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ELECTROPHORESIS

RESEARCH ARTICLE

Separation mixed semen of two individuals using magnetic beads coupled ABH blood group antibody

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Abstract

In sexual assault cases, one of the most common samples collected is a mixed semen stain, which is often found on the vagina, female underwear, or bed sheets. However, it is usually difficult to identify the perpetrator based on this sample alone. One technique that has been developed to address this issue is magnetic bead-based separation. This method involves using modified magnetic microspheres to capture and enrich specific target cells, in this case, sperm cells. In this study, we utilized magnetic beads coupled with ABH blood group antibody to isolate sperm cells from an individual of a single ABO blood type. Subsequently, polymerase chain reaction amplification and capillary electrophoresis were employed to perform the genotyping the short tandem repeat (STR) loci. This approach allows for the identification of different individuals in a mixed seminal stain sample from two individuals, by first separating sperm cells based on ABH antigen differences and subsequently utilizing autosomal STR typing on the enriched single blood group cells.

KEYWORDS
ABO blood grouping, magnetic bead, mixed semen stain, personal identification, rape cases

1 | INTRODUCTION

In rape cases, one of the most common types of samples collected is a mixed stain, typically found on the vagina, female underwear, or bed sheets. The male component can be obtained after the differential digestion of female constituents [1]. However, in some cases, the sperm cells of multiple male offenders are often mixed together, posing challenges in separating the male components from the large number of female epithelial cells using the traditional extraction methods [2]. Even if the typing results are obtained, analyzing the results can be complicated due to the presence of multiple mixed components or the dominance of the female component [3]. In the statistical analysis of peak height and peak area of the short tandem repeat (STR) typing analysis, factors such as DNA concentration and the proportion of mixed samples can impact the results, making it difficult to eliminate the influence of shadow bands, nonspecific bands, amplification imbalance, and allele imbalance [4, 5]. Through the statistical analysis of mixed DNA profiles, it becomes possible to interpret the potential STR genotypes of each individual [5, 6]. Additionally, Y-STR profiles can be assessed using a likelihood ratio method [7], although personal identification is not always possible.

Abbreviation: RFU, relative fluorescence units.

Jun Yao and Atif Adnan contributed equally and should be considered first authors.

Magnetic bead-based separation is a technique used for target capture and enrichment through modified magnetic microspheres. This method finds widespread application in immune detection, cell separation, biomacromolecule purification, and molecular biology [8, 9]. Magnetic bead coupling offers cost-effectiveness by utilizing only 25% of the normal antigen amount and 50% of the beads compared to the non-magnetic beads [10]. When combined with other methods like quantum-dot fluorescence labeling and immunoaffinity separation, the magnetic bead-based assay showed high specificity and sensitivity [11]. Moreover, by sequentially incubating the prepared cell mixtures with biotinylated MOSPD3 antibody and avidin-coated magnetic beads [12], the detection accuracy rate for each STR locus of the sperm cells was 100% in both flocked and cotton swabs preserved for 1 day, 87.5% in flocked swabs and 40% in cotton swabs preserved for 3 days, and 40% in flocked swabs and 16.67% in cotton swabs preserved for 10 days. Another separation technique proposed by Yano et al. combines antihuman leukocyte CD45 antibody and ABO blood group antibody [13]. It involves magnetic separation utilizing CD45 antibody-coated microbeads specific to leukocyte and centrifugal separation of leukocyte agglutination using ABO antibody. Even when the mixing ratio is as low as 1:512, the partial loci of low components in the mixed sample can still be detected.

The surface of sperm cells is characterized by the presence of ABH antigens, which correspond to individual ABO blood types. The expression of ABH antigen in sperm cells is regulated by the ABH gene, which is influenced by secretor status [14]. In this study, we utilized immunomagnetic beads and monoclonal antibodies to exploit the ABO antigen difference on the surface of sperm cells. This allowed us to separate the sperm cells in mixed sample containing two male individuals and subsequently proceed with STR typing detection. Specifically, we employed immunomagnetic beads and monoclonal antibodies to target the inherent antigen on the surface of sperm cells. This interaction led to the formation of a stable sperm-antibody-biotin complex. To enhance the recovery of sperm cells, this complex was then enriched using magnetic beads, resulting in a more rapid, convenient, and efficient method for isolating sperm cells from different donor sources in a mixed sample.

2 | MATERIALS AND METHODS

2.1 | Sample collection

The study protocol received approval from the Ethics Committee of China Medical University and all the participants provided written informed consent after receiving a thorough explanation of the study procedures. The ABO blood type and the secretor status of the participants were determined based on a previous study [15, 16]. Secretor individuals possess at least one functional FUT2 allele (Se), whereas nonsecretors are homozygous for nonfunctional FUT2 (nonsecretor) alleles (se). High-resolution melting analysis of rss601338 (428G>) was employed to determine the genotyping of secretor status [16]. Four secretor individuals, representing A, B, AB, and O blood groups, were selected to provide semen samples, while one female individual was chosen to provide the vaginal fluid sample. Within 30 min of collection, the semen samples were washed three times with phosphate-buffered saline (0.01 mol/L, pH 7.2) and then diluted to a density of 1.0×10^6 cell/mL using a cell counter (Countess 3, Thermo Fisher Scientific). Subsequently, the sperm cells from the four different ABO blood groups were mixed in equal volume in pairs, resulting in a total of six mixed semen samples. Furthermore, mixed samples were prepared at three different ratios (1:10, 1:100, and 1:1000) by combining a sperm cell suspension with A blood group (10^3 cells/mL) and a cell suspension with B blood group (10^4, 10^5, or 10^6 cells/mL). Mixed semen and vaginal fluid specimens were also created by combing equal amounts of cells.

2.2 | ABO blood group antibody labeled with biotin

The process followed the methodology outlined in our previous study [17]. To label the ABO blood group antibody with biotin, 50 μL of ABO blood group antibody (Abcam) was added to a 2 mL reaction tube equipped with magnetic stirring columns. Subsequently, 5 μL of freshly prepared NaHCO3 solution was added to the tube. In a separate container, the DSB-X biotin labeling buffer (Thermo Fisher), was mixed with 40 μL dimethyl sulfoxide, and 3 μL biotin labeling buffer mixture was added to the reaction tube. The solution was thoroughly mixed and stirred for 1–1.5 h at room temperature. The liquid was then transferred to a centrifuge tube containing a filter column and centrifuged at 1100 × *g* for 3 min. This process was repeated once more, resulting in the obtainment of pure biotin-labeled antibody.

2.3 | Sperm cells separated by magnetic beads

For the separation of sperm cells, the differential lysis was employed to eliminate female epithelial cells [18]. First, 25 μL of the biotin-labeled ABO blood group antibody

TABLE 1 Short tandem repeat (STR) typing of four ABO blood type individuals.

Sample	Blood type A	Blood type B	Blood type O	Blood type AB
D8S1179	14/14	12/13	14/14	14/16
D21S11	32.2/32.2	29/32.2	30/31	30/31.2
D7S820	10/11	11/11	8/10	10/12
CSF1PO	15/15	11/11	10/12	10/11
D3S1358	15/15	14/15	15/17	15/16
TH01	7/9	6/9.3	6/9	7/9
D13S317	8/8	11/12	10/11	8/10
D16S539	11/11	9/12	11/12	9/12
D2S1338	18/21	19/19	20/22	23/27
D19S433	14/14	15.2/15.2	13/14	13/16.2
VWA	17/17	14/14	14/16	14/17
TPOX	11/11	8/8	8/8	8/9
D18S51	14/21	15/15	16/16	13/13
D5S818	14/14	9/14	10/11	11/13
FGA	21/23	24/24	19/21	22/25

was added to 500 μL of mixed sperm cell suspension and incubated for 10 min in 4°C. The sperm cells were subsequently washed with 2 mL of separation buffer. After centrifugation at 350 × *g* for 10 min at 4°C, the supernatant was discarded. Next, 1 mL of precooled separation buffer was added to resuspend the cells, and 75 μL of magnetic beads (Thermo Fisher) were added and mixed with the cells for 15 min at 4°C. The cells were mixed with 1 mL separation buffer for 2 min, and the supernatant was carefully removed by placing the tube on a magnetic frame. The sperm cells were washed with separation buffer more than 10 times, and 1 mL of release buffer (Thermo Fisher) was added. The tube was gently shocked at 4°C for 10 min, placed on the magnetic frame for 1 min, and the cells without magnetic beads were transferred to a new tube.

2.4 | DNA extraction and STR typing

The genomic DNA of the enriched sperm cells was extracted using Chelex 100 method [19]. Autosomal STR loci were amplified utilizing the AmpFLSTR Identifiler polymerase chain reaction (PCR) amplification kit (Applied Biosystems) on a GeneAmp PCR System 9700 (Applied Biosystems) according to manufacturer's instructions. Subsequently, PCR products were subjected to capillary electrophoresis on 3500xl Genetic Analyzer, and raw data were analyzed using GeneMapper *IDX* 1.5 software (Applied Biosystems). The reference profile for the blood samples from the volunteers were utilized. A threshold value of 100 relative fluorescence units (RFU) was employed to confirm successful genotyping.

3 | RESULTS

The profiles of the four ABO blood group samples, used as references, are presented in Table 1 and Figure 1.

3.1 | Autosomal STR typing of two mixed male samples

The semen mixture from two individuals exhibited four peaks at the TH01, D2S1338, and D18S51 loci and three peaks at D8S1179, D21S11, D13S317, D16S539, vWA, D5S818, and FGA loci (Figure 2). Since the locus allele of each individual was not determined, personal identification could not be performed.

3.2 | Autosomal STR typing of sperm enriched by magnetic beads coupled ABH antibody

Following the differential lysis to remove the female epithelial cells, the mixed samples of individuals with blood type A and B were enriched using by anti- A and anti- B antibodies, respectively. However, complete STR typing results for single blood group individuals were not obtained after the enrichment using magnetic beads coupled with blood type B antibody (Figure 3). Notably, the RFU of blood type A individual significantly decreased.

Subsequently, we increased the elution times and the temperature of the eluent buffer from 4 to 37°C. At 37°C, the sperm cells were separated using magnetic beads coupled with type B blood group antibody and type A blood

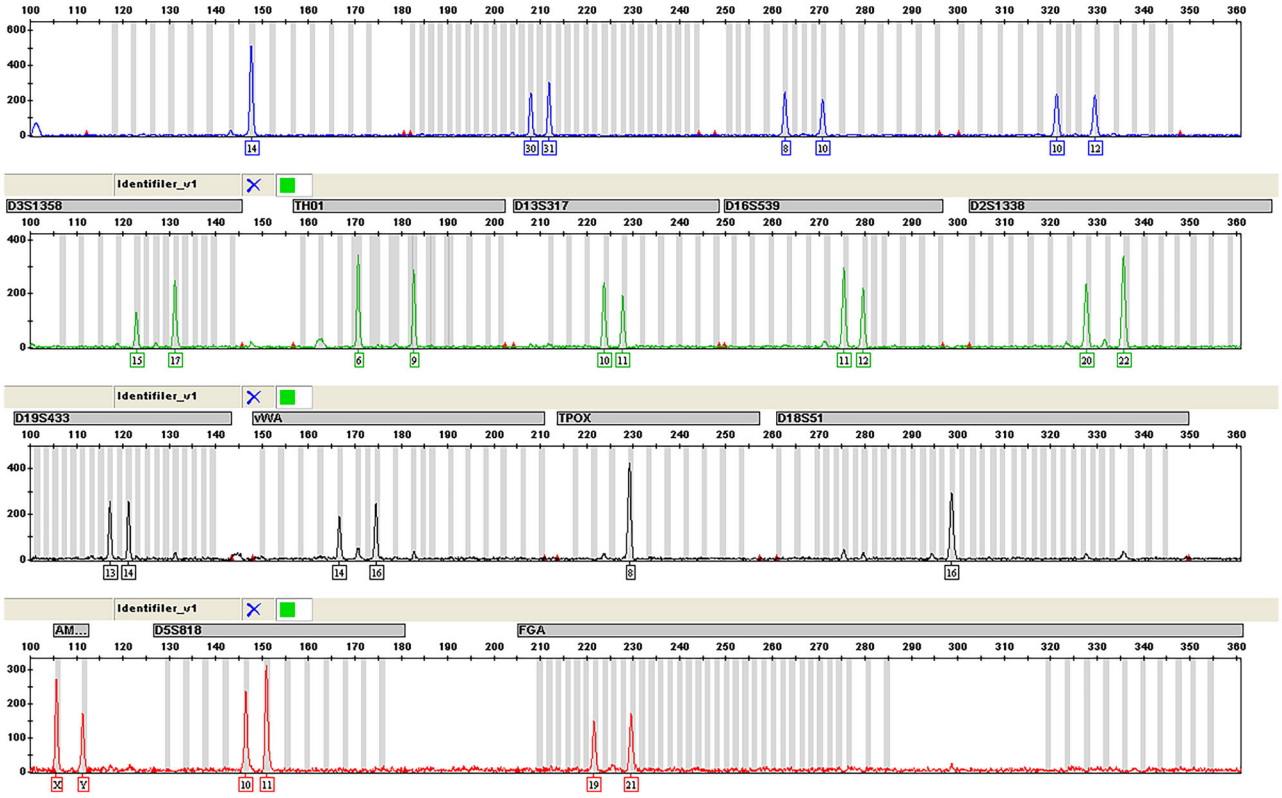

FIGURE 1 Short tandem repeat (STR) typing of blood type O sample.

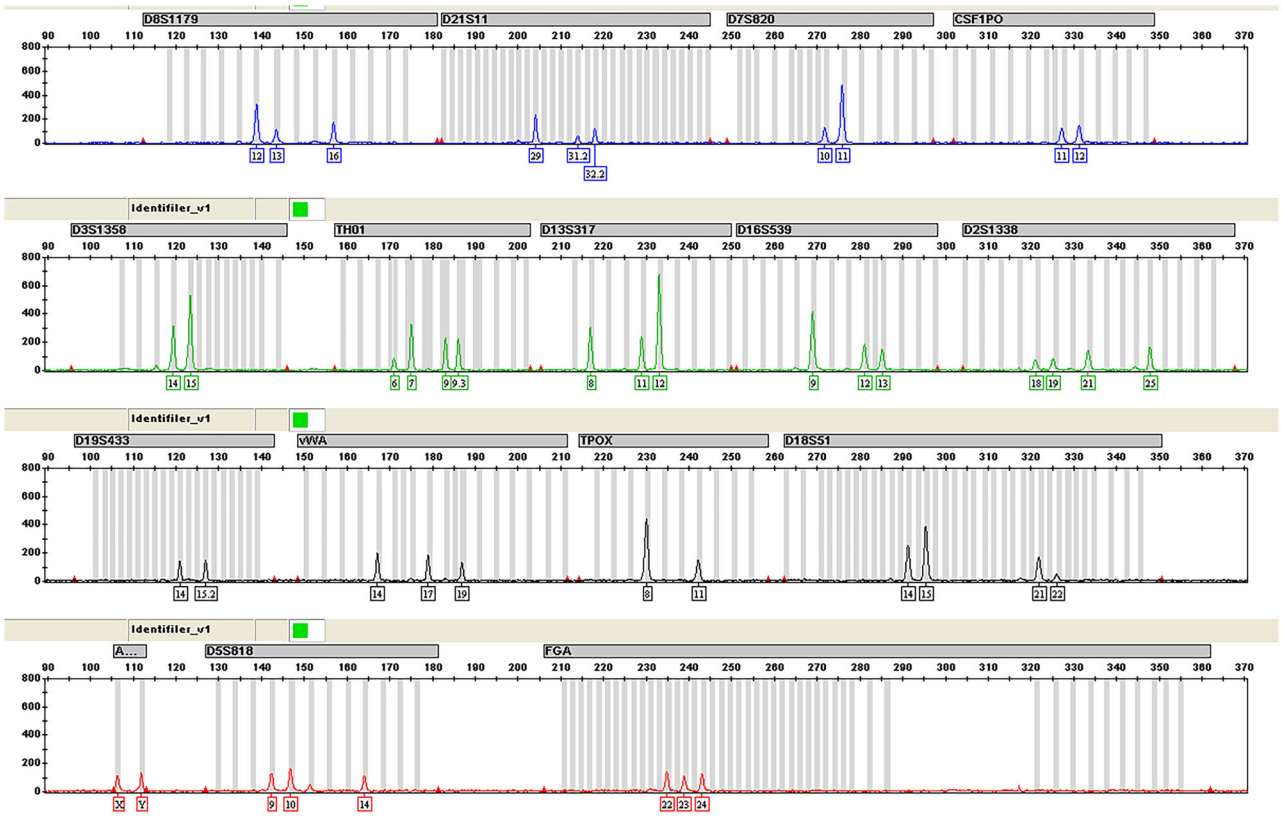

FIGURE 2 Short tandem repeat (STR) typing of the mixed semen in individuals of blood type A and type B.

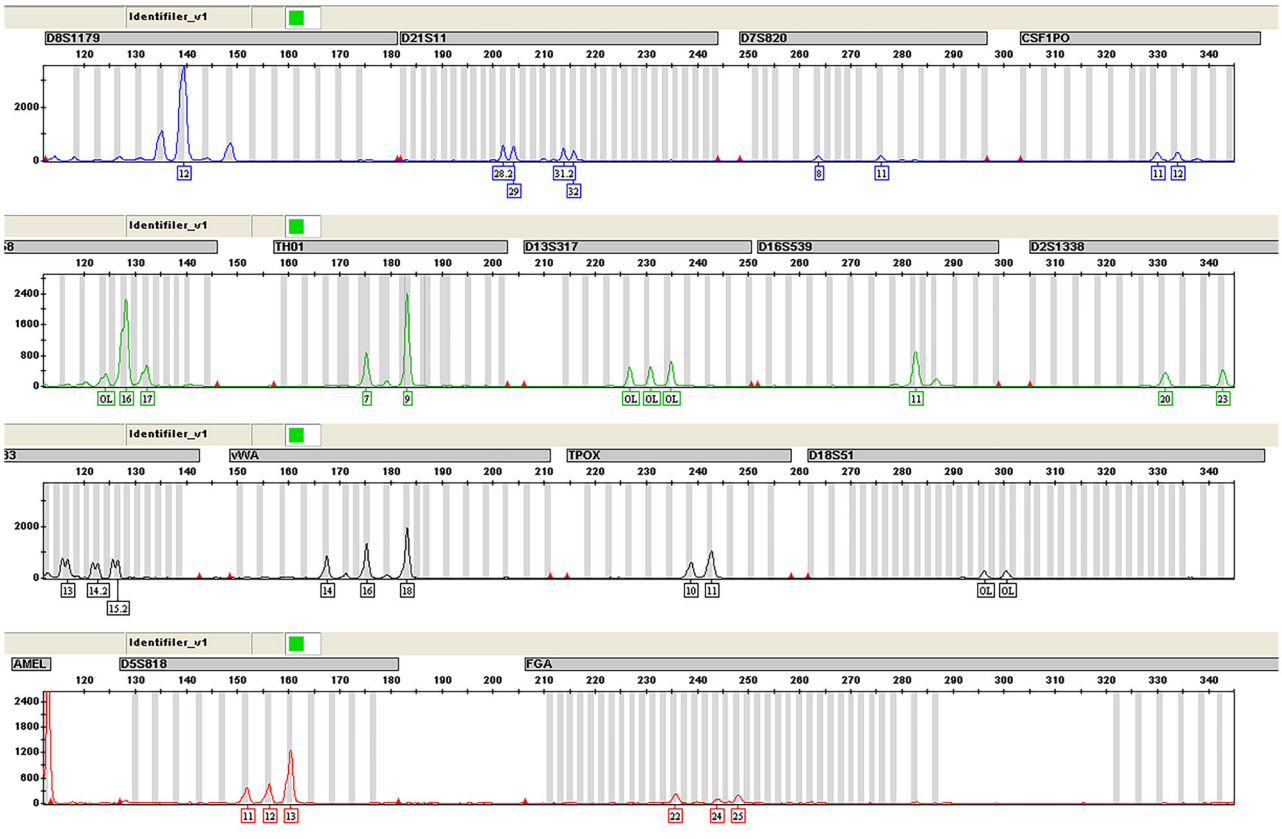

FIGURE 3 Short tandem repeat (STR) typing of single-source B-type sperm cells (4°C elution).

group antibody, respectively. Accurate STR genotyping results were obtained for each locus (Figures 4 and 5). We also separated mixed samples of individuals with blood type AB and O, which were enriched using anti-A and anti-B antibodies. Complete genotyping for blood type AB individuals could be achieved (Figure 6); however, complete genotyping of blood type O individuals could not be attained.

Furthermore, the efficiency of separation using the proposed approach was evaluated in mixed samples with three different ratios (A blood group cells vs. B blood group cells in 1:10, 1:100, and 1:1000 ratios). After 10 repetitions with mixtures of varying proportions, the number of successfully genotyped STR loci is presented in Table 2. The results indicated a 90% success rate in obtaining genotyping for 15 STR loci in the 1:10 ratio, whereas a full profile could not be obtained in the 1:100 and 1: 1000 ratios.

4 | DISCUSSION

The sperm cells of different blood types can be obtained by using the corresponding ABO blood group antibody for specific enrichment, followed by performing STR genotyping to obtain the profile of each individual.

Immunomagnetic beads, which are uniform and suitable-size polystyrene microspheres, are effective in isolating cells through magnetization and their connection with antibodies [20, 21]. Their main characteristics include: (i) fast separation speed, high efficiency, and good repeatability; (ii) simple operation without the need for expensive equipment; (iii) minimal impact on the biological characteristics and functions of the isolated cells; (iv) specific separation achieved by coupling specific antibodies and probes [22]. Magnetic bead-based separation has been widely used in clinical diagnosis, isolation and detection of various tumor cells, bacteria, and other microorganisms due to its high efficiency, rapidity, simplicity, non-toxicity, low cost, high separation purity, and retention of cell activity [23–25]. In our study, we utilized biotin–avidin coupling between antibodies and magnetic beads, resulting in strong covalent bonds [26, 27]. The process of separating sperm cells based on blood type antigens involves two steps. The first step involves the specific binding of immunomagnetic beads to cells through a specific immune binding reaction between antigens and antibodies. The second step involves the qualitative movement of the antigen–antibody–magnetic beads immune complex under an external magnetic field to separate the cells [28]. Most of the ABO blood group

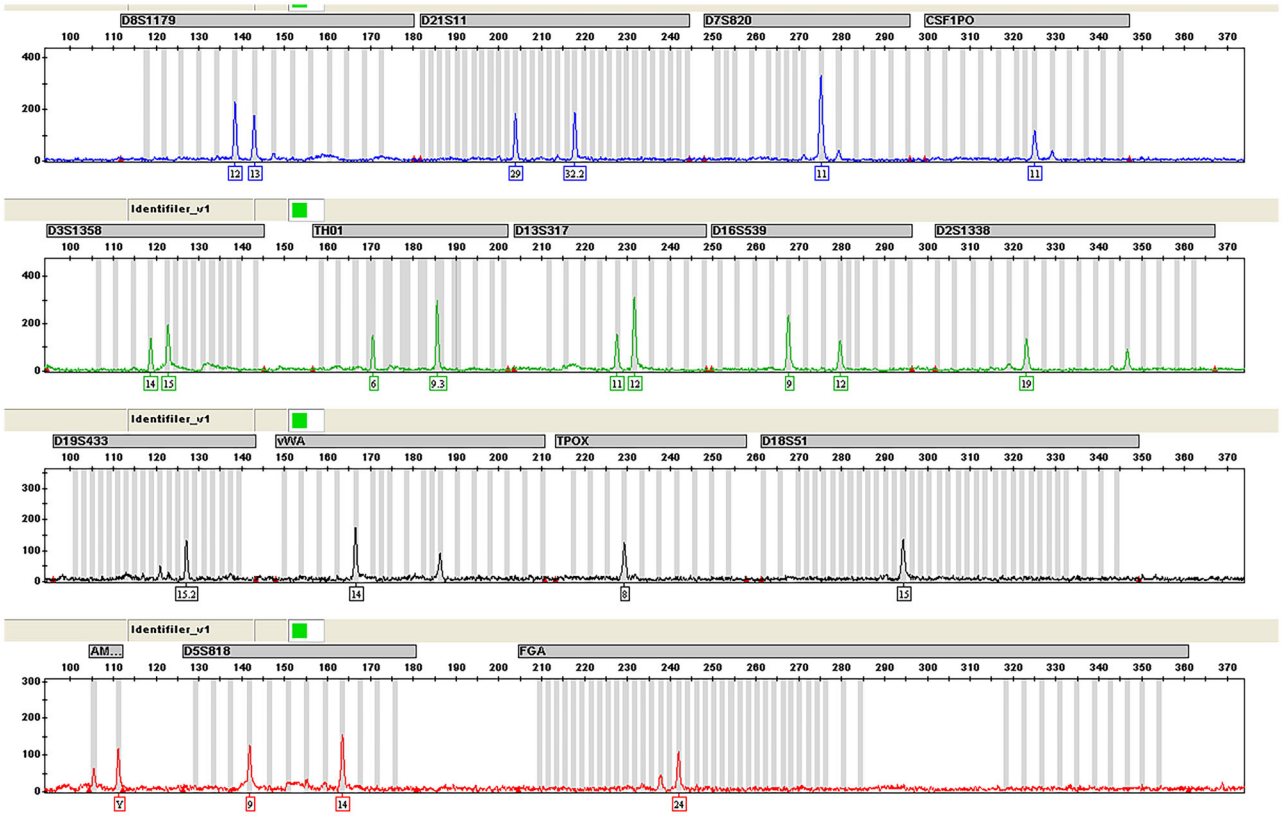

FIGURE 4 Short tandem repeat (STR) typing of single-source B-type sperm cells (37°C elution).

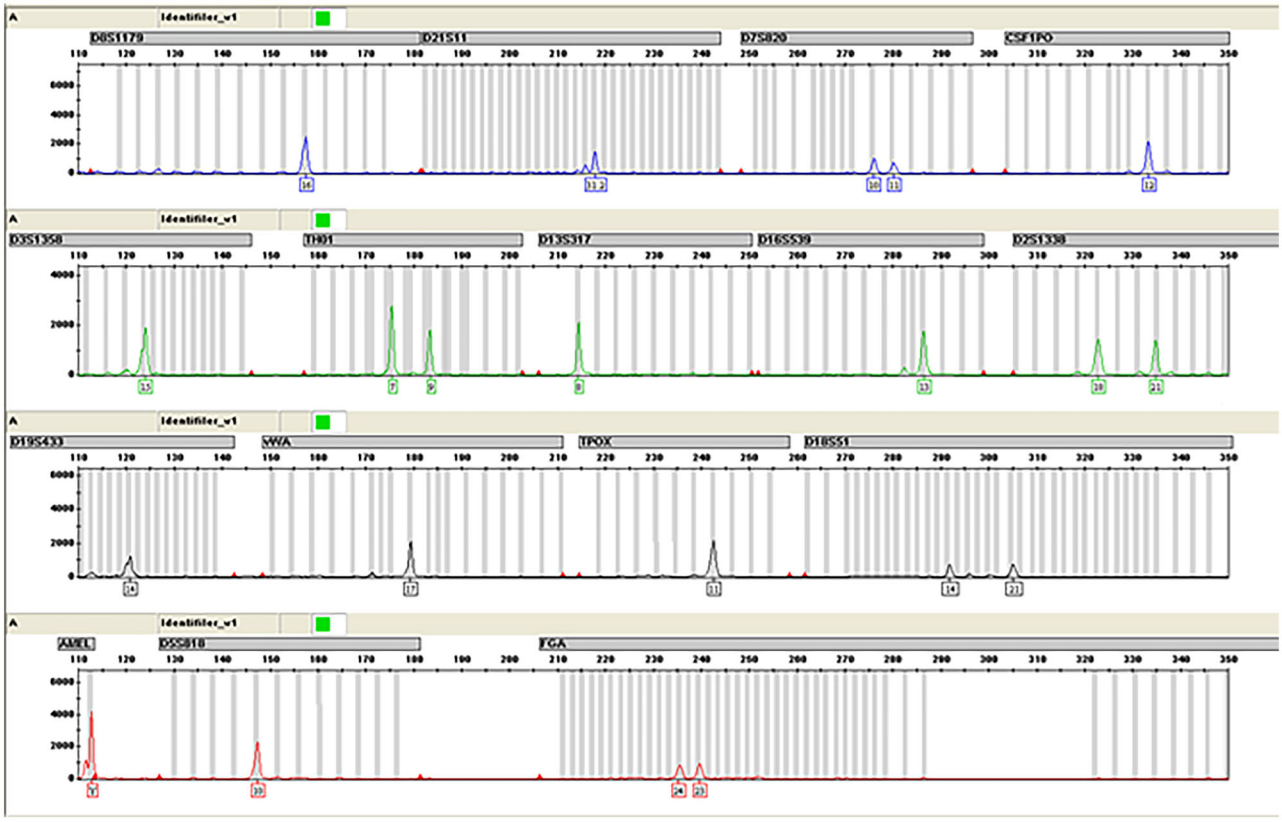

FIGURE 5 Short tandem repeat (STR) typing of single-source A-type sperm cells (37°C elution).

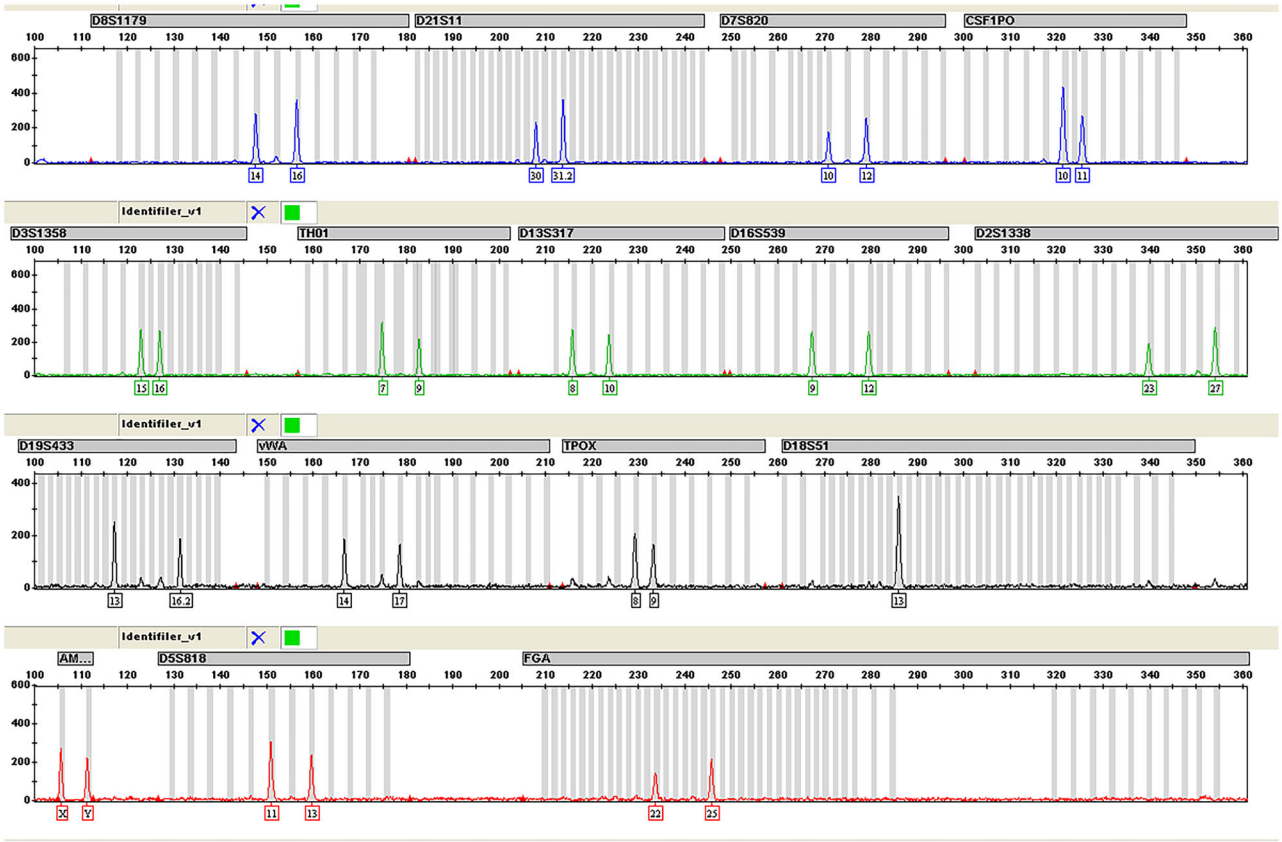

FIGURE 6 Short tandem repeat (STR) typing of single-source AB-type sperm cells (37°C elution).

TABLE 2 Number of short tandem repeat (STR) loci successfully genotyped of A blood group sperm cells in mixed samples comprising three ratios (1:10, 1:100, and 1:1000).

Mixed sperm cells (A blood group sperm:B blood group sperm) (cells/mL)	STR loci successfully genotyped of A blood group sperm cells			
	15	13–15	10–12	<10
10^3:10^4	9	1	0	0
10^3:10^5	0	3	5	2
10^3:10^6	0	1	1	8

antigens are located in the head of the sperm cells [2, 14]. This means that even sperm cells that have lost their tail or have broken bodies can still be captured by magnetic beads using antibodies against ABO blood group antigens.

To elute nonspecifically bound sperm cells, increasing the temperature of the eluent buffer ensures higher specificity. Results have shown that increasing the elution temperature from 4 to 37°C can prevent the effect of other nonspecific bindings. This temperature improvement enhances elution efficiency and removes nonspecifically bound sperm cells without affecting the binding between antigens and antibodies [29, 30]. Monoclonal antibodies, with an appropriate temperature of 37°C, are used in this process. It is also crucial to wash the samples more than 10 times under the action of the magnetic frame to completely elute sperm cells without antibodies and prevent interference with later STR analysis, particularly during capillary electrophoresis detection of fluorescent products.

The mixed samples of blood type A and blood type B can be well separated, and complete STR typing can be obtained by using anti- A or anti- B antibodies in the isolation of individuals with blood type AB. However, in the mixed detection of blood type O individuals with blood type A, B, or AB individuals, only sperm cells from blood type A or B sources can be obtained through anti-A or anti-B antibodies, but not from blood type O sources. The H antigen, a precursor of the A and B blood group antigens, is defined by a terminal fucose residue found on red blood cells in a secretory individual [31]. H antigens

can also be weakly expressed in sperm cells of individuals with blood type A, B, or AB, especially in individuals with AO or BO genotypes. Therefore, to avoid interference from weakly expressed H antigens, it is better not to use anti-H antibodies to obtain sperm cells from blood type O sources. Although the method established in this experiment has not been successfully used to detect sperm cells from blood type O source, the exploration and optimization of the experimental conditions can help to address this issue in future studies. However, the limited combination of ABO blood groups may restrict the application of this experimental method, which is a limitation of the study. In subsequent studies, additional blood group antibodies, such as MN blood type and Lewis blood type, will be incorporated to improve the separation capability [32, 33].

It is important to note that this study only focused on separating semen from two different individuals with the same volume. However, in practical cases, the mixing ratio of semen samples varies greatly due to various factors. Additionally, the experimental sample may be influenced by the surrounding environment, which can impact the antigen–antibody recognition ability. Therefore, future experiments should aim to effectively acquire sperm cells with low-component male samples and enhance the method's sensitivity.

5 | CONCLUDING REMARKS

In conclusion, magnetic bead-based separation emerges as a promising technique for isolating sperm cells from mixed semen stains in sexual assault cases. By targeting specific antigens present on the surface of sperm cells, such as ABH antigens corresponding to ABO blood types, and utilizing PCR amplification and capillary electrophoresis for genotyping at STR loci, it becomes possible to identify different individuals within a mixed seminal stain sample originating from two individuals. This method proves to be cost-effective, specific, and efficient, offering valuable information for personal identification in sexual assault cases. The study protocol received approval from the Ethics Committee of China Medical University and all the participants provided written informed consent. The study successfully demonstrates the feasibility of this method for isolating sperm cells from mixed semen stains in sexual assault cases, and further research will be needed to optimize the method and apply it in forensic practice.

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CONFLICT OF INTEREST STATEMENT

The authors have declared no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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