Development of cost-effective and accurate allele-specific PCR for determination of *M2/ANXA5* haplotype in recurrent pregnancy loss

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Abstract. Repeated Pregnancy Loss (RPL) affects approximately 1 out of 20 pregnant women globally; it is traumatic for parents seeking parenthood with ensuing anxieties for the next pregnancy. *M2/ANXA5* haplotype is a hereditary predisposition gene for thrombophilia-associated RPL; the association between *M2/ANXA5* haplotype and RPL is further ascertained in a recent meta-analysis. Precision treatments have been proposed for RPL women with *M2/ANXA5* haplotype. Therefore, screening for *M2/ANXA5* haplotype is highly recommended as a panel of laboratory investigations for idiopathic RPL. To date, direct sequencing of PCR products is the most common method for the determination of *M2/ANXA5* haplotype; this method is however tedious, expensive and time-consuming. Hereby, we demonstrate a simple and robust allele-specific PCR (AS-PCR) that detects two inherent SNPs in a single tube, which could serve as a routine genotyping tool for *M2/ANXA5* haplotype. This test is rapid, only taking maximum 4 working hours to complete the analysis. Validation of the assay by 105 clinical DNA samples yields 100% concordance rate with the DNA sequencing results.

Keywords: *M2/ANXA5* haplotype, repeated pregnancy loss, allele-specific PCR

INTRODUCTION

Repeated Pregnancy Loss (RPL) also named 'recurrent pregnancy loss' or, 'repeated spontaneous abortion' (RSA), is defined as two or more failed pregnancies (Practice Committee of the ASRM, 2008). RPL is a traumatic event for couples seeking parenthood (Sutan, Miskam, 2012). It affects approximately 5% of clinically defined pregnant women globally (The Practice Committee of the American Society for Reproductive, 2012). The etiology of RPL is complex which usually involved in polygenic background. Half of these cases have no apparent

causes, termed idiopathic RPL (Ford & Schust, 2009). Among the known causes, heritable thrombophilia is a major cause of idiopathic RPL. Factor V Leiden (FVL) and Prothrombin G20210A mutation (PTm) are the two most common predisposition factors for hereditary thrombophilia. However, their prevalence is rare in African and Asian descendants (Kujovich, 2010; Rosendaal *et al.*, 1998).

In 2007, *M2/ANXA5* haplotype, nucleotide substitutions in the ANXA5 promoter gene, has emerged as third hereditary predisposition gene

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for thrombophilia-associated RPL. Annexin A5 (ANXA5), a Ca^{2+} dependent anticoagulant protein, forms a highly ordered two-dimensional crystal structure at the surface of syncytiotrophoblasts of the chorionic villi to prevent coagulation activation. *M2/ANXA5* haplotype will result in reduced ANXA5 mRNAs (Chinni *et al.*, 2009; Markoff *et al.*, 2010) and its protein levels (Ota *et al.*, 2013) in placentas; this would cause insufficient coverage of PS and trigger thrombotic events leading to RPL (Bogdanova *et al.*, 2012). A recent meta-analysis across different geographic areas ascertains the association between *M2/ANXA5* haplotype and RPL, with the odds of 1.54 (95% CI: 1.08 to 2.20) (Ang *et al.*, 2019). Therefore, *M2/ANXA5* haplotype screening test is highly recommended for idiopathic RPL (Ang *et al.*, 2019).

Direct sequencing of PCR products is the gold standard for determination of *M2/ANXA5* haplotype. This method typically requires outsourcing to external sequencing facilities, causing a longer turnaround time up to a few days. Direct sequencing is tedious, laborious and time consuming. Probe-based qPCR and PCR with restriction enzyme (PCR-RE) have been reported as alternatives (Hayashi *et al.*, 2013; Nagirnaja *et al.*, 2015). Nevertheless, probe-based qPCR requires a real time PCR machine and expensive fluorescence reagents. PCR-RE is comparatively cheap as no sophisticated machine is used. However, there are challenges in adopting this method such as issues regarding the efficacy of RE and requirement of a specific short nucleotide recognition site that matches to the targeted single nucleotide polymorphisms (SNPs). Taking these into considerations, there is a need to develop an alternative method that is simple, cheap, and rapid but accurate for *M2/ANXA5* haplotype determination.

To achieve the desirous characteristics of the determination methodology for *M2/ANXA5* haplotype, this study aimed to develop an allele-specific PCR (AS-PCR) that specifically and simultaneously analyses two crucial inherent SNPs characterizing *M2/ANXA5* haplotype. Accuracy and specificity of the newly developed method was subsequently evaluated and validated by using clinical DNA samples against the gold standard direct sequencing method.

MATERIALS AND METHODS

Samples and genomic DNA extraction

The study was approved and complied with the ethical guidelines of the Human Ethics Research Committee of the Universiti Sains Malaysia (reference USM/KK/PPP/JEPeM [245.3. (2)]) and Malaysian National Medical Research Register (NMRR), ID: NMRR-11-1044- 9519. Subjects were recruited at the Universiti Sains Malaysia, Penang campus from January 2011 to May 2013 with appropriate informed consent. All subjects were of Malay origin verified across at least three generations that did not have intermarriage. They were healthy individuals with no history of medication therapy. Data of previous pregnancies and pregnancy losses were not recorded. Venous blood samples were collected and commercially available kit from Omega Bio-tek Inc., Norcross, USA was used to extract genomic DNA.

Design of allele-specific PCR

AS-PCR for *M2/ANXA5* haplotype was designed based on the principle of nested PCR. The first PCR primers were reported by Bogdanova *et al.* (2007). The second PCR reaction comprised of two independent allele-specific primers (AS-primers) for discriminating 'Normal' and *M2/ANXA5* haplotype, respectively.

Conditions of allele-specific PCR

The first PCR reaction was carried out as described in Bogdanova *et al.*, 2007 with minor modification. A total volume of 25 μL PCR reactions contained: 1 $\times$ PCR reaction buffer (Biotools, Spain); 0.2 μM of each primer (forward: ANXA5.P + ex1.Fwd and reverse: ANXA5.P + ex1.Rv); 200 μM dNTPs; 100 ng genomic DNA and 1 U *Taq* polymerase (Biotools, Spain). Concentrations of dimethyl sulfoxide (DMSO) ranged from 5 to 7.5%, and MgCl_2 (Biotools, Spain) was 1.0 mM to 1.5 mM. The initial denaturation was performed at 95°C for 5 min; followed by 25 cycles of denaturation at 95°C for 30 s; gradient annealing from 55°C to 65°C for 30 s; and extension at 72°C for 30 s. The final extension was performed at 72°C for 5 min.

Next, the second PCR reactions were carried out using 'Normal' primers (Nor -19G Fwd and Nor 76G Rv New) and M2/ANXA5 primers (M2 -19A Fwd and M2 76A Rv New), respectively, in the separate tubes to discriminate 'Normal' and M2/ANXA5 haplotype. The reaction mixture consisted of 2 µL of 10 × diluted first PCR product as template, 1× PCR reaction buffer (Biotools, Spain), 0.2 µM of each primer, 5 % DMSO and 1.25 U *Taq* polymerase. Concentrations of MgCl₂ (Biotools, Spain) ranged from 0.5 mM to 1.0 mM. The PCR cycling condition was similar to the first PCR reaction. Finally, the second PCR product was analyzed on 2% agarose gel in 1 × TAE at 100 V for 25 min. The amplicon size was 139 bp.

DNA sequencing

Verification of AS-PCR results was confirmed by DNA sequencing analysis. BigDye Terminator Cycle Sequencing Ready Reaction Kit (ABI/Perkin-Elmer, Weiterstadt, Germany) was used in the first PCR products from 105 clinical DNA samples and sequencing reactions were analyzed on an ABI PRISM[®] 3700 DNA Analyzer (ABI/Perkin-Elmer, Weiterstadt, Germany). FinchTV version 1.4 (Geospiza Finch Suite, Perkin Elmer) was used to view the analyzed DNA sequences.

RESULTS

Sequence specific primer for M2/ANXA5 haplotype

The reference sequence of *Homo sapiens* annexin A5 (ANXA5), was first retrieved from the Gen Bank (accession no. NG_032042.1). The nucleotide 1 is at the first transcription start point of the gene, tsp 1, which corresponds to nucleotide of 4956 of NG_032042.1; whereas the 5'-untranslated region (UTR) begins at -1 and proceeds in the negative direction (upstream from nucleotide 1). Figure 1 shows the constellation of four consecutive nucleotide substitutions of M2/ANXA5 haplotype. The first set of PCR primers (forward: ANXA5.P+ex1.Fwd and reverse: ANXA5.P+ex1.Rv) that span the region from promoter to 5'-UTR yielding an amplicon of 496 bp have been previously designed (Bogdanova *et al.*, 2007). The second set of AS-PCR primers were newly designed to detect the 3'-nucleotide mismatches at -19 G → A, on rs112782763 (Nor-19G-Fwd and M2-19A-Fwd) and at 76 G → A, on rs113588187 (Nor-76G-Rv New and M2-76A-New). The sequences of the primers and their expected amplicon sizes are listed in Table 1 and Figure 2. Then, 'Basic Local Alignment Search Tool' (BLAST) program was used to confirm the specificity of AS-primers.

Table 1. Primers for the AS-PCR of M2/ANXA5 haplotype.

Primer Name	Primer Sequence	Size	T _m	Amplicon size
ANX5.P +ex1.Fwd	5'- CCGAGCCCTGGACAGCTCCCCA-3'	22 bp	67.9 °C	439 bp
ANX5.P+ex1.Rv	5'- GCCCCGCGACCACGCTCTCCTCT- 3'	23 bp	69.5 °C	
Nor-19G-Fwd	5'-TGGCGCGGCCGGCCTGCGGTGG- 3'	23 bp	75.6 °C	139 bp
Nor-76G-Rv New	5'-GAGATGCAGACGCTGAAGGATCC- 3'	23 bp	59.6 °C	
M2-19A-Fwd	5'-TGGCGCGGCCGGCCTGCGGTGA- 3'	23 bp	74.7 °C	139 bp
M2-76A-Rv New	5'- GAGATGCAGACGCTGAAGGATCT-3'	23 bp	58.7 °C	

1 CCGAGCCCTG GACAGCTCCC CAGGCCCTTC CCGCGGCGCG AGGACAAGAG
 51 GTCTCCGGGG CCCTCGGGGG AGCGGCGCCT CCTCCTGGTT CCAGCAGCTC
 101 TGC GGCCGCT CCCACCCAG GCCCGCGAGA CCAGCGGGAC AGTCCGCGCC
 151 GCGGGAGACC AACTGGGACG AGCCGCGACC CACGCAGGCG CGCTGAGGCC
 201 GGGGCAGGGG CGGGCCCCGGC TGGCGCGGCC GGCCTGCGGT TG**GGG**CCCTG
 251 GCGGGGGTGG **G**ACGGGCCAA GCCGGGCAGG GCCGGGGTGG GGCCGCTGGC
 301 GTTTCGTTG CTTGGATCAG TCTAGGTGCA GCTGCCGGAT CCTTCAGCGT
 351 CTGCATCTCG GCGTCGCCCC GCGTACCGTC GCCCGGCTCT CCGCCGCTCT
 401 CCCGGGGGTT CGGGGCACTT GGGTCCCACA GTCTGG

Figure 1. Sequence of the *ANXA5* gene core promoter region. Circle is the *tsp1*. Bold capital letters are the constellation of 4 single nucleotide polymorphisms. *M2/ANXA5* haplotype is -19 G → A, rs112782763; 1 A → C, rs28717001; 27 T → C, rs28651243; and 76 G → A, rs113588187).

Figure 2. Schematic illustration of AS-PCR.

PCR conditions for M2/ANXA5 haplotype

Although the first set of PCR primers has been reported by Bogdanova *et al.*, 2007, optimization was carried out. As a high GC content (75.8%) was observed in the core promoter region of *ANXA5*; DMSO, a PCR enhancing agent, was added at 5 % and 7.5 % respectively. Five percent DMSO was determined as the optimal concentration to disrupt base pairing of gDNA and to ensure the primers hybridize specifically in this region (Figure 3A). Secondly, annealing temperature was determined at 65°C. The annealing temperature was increased gradually from 55°C to 65°C to eliminate non-specific products (Figure 3B) Increasing the concentration of MgCl₂ enhanced the processivity and efficiency of *Taq* DNA polymerase but had the drawback of producing non-specific amplification. The optimum amount of MgCl₂ in first PCR reaction was found to be 1.0 mM [Figure 3C(i)].

A similar amplification condition was applied for second PCR reaction, except the concentration of MgCl₂. The concentration of MgCl₂ was critical in second PCR reactions to ensure the allele-specific primers hybridize perfectly to SNPs characterizing 'Normal' and M2/ANXA5 haplotype. The optimum concentration of MgCl₂ in second PCR reaction was found to be 0.5 mM [Figure 3C(ii)], whereby only the designed AS-PCR primers yielded the expected PCR products in the presence of corresponding haplotypes and vice versa.

The final optimized condition for AS-PCR were as follows: first PCR reaction: 1x PCR reaction buffer (Biotools, Spain); 1 mM MgCl₂ (Biotools, Spain); 5% DMSO; 0.2 µM of each primer (forward: ANXA5.P + ex1.Fwd and reverse: ANXA5.P + ex1.Rv); 200 µM dNTPs; 100 ng genomic DNA and 1 U *Taq* polymerase (Biotools, Spain). Cycling conditions were as follows: initial denaturation at 95°C for 5 min; followed by 25 cycles of amplification; 95°C for 30 s; 65°C for 30 s; 72°C for 30 s. The final extension was performed at 72°C for 5 min; second PCR reaction: two independent allele-specific reactions ('Normal' and M2/ANXA5 haplotype) were carried out using 'Normal'

primers (Nor -19G Fwd and Nor 76G Rv New) and M2/ANXA5 primers (M2 -19A Fwd and M2 76A Rv New), respectively, in two separate PCR tubes. The final optimized protocol included 2 µL of 10 × diluted first PCR product as template, 1× PCR reaction buffer (Biotools, Spain), 0.5 mM MgCl₂ (Biotools, Spain), 0.2 µM of each primer, 5% DMSO and 1.25 U *Taq* polymerase with similar PCR cycling condition as mentioned above.

To determine the haplotype of a sample, the two second PCR products were analyzed on 2% agarose gel in 1× TAE at 100 V for 25 min. The amplicon size was 139 bp. Figure 3D shows the result of AS-PCR. Each sample consists of two lanes, the first lane is for 'Normal' haplotype allele-specific reaction whereas the second lane is for M2/ANXA5 haplotype allele-specific reaction. A 'Normal' haplotype sample has PCR products only at the first lane; M2/ANXA5 haplotype homozygous only at the second lane; and M2/ANXA5 haplotype heterozygous in both lanes.

The accuracy and specificity of our established AS-PCR was further validated by using 105 clinical DNA samples. Table 2 shows 100% concordance rate between AS-PCR results as compared to the gold standard of direct sequencing of PCR products results. Figure 4 shows some examples of the sequencing result for samples determined to be A) Homozygous 'Normal' haplotype; B) Heterozygous; C) Homozygous M2/ANXA5 haplotype based on AS-PCR. The AS-PCR could discriminate M2/ANXA5 haplotype for DNA samples derived from lysed blood.

Table 2. Result of AS-PCR assay and DNA sequencing from 105 DNA samples.

Genotype	AS - PCR	DNA sequencing
N/N	68	68
N/M2	32	32
M2/M2	5	5

Figure 3. **A)** Optimization of the concentration of DMSO for first AS-PCR reaction; **B)** Optimization of the annealing temperature for first AS-PCR reaction; **C(i)** Optimization of MgCl₂ for first AS-PCR reaction; **(ii)** for second AS-PCR reaction by using *M2/ANXA5* primers for ‘Normal’ sample. Note, at 0.5 mM MgCl₂, the expected “negative” result was obtained for *M2/ANXA5* primers in ‘Normal’ haplotype; **D)** Agarose gel visualizing the products of AS-PCR.

DISCUSSION

RPL is a common clinical problem in reproductive health whereby as many as 50% of cases remain unexplained (Ford, Schust, 2009). *M2/ANXA5* haplotype, the third hereditary predisposition gene for thrombophilia-associated RPL (Ang *et al.*, 2017), has been shown to be associated with unexplained RPL cases. The prevalence ranges from 11 % to 42 % among healthy individuals (Ang *et al.*, 2017; Aranda *et al.*, 2018; Bogdanova *et al.*, 2007; Demetriou *et al.*, 2015; Hayashi *et al.*, 2013; Miyamura *et al.*, 2011; Nagirnaja *et al.*, 2015; Rogenhofer *et al.*, 2012;

Tiscia *et al.*, 2009; Tüttelmann *et al.*, 2013). A recent meta-analysis demonstrates that a *M2/ANXA5* woman carrier has 1.54 times the odds to experience RPL. Male partners with *M2/ANXA5* haplotype are also implied to carry similar risk for RPL (Ang *et al.*, 2019). Recent studies have also proposed that low-molecular-weight heparin (Ang *et al.*, 2017; Fishel *et al.*, 2016; Rogenhofer *et al.*, 2017) and micromolar zinc intake (Danisik *et al.*, 2019) could be the precision anticoagulant interventions for *M2/ANXA5* haplotype-associated RPL. Therefore, screening for *M2/ANXA5* haplotype for patients afflicted by RPL is recommended as a panel of laboratory investigations (Ang *et al.*, 2019).

Figure 4. Result of AS-PCR and DNA sequencing **(A)** Homozygous ‘Normal’ haplotype, U042; **(B)** Heterozygous, U048; **(C)** Homozygous *M2/ANXA5* haplotype, U070.

Although there are several methods reported over the years to determine *M2/ANXA5* haplotype, namely direct sequencing of PCR products, probe-based qPCR and PCR-RE, these methods have their inherent strength and drawback. As aforementioned, from the economical, logistic and technical perspectives, direct sequencing of PCR products and probe-based qPCR are only available in well-equipped laboratories with considerable cost per test. Although PCR-RE is relatively cheap, it requires proper storage of RE, especially those isolated from mesophilic bacteria that are temperature-sensitive for their activities. Succeeding, a high amount of PCR products was used; partial RE digestion may occur and lead to false-negative results (which showed undigested PCR products on gel electrophoresis). Therefore, to address the issue of RPL, a simple, cheap, and rapid but accurate for *M2/ANXA5* haplotype determination test is desirous in order that the test could be widely and readily applied. In this study, allele-specific PCR has been developed, fulfilling the desirous cost-effectiveness and diagnostic accuracy.

PCR is the common and affordability technique for the detection of genetic polymorphism. In general, it demands the optimization for its specificity and sensitivity for basic research and

clinical application. In this study, the region of *M2/ANXA5* haplotype is rich in GC content (75.8 %); thus, a stable secondary structure is formed. The nature of the sequence also resulted multiple bands on a stained agarose gel were observed in first PCR reaction. We tried but failed to adjust by the concentration of standard PCR components or cycling conditions; therefore, a PCR enhancing agent is needed in PCR amplifications to increase yield, specificity and consistency. DMSO, glycerol, formamide, N,N,N-trimethylglycine (betaine), non-ionic detergents, bovine serum albumin, polyethylene glycol and tetramethylammonium chloride are the commonly used PCR enhancing agents. Among these, DMSO enhances the specificity and productivity of PCR reaction, via decreasing the reannealing inter- or intra-strand and formation of the secondary structure (Susan Frackman, Gary Kobs, Simpson, Storts, Corporation, 1998), by reducing the melting temperature of the primers and expedites the strand separation, providing more efficient amplification. In this study, optimal concentration of 5 % DMSO was determined, it was within the recommended range of 1 - 10 % (Grunenwald, 2003, Kolmodin, Birch, 2002).

Secondly, an adequate annealing temperature is an important factor for consideration. However,

there is no proper method to determine annealing temperature of a PCR reaction. In general, annealing temperature is set 5 °C below the T_m of the primer. In spite of that, the annealing temperature is mostly determined empirically (Michael, Simon, 2010). A temperature of 55 °C is commonly used, hence the gradient temperature was set from 55 °C to 65 °C in this study. Reactions at the annealing temperature from 55 °C to 61 °C showed unspecific amplification whereas 63 °C to 65 °C showed specific amplification with little smearing. Therefore, the annealing temperature was set at 65 °C.

Thirdly, adjusting the concentration of $MgCl_2$ was the first to consider eliminating the slight smearing below the targeted band. It is known to influence the specificity and efficiency of PCR amplification (Michael, Simon, 2010) by the interaction of both magnesium-binding reaction components, such as template DNA, primers, and dNTPs, and the residues of chelators, e.g. EDTA in PCR reaction (Grunenwald, 2003). In general, the concentration of $MgCl_2$ in PCR reactions ranges between 0.5 and 2.5 mM; too high or too low concentration of $MgCl_2$ in the reaction, unspecific amplification or little to no amplification will be observed in stained agarose gel. To avoid unspecific PCR products that will affect the second PCR reaction, the concentration of $MgCl_2$ was adjusted from 1.5 mM to 1.0 mM without affecting the amplification.

In addition, $MgCl_2$, an essential cofactor, may affect primer annealing, the activity and fidelity of *Taq* DNA polymerase and melting temperatures as well as the formation of artifacts (Grunenwald, 2003, Kolmodin, Birch, 2002). Therefore, optimization of the concentration of $MgCl_2$ in the second PCR reactions was a prerequisite to obtain accurate AS-PCR reaction. A range of magnesium molarities in separate reactions were tested to yield the expected AS-PCR products in the presence of corresponding haplotypes. The concentration of $MgCl_2$ in the second PCR reaction was 0.5 mM, this was the best concentration to discriminate two SNPs in *M2/ANXA5* haplotype in a single tube without affecting the PCR performance. A conceivable explanation of requiring extremely low $MgCl_2$ concentration is the spurious annealing of primers

to incorrect template sites is greatly reduced and thus the increased specificity and the desired PCR products (Lorenz, 2012). A 10 × dilution of first PCR product was needed to eliminate all non-specific bands in second PCR reaction.

In conclusion, AS-PCR based on the principle of nested PCR was successfully developed to determine *M2/ANXA5* haplotype. It is simple, cheap and rapid. Validation by 105 clinical samples showed 100% concordance rate to DNA sequencing method. The developed AS-PCR would greatly enhance the availability and affordability to determine *M2/ANXA5* haplotype for couples suffered from RPL and thus provide better management for these patients.

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