Colorimetric Detection by Gold Nanoparticle DNA Probes for Miltenberger Series (GP.Mur, GP.Hop, and GP.Bun) Identification

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Background: Miltenberger (Mi) series are the collective glycophorin hybrids in the MNS blood group system. Mi series are composed of several subtypes, for examples, GP.Mur, GP.Hop, and GP.Bun. The incompatibility of Mi series blood transfusion poses the risk of hemolysis. Due to the lack of standard antibodies for Mi series blood typing, colorimetric gold nanoparticle (AuNP) DNA probes were therefore explored for Mi series identification. **Methods:** AuNPs were synthesized and conjugated to an RvB (test) probe and an RvA2 (control) probe. Each of the AuNP DNA probes was tested against the amplified products of Mi(+) (GP.Mur/Hop/Bun), Mi(–), and the blank (no amplified product). The change in color was observed by visual inspection and UV-Vis spectroscopy. **Results:** The

amplified product of the Mi(+) sample retained the color on both probes (test+/control+). The amplified product of the Mi(–) sample retained the color only on the control probe (test–/control+) and the amplified product of the blank turned clear on both probes (test–/control–). The results by optical density absorbance measurement were concordant with the results by visual inspection. Both probes were validated with the amplified products of the ten Mi(+) and ten Mi(–) samples. All of the samples were correctly identified. **Conclusion:** AuNP DNA probes (RvB and RvA2) could be applied to distinguish the amplified products of Mi(+), Mi(–), and the blank by visual inspection and/or OD absorbance measurement. *J. Clin. Lab. Anal.* 30:880–887, 2016. © 2016 Wiley Periodicals, Inc.

Key words: Miltenberger series; blood group; genotyping techniques; gold nanoparticles; DNA probes

INTRODUCTION

The MNS blood group is the second blood group system by the International Society of Blood Transfusion (ISBT 002) (1). The “Miltenberger (Mi) series” consists of the collective glycophorin hybrid antigens in the MNS blood group system (2, 3), and there are several subtypes with individual genetic terminology related to the blood group antigens in the ISBT system (3, 4). The Mi series was supposed to have originated from the recombination of the glycophorin A (*GYP A*) and glycophorin B (*GYP B*) genes. GP.Mur is the most prevalent subtype in some regions, such as Taiwan (5–7), Hong Kong (8), and Thailand

(9, 10), and less-common subtypes, including GP.Hop and GP.Bun, are also found in Thailand (9).

Identification of Mi series blood in high-prevalence populations is necessary because the incompatibility of

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TABLE 1. Primer and Probe Details

Primers		Primer pair	PCR product	PCR product size (bp)
FwVwA	5'-AGTTTGTCTTTCATAATACGCTC-3'	Fw1N, RvNN	<i>GP.Mur/Hop/Bun</i>	137
Fw1N ^a	5'-TCTCAACTTCTCTTATATGCAGATAA-3'	FwVwA, RvNN	<i>GYP A</i> (control)	197
RvNN ^a	5'-CTATTTAAAACTAAGAACATACCGG-3'			
Thiol-modified probes				PCR product target
RvB	5'-ATTTCTGAAACTTCATTAGCAGTATG AGCTGGAAAAAAAAAAAA-3'-Thiol			<i>GP.Mur/Hop/Bun</i>
RvA2	Thiol-5'-AAAAAAAAAATTAATACTA AGAACATACCGGTTTCCTCTCT-3'			<i>GP.Mur/Hop/Bun</i> and <i>GYP A</i>

^aFw1N and RvNN were modified from F2 and Rccgg, respectively (10).

Mi series blood transfusions can cause life-threatening hemolytic transfusion reactions (11). Due to the lack of standard antibodies for Mi series blood typing (serotyping) and the inconsistent expression of Mi^a antigens on red blood cells (RBCs) (11, 12), molecular blood group typing (genotyping) is a better alternative for Mi series blood identification (13).

Gold nanoparticles (AuNPs) could be synthesized into different sizes and shapes with unique light-absorption properties (optical density, OD) in the colloidal stage (14, 15). When colloidal AuNPs aggregated, the color of the solution changed from purple-red to purple-blue and from purple-blue to clear. The OD absorbance peak also shows a right-shifted and flattened-down pattern (16). This property has been utilized for the detection of complementary targets by AuNP conjugated oligonucleotide probes in molecular diagnosis (17–19). In this study, simple colorimetric detection by NaCl-induced aggregation of AuNP oligonucleotide probes (20, 21) was applied for Mi series identification.

MATERIALS AND METHODS

Primer and Probe Design

Primers and probes were based on the reference sequences *GYP A* gi|297632354, *GYP B* gi|299782564, and *GYP E* gi|218505794 from the National Center for Biotechnology Information (NCBI) database. The hybrid glycophorin sequences (*GP.Mur (MiIII)* gi|3639078 (22), *GP.Hop (MiIV)* gi|61696953 (23), and *GP.Bun (MiVI)* gi|183553 (22)) were acquired from the Blood Group Antigen Gene Mutation Database (BGMUT) of dbRBCs of the NCBI (24). Sequence alignment was performed using the BioEdit Sequence Alignment Editor (Tom Hall) software, version 7.0.9. Primer targets and product sizes were evaluated using the Primer-BLAST online tool from the NCBI. The free thiol-modified probes with (A)₁₀ spacer oligonucleotide were synthesized (ready-to-use, free thiol end) according to the instructions (BioBasic, Canada) and were designed in the reverse-sequence manner. The primer and probe details are shown in Table 1,

and targets of the sequence alignment are shown in Figure 1c.

Blood DNA Samples

Twenty blood samples, including ten Mi(+) and ten Mi(−) samples identified by serology, were collected from repeat donors at the blood bank of Ramathibodi Hospital. The samples were obtained from EDTA tubes left over from blood donation testing. The protocol was approved by the Ramathibodi Hospital ethics committee.

DNA was extracted from the blood samples using the QIAamp DNA Blood Mini Kit (spin protocol), and it was eluted in AE buffer (eluting buffer). Then, the quality was evaluated by NanoDrop (NanoDrop spectrometry, nucleic acid mode), and the remaining samples were stored at −20°C.

PCR

The PCR reagent (i-Taq DNA Polymerase kit, iNtRON Biotechnology, Kyungki-Do, 462-120, Republic of Korea) was mixed to a final reaction volume of 20 µl. The reaction consisted of 0.3 µl of i-Taq DNA Polymerase (1.5 U), 2 µl of 10× PCR buffer, 2 µl of 10× MgCl₂-free PCR buffer, and 2 µl of 10 mM dNTPs, with the addition of primers, DNA template, and H₂O for a volume of 20 µl. Three primers were included in the reaction: 150 nM of FwVwA, 400 nM of Fw1N, and 150 nM of RvNN. The blood DNA template was in the range of 20–60 ng per reaction (no DNA template for blank). The PCR protocol began with one cycle of 94°C for 3 min, followed by 40 cycles of 94°C for 30 s, 58°C for 20 s, and 72°C for 40 s, and then completed with one cycle of 72°C for 5 min.

The amplified products of Mi(+), Mi(−), and the blank were electrophoresed on a 4% agarose gel with a 100-bp DNA ladder in 1× TBE buffer at 100 V for 45 min. The gel was visualized and photographed under UV light using a UV lamp. The remaining amplified products were stored at −20°C before testing with AuNP DNA probes.

Fig. 1. (a) The experimental flow chart, (b) gel electrophoresis, and (c) targets of the primers and probes. (a) The three steps of the experiment: (1) the preparation; (2) the optimization; and (3) the validation. (b) The Mi(+) (GP.Mur/Hop/Bun) sample showed the amplified products of both GP.Mur/Hop/Bun (137 bp) and GYPA (197 bp), Mi(-) sample showed only the amplified products of GYPA (197 bp, internal control), and the blank (no DNA template) did not show any products. (c) The sequence alignment of GP.Mur, GP.Hop/Bun, GYPA, GYPB, and GYPE; each target of the primers was underlined with the name above (FwVwA, Fw1N, and RvNN), and each target of the probes was shaded with the name above (RvB and RvA2).

AuNP DNA Probes

The AuNP preparation was prepared by citrate reduction, according to Enustun and Turkevich (14, 25). Fifty milliliters of 0.01% HAuCl₄ (0.3 mM) were boiled and stirred continuously. When the solution came to a boil, 1 ml of 1% trisodium citrate (38.7 mM, pH 8) was added. The solution was continuously boiled and stirred for 15–20 min until it turned a stable purple-red color; then, it was left to cool to room temperature. Conjugation of DNA probes to AuNPs was completed according to Hill and Mirkin (26).

In the first step (the conjugation of DNA probes to AuNPs), 1 ml of AuNPs (from the AuNP preparation section) was pipetted into a 2 ml tube for two tubes, followed by addition of 10 µl of 100 µM free thiol-modified RvB probe (ready-to-use probe) to one tube and 10 µl of 100 µM free thiol-modified RvA2 probe (ready-to-use probe) to the other tube (RvB and RvA2 probes were prepared in separate tubes). The tubes were wrapped in foil and shaken overnight. Then, 104 µl of 0.1 M sodium phosphate buffer (pH 7) and 11.5 µl of 10% SDS solution were added to each tube, and the

tubes were re-wrapped in foil and shaken for 30 min. Next, each tube had 29 µl of 2 M NaCl added to it every 8 h over a 48-h period (total six times, 174 µl of 2 M NaCl), and the tubes were re-wrapped in foil and continuously shaken each time. When this step was complete, the DNA probes were conjugated with the AuNPs into “AuNP DNA probes.” However, an excess of the unconjugated DNA probes remained in the solution; thus, the AuNP DNA probes required washing before use.

In the second step (washing of the AuNP DNA probes), the tubes were centrifuged at 13,000 × g for 20 min to sediment the AuNP DNA probes, the supernatant (the unconjugated DNA probes) was removed, and the pellet (the AuNP DNA probes) was washed with 1 ml of PBS buffer (pH 7.4) in each tube. The sedimentation and washing process was repeated a total of three times with the same PBS volume and centrifugation rate. After the last washing, the supernatant was removed, and 250 µl of PBS buffer (pH 7.4) was added to each tube for re-suspension of the AuNP DNA probes. The probes were stored at 4°C in foil before testing with the amplified products from PCR.

The Mi Series Identification by AuNP DNA Probes

Colorimetric detection by NaCl-induced aggregation of AuNP DNA probes was performed (20, 21). In 0.2 ml tubes, 5 μ l of RvB or 5 μ l of RvA2 AuNP DNA probe was added to the separate tubes, followed by 1 μ l of the amplified product (the PCR product) in each tube. The tubes were heated at 95°C for 2–3 min (denaturation step) and left to cool at room temperature for 15 min (hybridization step). Then, 6 M NaCl and H₂O were added to obtain the desired NaCl concentration at the 15 μ l final volume (aggregation step). After a waiting period of 40–60 min, the results were observed by visual inspection and/or OD absorbance measurement (NanoDrop spectrometry, UV-Vis mode).

Three final NaCl concentrations (2, 2.8, and 3.6 M) were used to determine the optimal NaCl concentration for each probe. To obtain the final 2 M NaCl, 5 μ L of 6 M NaCl and 4 μ L of H₂O were added. To obtain the final 2.8 M NaCl, 7 μ L of 6 M NaCl and 2 μ L of H₂O were added, and to obtain the final 3.6 M NaCl, 9 μ L of 6 M NaCl was added.

For the Mi series identification, the final 2.8 M NaCl concentration was applied for the RvB probe and the final 3.6 M NaCl concentration was applied for the RvA2 probe. The amplified products of the ten Mi(+) and ten Mi(–) samples were validated under these conditions. The overall process of the experiment is summarized in Figure 1a.

RESULTS AND DISCUSSION

Three primers of FwVwA, Fw1N, and RvNN were combined in the PCR reaction. The first pair, Fw1N and RvNN, targeted *GP.Mur*, *GP.Hop*, or *GP.Bun* (*GP.Mur/Hop/Bun*) and produced the 137-bp product. The second pair, FwVwA and RvNN, targeted *GYP A* and produced the 197-bp product (internal control), shown in Table 1. The PCR product of the Mi(+) (*GP.Mur/Hop/Bun*) sample showed both of the amplified products at 137 and 197 bp. The PCR product of the Mi(–) sample showed only the amplified product at 197 bp, and the PCR product of blank (no DNA template) showed no amplified product, as shown in Figure 1b. The Fw1N and RvNN primer pair also targeted *GP.Hut* or *GP.HF* (*GP.Hut/HF*) and produced the 140-bp product (10). However, *GP.Hut* has only rarely been found, and *GP.HF* has never been reported in the Thai population (9). Therefore, the probes in this study were designed to detect only the amplified product of *GP.Mur/Hop/Bun*, but they could not detect the amplified product of *GP.Hut/HF*.

Both the RvB and RvA2 probes were designed in the reverse sequence and hybridized to the forward strand of

the amplified product. The specificity of each probe was based on the sequence next to the (A)₁₀ spacer and was not related to either the 3' or 5'-thiol modification. The sequence of the RvB probe was composed of the same sequence as the *GYP B* gene, and the complementary target was present only in the amplified product of Mi(+) (*GP.Mur/Hop/Bun*). The sequence of the RvA2 probe was composed of the same sequence as the *GYP A* gene and the complementary target was present in both the amplified products of Mi(+) (*GP.Mur/Hop/Bun*) and Mi(–), as shown in Figure 1c. Therefore, the amplified product of Mi(+) could hybridize to both the RvB and RvA2 probes, the amplified product of Mi(–) could hybridize to only the RvA2 probe, and the blank (no amplified product) could not hybridize to any probes.

The color of the AuNP DNA probes was purple-red before NaCl-induced aggregation, with the OD absorbance peaks at approximately 530–540 nm. Based on the principle that AuNP DNA probes would increase their aggregating resistance (retaining the color or retaining the OD peak) when hybridizing with the complementary target, the optimal NaCl was therefore an important factor for distinguishing among the complementary target, the noncomplementary target, and no target. To determine the optimal NaCl concentration that could distinguish the amplified products of Mi(+) (*GP.Mur/Hop/Bun*), Mi(–), and the blank (no amplified product), three final NaCl concentrations (2, 2.8, and 3.6 M NaCl) were tested on each probe. At the concentration of 2 M NaCl, the RvB probe showed a slightly differentiated result for Mi(+), while the RvA2 probe showed no differentiated result, as shown in Figure 2a. At the concentration of 2.8 M NaCl, the RvB probe showed an obviously differentiated result for Mi(+), while the RvA2 probe showed a slightly differentiated result for the blank, as shown in Figure 2b. At the concentration of 3.6 M NaCl, the RvB probe also showed an obviously differentiated result for Mi(+) (but not as good as at 2.8 M NaCl), while the RvA2 probe showed an obviously differentiated result for the blank, as shown in Figure 2c. Therefore, the optimal NaCl concentration for the RvB probe was at 2.8 M NaCl, and for the RvA2 probe, it was at 3.6 M NaCl. The three factors that determined the vulnerability of AuNP aggregation were the final NaCl concentration, the sequence of the DNA probe, and the hybridizing target.

The RvA2 probe was required at the higher NaCl concentration (3.6 M NaCl) to differentiate the result of the blank (no amplified product) when compared with the RvB probe (2 M NaCl), as shown in Figure 2; thus, the RvA2 probe was considered to have greater aggregating resistance than the RvB probe when interacting with the primers in the blank. The two factors that contributed to the difference in the aggregating resistance were the

Fig. 2. The optimization of NaCl concentration for AuNP DNA probe detection by OD absorbance measurement. (a), (b) and (c) were the RvB and RvA2 probes at final concentrations of 2, 2.8, and 3.6 M NaCl, respectively, and each condition was tested with the amplified products of Mi(+) (GP.Mur/Hop/Bun), Mi(-), and the blank (no amplified product). The optimal NaCl concentration for the RvB probe was 2.8 M NaCl, and for the RvA2 probe, it was 3.6 M NaCl.

sequences of each probe (27) and the hybridizing and/or adsorbing primers with each probe (28). The time required to read the result was another key factor. After inducing aggregation, the color intensity

gradually decreased with time, and the color also gradually changed from purple-red to purple-blue and from purple-blue to clear. Even if the probe was hybridized with the complementary target, aggregation could still

Fig. 3. The Mi series identification by AuNP DNA probes: the RvB (test) and RvA2 (control) probes were performed at final concentrations of 2.8 and 3.6 M NaCl, respectively. (a) By visual inspection; (left) the amplified product of the Mi(+) (GP.Mur/Hop/Bun) sample showed the purple-red color on both probes, (middle) the amplified product of the Mi(-) sample showed the purple-red color only on the RvA2 probe, and (right) the blank (no amplified product) turned clear on both probes. (b) By OD absorbance measurement; (left) the Mi(+) retained the peaks on both probes, (middle) the Mi(-) retained the peak only on the RvA2 probe, (right) the blank (no amplified products) flattened the peaks on both probes.

occur, but at a slower rate than with the noncomplementary target or no target. Therefore, the results should be read between 40 min and 8 h after induced aggregation. The color should not be read before 40 min because the color is not settled, and the result read after 8 h should be ignored (having turned clear). The best time for detecting a clear-cut color was approximately 1 to 4 h after inducing aggregation. The higher NaCl concentration could be applied to accelerate the reaction—the RvB probe at 3.6 M NaCl and the RvA2 probe at 4 M NaCl—but the results should be read between 15 min and 2 h after inducing aggregation.

For the Mi series identification, both probes were necessary; the “RvB” probe served as the “test” probe to determine the Mi series status, while “RvA2” served as the “control” probe to verify the success of amplification. The results would be neglected if the RvA2 probe showed a negative result (indicated amplification failure). The amplified product of the Mi(+) (GP.Mur/Hop/Bun) sample retained the purple-red color on both the RvB and RvA2 probes (test+/control+). The amplified product of the

Mi(-) sample retained the purple-red color only on the RvA2 probe (test-/control+). The blank (no amplified product) turned clear color on both the RvB and RvA2 probes (test-/control-), as shown in Figure 3a. The results by OD absorbance measurement were concordant with the results by visual inspection. The Mi(+) sample retained the peaks on both probes, the Mi(-) sample retained the peaks only on the RvA2 probe, and the blank flattened the peaks on both probes, as shown in Figure 3b.

For validation of the Mi series identification, the amplified products of the ten Mi(+) (GP.Mur/Hop/Bun) and ten Mi(-) samples were tested. The results showed that the Mi series status was identified correctly in all of the samples. The RvB probe showed positive results (retaining the OD peak) in all of the Mi(+) samples, as shown in Figure 4a, while the RvA2 probe showed positive results in all of the samples (both Mi(+) and Mi(-)), as shown in Figure 4b.

According to the conventional PCR process, gel electrophoresis is a labor-intensive and time-consuming step.

Fig. 4. Validation of the Mi series identification by AuNP DNA probes with the amplified products of the ten Mi(+) (GP.Mur/Hop/Bun) and ten Mi(-) samples under OD absorbance measurement. (a) The RvB (test) probe retained the peaks in all of the Mi(+) samples. (b) The RvA2 (control) probe retained the peaks in all of the samples (both Mi(+) and Mi(-)).

AuNP DNA probe detection could replace gel electrophoresis, thus simplifying the conventional PCR process. AuNP DNA probe detection requires only NaCl solution and a water bath (or dry bath), in contrast with gel electrophoresis, which requires large amounts of equipment and reagents. Moreover, the specificity of the RvB probe distinguishes GP.Mur/Hop/Bun from GP.Hut/HF, whereas the gel electrophoresis could not. Therefore, the application of AuNP DNA probes in the conventional PCR process could improve the performance of analytical assay.

In conclusion, colorimetric detection by AuNP DNA probes was a simple technique for detecting complementary targets. The Mi series status could be identified by color alone under visual inspection and/or OD absorbance measurement. Furthermore, the AuNP DNA probes could be applied to a broad range of the analytical platforms (29, 30) and could serve as another tool for blood group genomic studies.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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