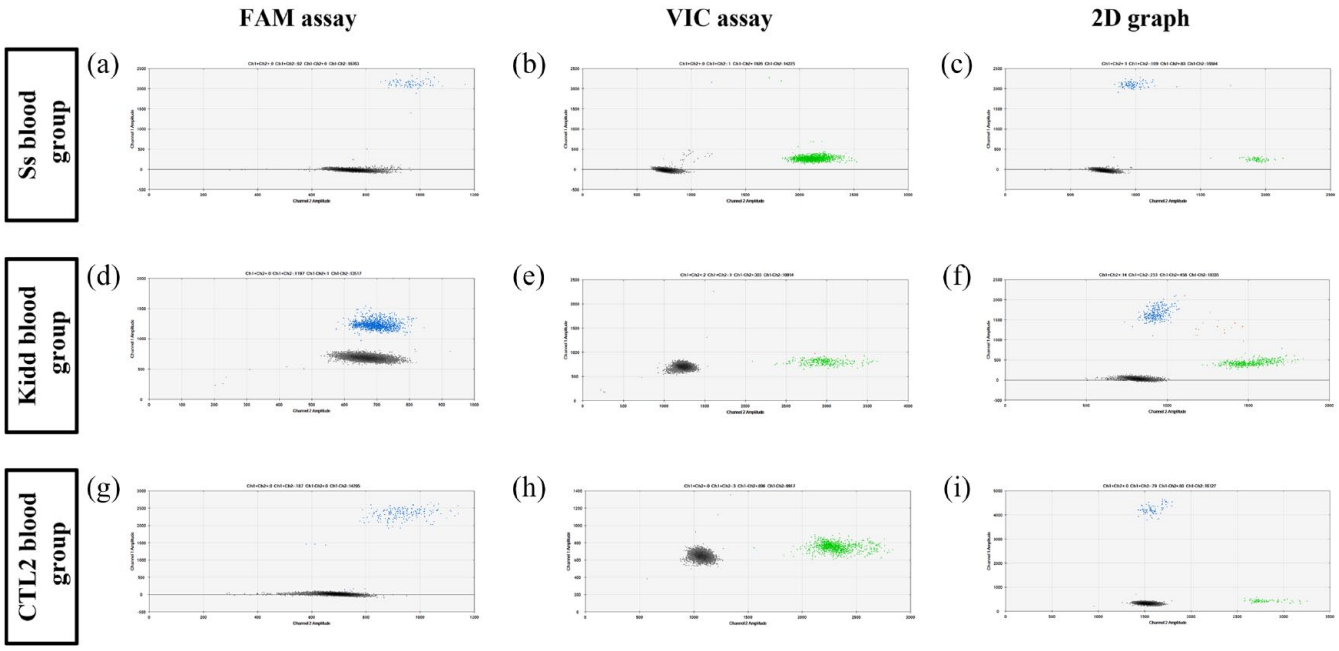

Figure 1. Duplex assay droplet separation results in one-dimensional (1D) and two-dimensional (2D) amplitudes. X- and Y-axes represent the fluorescence intensity of the tested sample. (a) S blood group sample. (b) s blood group sample. (c) Ss blood group sample; (d) JKa^+JKb^- blood group sample. (e) JKa^-JKb^+ blood group sample. (f) JKa^+JKb^+ blood group sample. (g) HNA-3A blood group sample. (h) HNA-3B blood group sample. (i) HNA-3A/B blood group sample.

Results

Blood group genotyping by duplex ddPCR

For the duplex assays, FAM is S, Jk^a, and HNA-3A; VIC is s, Jk^b, and HNA-3B; and FAM and VIC are both antithetical antigens.

A pair of primers and probes that recognize Ss antigen point mutations in the MNS blood group were used to detect S, s, and Ss blood group samples. Positive signals were observed in the FAM channel of the S blood group sample (Figure 1(a)), but not in the VIC channel, resulting in a two-droplet amplitude partition in the 2D channel. The opposite was true in the s blood group sample (Figure 1(b)). When an Ss blood group sample was read, signals were observed in both the FAM and VIC channels (Figure 1(c)).

Furthermore, when the Jk(a+b–) blood group sample was read, positive signals were only observed in the FAM channel (Figure 1(d)). When Jk(a–b+) blood group samples were read, positive signals were observed in the VIC channel (Figure 1(e)). Signals in both the FAM and VIC channels were observed when Jk(a+b+) blood group samples were read (Figure 1(f)). In addition, in the CTL2 blood group, positive signals were observed in the FAM channel when the HNA-3A blood group sample was read (Figure 1(g)), and positive signals were observed in the VIC channel when the HNA-3B blood group sample was read (Figure 1(h)). When the HNA-3A/B blood group sample was read, signals were observed in both the FAM and VIC channels (Figure 1(i)).

Blood group genotyping by quadruplex amplitude-based ddPCR

We confirmed that the Ss and CTL2 blood groups can be detected by quadruplex ddPCR. Because the fluorescence values of Kidd blood group droplet cluster are similar to those of Ss and CTL2 blood groups, the values cannot be accurately read, and we give up adding Kidd blood group to quadruplex ddPCR detection. Then, two pairs of primers and probes were used to identify Ss antigens in the MNS blood group and HNA-3A/B antigens in the CTL2 blood group. The Quanta Soft™ software used for reading the droplets could not directly classify the

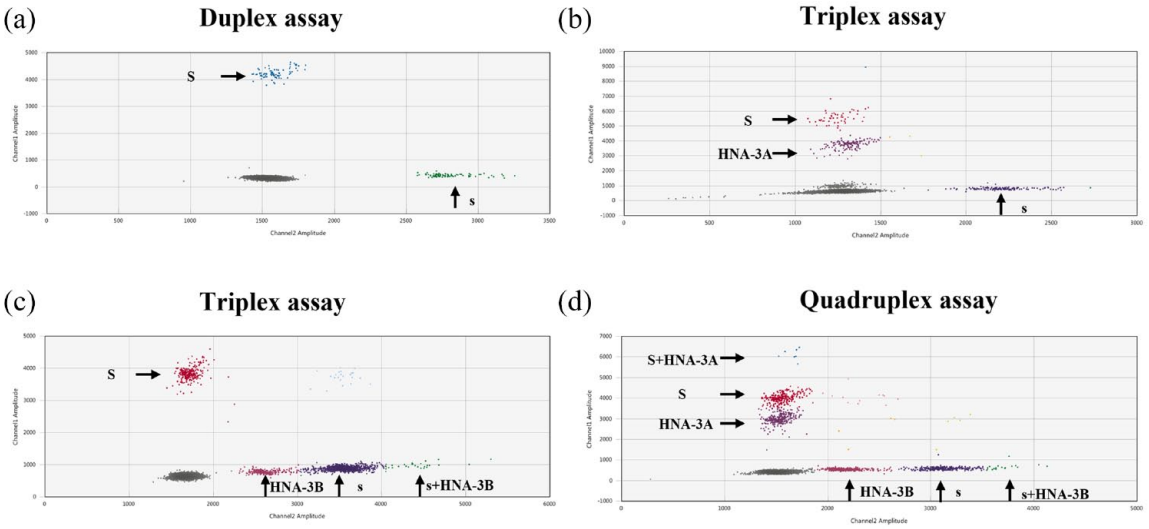

Figure 2. Droplet separation in triplex and quadruplex amplitude-based assay. X- and Y-axes represent the fluorescence intensity of the tested sample. (a) Ss blood group sample. (b) Ss and HNA-3A blood group sample. (c) Ss and HNA-3B blood group sample. (d) Ss and HNA-3A/B blood group sample.

clusters as seen in Figure 1; hence, the Quanta Soft™ Analysis Pro software was used. We used different blood groups to test for cross-reactions between probes. Droplet aggregation was: quadruple negative (gray aggregation at lower left); HNA-3A [single positive (FAM channel) was directly higher than quadruple negative]; S [single positive ([FAM channel) directly above HNA-3A]; S + HNA-3A [double positive (FAM channel) above quadruple negative]; HNA-3B [single positive (VIC channel) on the right side of quadruple negative]; s [single positive (VIC channel), located directly on the right side of HNA-3B]; and s + HNA-3B [double positive (VIC channel), on the right side of quadruple negative]. Clusters of double-positive and triple-positive droplets on different targets can be located at the intersection of the arrows. Different sample types also help to locate different clusters. From the amplitude results of the two-dimensional channel, the target droplet cluster remained at the same position even when different sample classes were used (Figure 2).

NIPT of fetal blood groups using ddPCR assays

Plasma samples from 26 pregnant women of different gestational ages were tested by ddPCR. For each sample, the maternal genotype was determined by blood group gene sequencing after genomic DNA was purified from blood samples. When the fetal blood group is consistent with the maternal blood group, accurate quantification of maternal plasma alleles using the RMD (Relative Mutation Dosage) method is described in single gene diseases. NIPT is required to assess whether there is a balanced proportion of alleles.[24] When the fetus is of the same genotype as the mother's (i.e. heterozygous), an allele balance is expected. Conversely, if the fetus is homozygous, an allele imbalance occurs. By using *SRY* and *RASSF1* genes,[25] which indicate fetal gene content, we can determine fetal genotypes.

The earliest point in a pregnancy that we were able to predict fetal blood group is 5 weeks. The percentage of fetal free DNA detected by ddPCR using *RASSF1* and *ACTB* probes was 2%.[25] Figure 3 shows the detection results of ddPCR. Table 3 shows 26 blood samples collected from pregnant women of different gestational ages (weeks 5–39) and the predicted and actual blood groups of the fetus, which all predicted prenatal blood groups that were confirmed after birth. According to statistical calculation, when $p = 0.05$, the confidence interval is 99% ± 3.82%.

Blood group gene frequency by ddPCR

The genotype frequency obtained by our experiment is shown in Table 4. Based on the average of

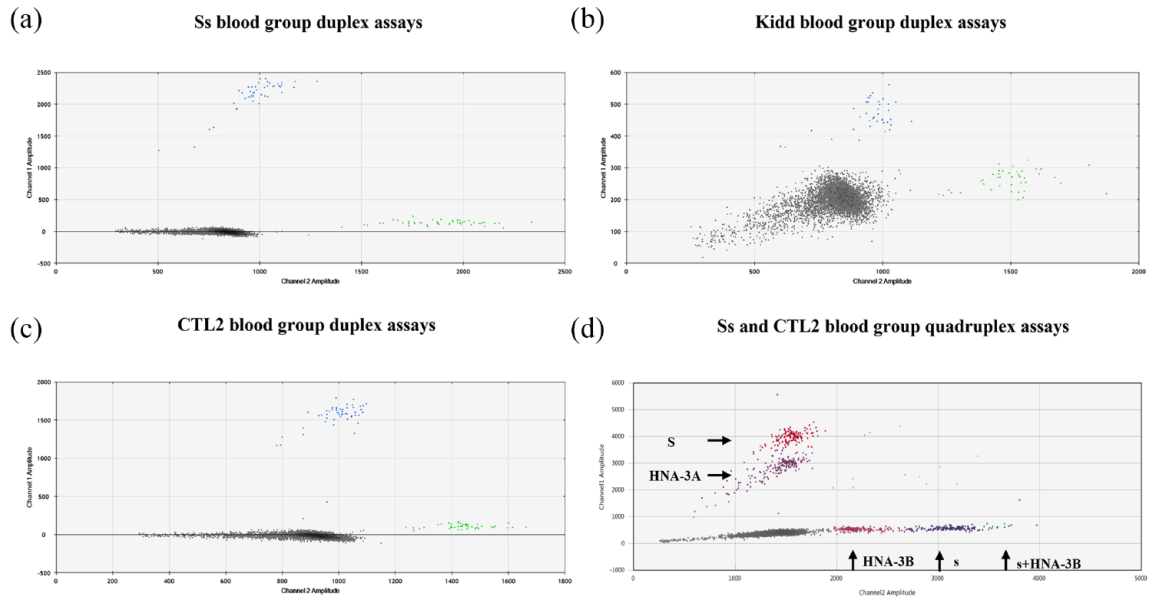

Figure 3. Droplet separation in two-dimensional (2D) and quadruplex amplitude-based assay. The sample was from a pregnant woman at 5 weeks of gestation and contained 2% free fetal DNA. (a) Ss blood group for the mother and s blood group for the fetus. (b) JKa^+JKb^+ blood group for the mother and JKa^+JKb^- blood group for the fetus. (c) HNA-3A/B blood group for the mother and HNA-3A/A blood group for the fetus. (d) Quadruplex amplitude-based assay for free fetal DNA.

Table 3. Fetal genotypes were detected by ddPCR for cell free fetal DNA (cffDNA) in the plasma of pregnant women.

Sample	Gestational Weeks	Ss		Kidd		CTL2		Fetal fraction, %	Diagnosis Outcome
		Parental genotypes	Fetal diagnosis	parental genotypes	Fetal diagnosis	Parental genotypes	Fetal diagnosis		
10	25	ss	ss	JKa^+JKb^-	JKa^+JKb^-	HNA-3A/B	HNA-3A/A	5.58	√
39	6	ss	ss	JKa^+JKb^+	JKa^+JKb^-	HNA-3A/B	HNA-3B/B	0.76	√
49	31	ss	ss	JKa^+JKb^+	JKa^-JKb^+	HNA-3A/A	HNA-3A/A	2.64	√
51	15	ss	ss	JKa^+JKb^+	JKa^+JKb^-	HNA-3A/B	HNA-3A/A	4.6	√
52	30	ss	ss	JKa^+JKb^+	JKa^-JKb^+	HNA-3A/A	HNA-3A/A	5.41	√
54	34	ss	ss	JKa^+JKb^+	JKa^-JKb^+	HNA-3A/B	HNA-3B/B	4.32	√
55	5	ss	ss	JKa^+JKb^+	JKa^-JKb^+	HNA-3A/B	HNA-3B/B	1.17	√
56	15	Ss	ss	JKa^+JKb^+	JKa^+JKb^+	HNA-3A/B	HNA-3B/B	3.82	√
57	35	Ss	ss	JKa^+JKb^+	JKa^+JKb^-	HNA-3A/B	HNA-3A/B	3.59	√
59	10	ss	ss	JKa^+JKb^-	JKa^+JKb^-	HNA-3A/B	HNA-3B/B	1.47	√
62	33	ss	ss	JKa^+JKb^+	JKa^-JKb^+	HNA-3A/B	HNA-3A/A	5.82	√
63	34	ss	ss	JKa^+JKb^+	JKa^+JKb^+	HNA-3A/B	HNA-3A/A	5.71	√

(Continued)

Table 3. (Continued)

Sample	Gestational Weeks	Ss		Kidd		CTL2		Fetal fraction, %	Diagnosis Outcome
		Parental genotypes	Fetal diagnosis	parental genotypes	Fetal diagnosis	Parental genotypes	Fetal diagnosis		
64	5	Ss	ss	JKa^+JKb^+	JKa^+JKb^-	HNA-3A/B	HNA-3A/A	2	√
65	36	Ss	ss	JKa^+JKb^+	JKa^+JKb^-	HNA-3A/B	HNA-3A/A	7.33	√
66	19	ss	ss	JKa^-JKb^+	JKa^-JKb^+	HNA-3A/B	HNA-3B/B	2.73	√
67	17	Ss	ss	JKa^+JKb^+	JKa^+JKb^-	HNA-3A/B	HNA-3B/B	1.72	√
68	6	ss	ss	JKa^+JKb^+	JKa^-JKb^+	HNA-3B/B	HNA-3B/B	0.46	√
69	27	ss	ss	JKa^+JKb^+	JKa^+JKb^-	HNA-3A/B	HNA-3A/B	4.53	√
70	22	ss	ss	JKa^+JKb^+	JKa^+JKb^+	HNA-3A/A	HNA-3A/A	4.81	√
72	19	ss	ss	JKa^+JKb^+	JKa^+JKb^+	HNA-3A/B	HNA-3B/B	2.17	√
73	29	ss	ss	JKa^+JKb^+	JKa^+JKb^-	HNA-3B/B	HNA-3B/B	3.92	√
74	24	ss	ss	JKa^+JKb^+	JKa^-JKb^+	HNA-3B/B	HNA-3B/B	3.69	√
75	33	ss	ss	JKa^-JKb^+	JKa^-JKb^+	HNA-3A/B	HNA-3B/B	3.36	√
76	35	ss	ss	JKa^+JKb^-	JKa^+JKb^-	HNA-3A/B	HNA-3A/A	5.66	√
77	30	ss	ss	JKa^+JKb^+	JKa^+JKb^+	HNA-3A/A	HNA-3A/A	7.28	√
78	8	ss	ss	JKa^+JKb^+	JKa^+JKb^-	HNA-3A/B	HNA-3B/B	1.03	√

ddPCR, droplet digital polymerase chain reaction.
For each maternal sample, gestational age, diagnosis based on ddPCR analysis, actual fetal genotype determined by DNA sequencing, fetal fraction, and diagnosis. √, confirmed. Fetal fraction represents the proportion of fetal free DNA to circulating free DNA in maternal plasma.

Table 4. The frequency of blood group in 20 blood pools was detected by ddPCR.

POOL	Ss blood group		Kidd blood group		CTL2 blood group	
	Ratio (FAM/VIC)	Genotype frequency	Ratio (FAM/VIC)	Genotype frequency	Ratio (FAM/VIC)	Genotype frequency
		SS:Ss:ss		JKa^+JKb^-:JKa^+JKb^+:JKa^-JKb^+		HNA-3A/A:HNA3A/B:HNA-3B/B
Pool 1	0.076	0.25:6.56:43.19	0.91	11.35:24.94:13.71	2.44	25.16:20.62:4.23
Pool 2	0.129	0.65:10.12:39.23	0.95	11.87:24.98:13.15	2.7	26.63:19.72:3.65
Pool 3	0.082	0.29:7.00:42.71	0.418	4.34:20.79:24.87	0.73	8.90:24.39:16.71
Pool 4	0.117	0.55:9.38:40.07	0.72	8.76:24.34:16.90	2.35	24.60:20.94: 4.46
Pool 5	0.078	0.26:6.71:43.03	0.98	12.25:25.00:12.75	2.35	24.60:20.94:4.46
Pool 6	0.081	0.28:6.93:42.79	0.72	8.76:24.34:16.90	2.36	24.67:20.90:4.43
Pool 7	0.061	0.17:5.42:44.42	1.05	13.12:24.99:11.90	1.72	19.99:23.25:6.76

(Continued)

Table 4. (Continued)

POOL	Ss blood group		Kidd blood group		CTL2 blood group	
	Ratio (FAM/VIC)	Genotype frequency	Ratio (FAM/VIC)	Genotype frequency	Ratio (FAM/VIC)	Genotype frequency
		SS:Ss:ss		JKa^+JKb^-:JKa^+ JKb^+:JKa^-JKb^+		HNA-3A/A:HNA3A/B:HNA-3B/B
Pool 8	0.05	0.11:4.54:45.35	1.07	13.36:24.97:11.67	2.72	26.73:19.66:3.61
Pool 9	0.071	0.22:6.19:43.59	1.67	19.56: 23.43: 7.01	1.65	19.38:23.50:7.12
Pool 10	0.111	0.50:8.99:40.51	0.93	11.61:24.97:13.42	1.17	14.54:24.85:10.62
Pool 11	0.207	1.47:14.21:34.32	0.87	10.82:24.88:14.30	1.71	19.91:23.28:6.81
Pool 12	0.064	0.18:5.65:44.17	1.17	14.54:24.85:10.62	1.49	17.90:24.03:8.06
Pool 13	0.047	0.10:4.29:45.61	1.36	16.60:24.42:8.98	1.9	21.46:22.59:5.95
Pool 14	0.231	1.76:15.24:33.00	0.56	6.44:23.01:20.55	1.51	18.10:23.97:7.94
Pool 15	0.152	0.87:11.45:37.68	0.81	10.01:24.72:15.26	2.26	24.03:21.27:4.70
Pool 16	0.082	0.29:7.00:42.71	1.01	12.62:25.00:12.38	2.6	26.08:20.06:3.86
Pool 17	0.159	0.94:11.84:37.22	1.18	14.65:24.83:10.52	1.7	19.82:23.32:6.86
Pool 18	0.078	0.26:6.71:43.03	0.66	7.90:23.95:18.14	2.26	24.03:21.27:4.70
Pool 19	0.115	0.53:9.25:40.22	1.1	13.72:24.94:11.34	2.06	22.66:22.00:5.34
Pool 20	0.06	0.16:5.34:44.50	0.65	7.76:23.88:18.37	2.16	23.36:21.63:5.01
Total	0.10255	9:168:823	0.9394	235:499:266	1.992	443:445:112

the ratios from 20 pools, the calculated gene frequencies were 9.30% for S, 90.70% for s, 48.43% for Jk^a, 51.57% for Jk^b, 66.57% for HNA-3A, and 33.43% for HNA-3B.

Discussion

The purpose of this study was to conduct NIPT of three blood groups in fetuses by multiplex ddPCR. In addition, the gene frequencies of three blood groups of 1000 people in northwest China were detected by ddPCR through the establishment of blood pools.

NIPT of fetal blood groups has always been an important focus of prenatal testing. However, previous NIPT has focused on common ABO and Rh blood groups. There is as yet no effective method for early screening for Ss, Kidd, and CTL2 blood groups. Therefore, we developed an NIPT for three blood groups based on ddPCR technology. Based on the fact that the use of ddPCR can establish multiple assay, we successfully established a multiplex ddPCR method to predict fetal Ss, Kidd, and CTL2 blood groups at 5 weeks' gestation.

This is not only the first BIPT for three blood groups based on ddPCR technology, but also the first multiplex ddPCR method for blood group point mutation detection. With the application of multiplex ddPCR, one blood group detected by a single well test was changed to two blood groups detected by a single well test. This not only saves testing materials but also saves testing labor costs.

In addition, ddPCR improves NIPT from 9 weeks of gestation to 5 weeks of gestation compared with next-generation sequencing.[21] This not only facilitates the monitoring and management of early pregnancy, but also provides guidance for the development of blood group tests in the future.

However, this study has some limitations. Our method could not determine whether the samples were Jk3 blood group or $S^{-}s^{-}$ blood group, as mutations at other sites could not be excluded. Fortunately, Jk3 and $S^{-}s^{-}$ blood groups are very rare, for example, accounting for only 0.63% and 2.53% of the population, respectively, among the Uighur people of Xinjiang, China.[26] The absence of the *SLC44A2* gene, which encodes the CTL2 blood group, is even rarer and has only been reported in rare cases.[27]

Furthermore, we used accurate quantitative characteristics of ddPCR to detect the gene distribution frequency of these blood groups. The frequency of the S blood group gene in the Chinese Han population as reported in 2016 was 9.99%, which is similar to the frequency of the S blood group of 9.30% found in our study.[28] The frequency of the Jk^{a} blood group gene in the Chinese Han population reported in 2012 was 51%, which is close to the frequency of 48.47% found in our study.[29] The frequency of the HNA-3A blood group gene reported in 2022 in northwest China population was 64.90%, close to the frequency of 66.57% in our study.[30] Therefore, we propose that it is feasible to use ddPCR to calculate the frequency of blood group–related genes in the human population. This will facilitate the management of blood banks.

In this study, the established ddPCR method was able to process at least 50 samples simultaneously in a single test at an average cost of $2.69 per sample ($134.5 per test). The TaqMan real-time PCR method is a common blood group typing method and costs approximately $51.92 per sample (three replicates).[30] The ddPCR method is cheaper than the TaqMan real-time PCR method. It changes the repeated detection of 1000 samples into the detection of 20 blood pool samples, which not only saves time cost and labor cost but also greatly improves the efficiency of blood group gene frequency detection.

Conclusion

This study demonstrated the non-invasive prenatal detection of Ss, Kidd, and CTL2 blood groups in 26 fetuses based on multiplex ddPCR. It not only saves detection cost but also provides guidance for establishing non-invasive prenatal detection point mutations based on multiplex ddPCR. In addition, the gene frequencies of three blood groups in 1000 northwestern China were detected by ddPCR. This will facilitate the management of blood banks.

Declarations

Ethics approval and consent to participate

Informed consent was obtained from all blood donors. This study was approved by the Ethics Committee of the Air Force Medical University in accordance with the declaration of Helsinki. Ethics approval number is 202207.

Consent for publication

The donors approve publication.

Author contributions

Yufeng Wang: Conceptualization; Data curation; Investigation; Methodology; Visualization; Writing – original draft.

Xiaoyue Chu: Conceptualization; Data curation; Formal analysis; Investigation; Visualization; Writing – original draft.

Xihui Chen: Methodology; Software; Validation.

Anan Yin: Methodology; Software; Validation.

Yan Yao: Methodology; Software; Validation.

Li Wang: Project administration; Resources; Supervision; Writing – review & editing.

Hua Xu: Project administration; Resources.

Fangfang Liu: Conceptualization; Project administration; Supervision; Writing – review & editing.

Kun Chen: Conceptualization; Project administration; Writing – review & editing.

Yuanming Wu: Conceptualization; Project administration; Writing – review & editing.

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Competing interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Availability of data and materials

All the data will be available upon request to the corresponding author.

ORCID iDs

Kun Chen https://orcid.org/0000-0002-4433-127X

Yuanming Wu https://orcid.org/0000-0002-5276-4382

Supplemental material

Supplemental material for this article is available online.

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