Original Article

Electrophoretic Techniques for the Detection of Human Microsatellite D19S884

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Submitted: 8 Jul 2014
Accepted: 24 Jan 2015

Abstract

***Background*: The detection and analysis of microsatellites is very important for the mapping of genetic diseases because they are commonly used as genetic markers. Microsatellite marker D19S884 has been associated with polycystic ovary syndrome (PCOS), the most common reproductive endocrine disease of women in their childbearing years. It is responsible for an estimated 70% of cases of anovulatory infertility. In this work, we detected microsatellites in DNA extracted from the blood of PCOS patients.**

***Methods:* DNA microsatellites were amplified by polymerase chain reaction (PCR) using a pair of specific primers tagged with fluorescence to yield products of 160–200 base pairs in length. Alleles were separated on 4% low-melting agarose gels; stained with a safe gel staining, GelRed™, which is an alternative to ethidium bromide; and visualised by ultraviolet illumination.**

***Results:* Bands were observed, but their base-pairs differences were difficult to distinguish. To identify each allele clearly, the PCR products were also analysed using capillary gel electrophoresis for fragment analysis where it was possible to discriminate even in case of difference between two pairs of bases between the alleles.**

***Conclusion:* In this article, we present a protocol that combines the use of gel electrophoresis and fragment analysis in the identification of genetic biomarkers for PCOS.**

Keywords: *microsatellite, D19S884, low-melting agarose gel, FBN3, GelRed, fragment analysis*

Introduction

Microsatellites are short repetitions of nucleotides in the genome. They are tandemly repeated segments of base pairs at a unique physical location in the genome, and tend to occur in non-coding deoxyribonucleic acid (DNA). A microsatellite varies among individuals as shown by patterns of inheritance tracked through families (1,2). Microsatellite analysis is being used in the study of genetic factors that determine complex diseases (3). Polycystic ovary syndrome (PCOS) is the most common reproductive endocrine disease of women in their childbearing years. It is responsible for an estimated 70% of cases of anovulatory infertility. The World Health Organization estimates that, as of 2010, it affected 116 million women worldwide (4). Although the mode of inheritance remains unclear, PCOS is likely a complex endocrine disorder involving several genes (5,6). Although more than 70 candidate genes have been studied, PCOS status has been associated through evidence with only one locus on chromosome 19p13.2 (D19S884) (7,8).

D19S884 is a dinucleotide repeat polymorphism ((CA)n) mapping to chromosome 19p13.2. It is located very close to the insulin receptor gene (INSR) (9). D19S884 is located exactly in intron 55 of the fibrillin 3 gene (FBN3). Women with PCOS and one or two alleles of allele 8 (FBN3+) have significantly elevated fasting insulin levels, and homeostasis model assessment of insulin resistance values indicates that they are more insulin resistant than affected women with all other alleles of D19S884 (FBN3-) (8). Several studies, which predominantly focus on Caucasians using independent patient cohorts, have investigated the association between PCOS and the D19S884 marker (8–10). The length polymorphism of a microsatellite marker is commonly detected through polymerase chain reaction (PCR) amplification using pairs of specific primers flanking tandem arrays of microsatellite repetitions and proceeded by electrophoresis (11). Horizontal electrophoretic analyses are commonly performed using agarose gels. However, denatured vertical polyacrylamide gels are often preferred due to their higher resolution (12).

Fragment analysis applications are those in which fluorescent fragments of DNA (produced by PCR using a pair of specific primers tagged with fluorescence) are separated using capillary

electrophoresis and sized according to a size standard. Usually, genetic marker analysis experiments rely on detection of changes in the length of a specific DNA sequence to indicate the presence or absence of a genetic marker. In this analysis, the sequence of the gene is not directly analysed, but the presence of a particular allele or mutant version of the allele of the gene is inferred from the presence or absence of a linked DNA sequence, which can serve as a marker for the allele. Genetic markers are usually polymorphic genetic sequences contained in or near an allele of interest, such as microsatellites or restriction fragment length polymorphisms which allow the chromosomal alleles to be distinguished (13,14). Here, we describe applications of low melting agarose (LMA) gel electrophoresis and capillary electrophoresis for D19S884 genotyping in PCOS patients.

Material and Methods

DNA extraction

For isolation of genomic DNA, patients' blood samples were collected aseptically in vacutainers containing anticoagulant solution. The genomic DNA was extracted from 200 µL of blood using QiaAmp Blood Mini Kit (Qiagen) according to the manufacturer's instructions. The quality of the DNA extracted was checked by agarose gel electrophoresis, and the quantity was checked by Multiskan™ GO Microplate Spectrophotometer (Thermo Scientific) and stored at -20°C. Ethics approval for this study was obtained from the Medical Research Ethic Committee, Ministry of Health of Malaysia (NMRR-13-206-15132).

Polymerase chain reaction

Microsatellite loci are amplified by PCR using fluorescently labeled forward primers and unlabeled reverse primers. A pair of microsatellite primers was custom synthesised at First BASE Laboratories Sdn Bhd (Selangor, Malaysia) and utilised in the study for amplification of PCOS markers. PCR primers for amplifying the D19S884 marker were 5'ACATTGGCTCACACAACTGCT -3' (forward) and 5'-GCTCAGGGTCATGTGTCTGTACT-3' (reverse, marked with FAM) (Table 1). PCR amplification was carried out in a total volume of 25 µL containing 150 ng template DNA, 0.2 µM of each primer, and 1.5 µL of PCR Master Mix (Type–It Microsatellite Kit, Qiagen). PCR was performed in a GeneAmp PCR System 9700 thermal cycler (Applied BioSystem) as follows: an initial denaturation step at 95 °C for 5 minutes, denaturation at 90 °C for 30 seconds, annealing at 60 °C for 90 seconds, and extension at 72 °C for 30 seconds. For 28 cycles, the extension temperature of 72 °C was held for 30 minutes.

Gel electrophoresis

First, the PCR products were run in 2%, 3%, and 4% (weight/volume) LMA gel in 1X Tris/Borate/EDTA (TBE) buffer at 100V at 90 minutes for gel electrophoresis optimisation. Then the PCR products were electrophoresed on a 4% LMA gel (Fisher Scientific) using 1X TBE buffer at 100 V for 1 hour and 25 minutes. O'Range Ruler 20 base pair DNA Ladder (Thermo Fisher Scientific Inc.) was used as the DNA standard. Bands were visualised using the Gel Doc EZ Imager (Bio-Rad Laboratories Inc.). All gels were stained with GelRed™ (Biotium, Hayward, CA, USA), a safe and sensitive alternative to ethidium bromide size determination in agarose gel electrophoresis. The images were processed and bands were analyzed with Image Lab Software 3.0 (Bio-Rad Laboratories Inc.) to obtain the size of the base pairs of the PCR products.

Capillary electrophoresis for fragment analysis

Each PCR product was analysed at First Base Laboratories (Selangor, Malaysia) for fragment analysis. The fluorescent PCR products were assayed by capillary electrophoresis and visually analysed using Applied Biosystem Genetic Analyzer with GeneMapper® v4.0 by Microsatellite Analysis Software.

Results

Using three random samples, we observed that LMA gel with 4% (w/v) run in 1XTBE buffer gave better separation of the bands compared to others (Figure 1). Next, gel electrophoresis was

Table 1: Primers used for PCR and microsatellite genotyping

Gene or locus	Genebank accession number	Location of amplicon	Forward primer (5' –3')	Reverse primer (5' –3')
D19S884	NC_000019	62 185–62 353	GGAGTTGCTCAGGGTC	TCCCTCAACCCCCCGAGTTC

carried out to analyse sixteen PCR products using 4% (w/v) LMA. As can be seen in figure 2, the PCR products migrated between the bands of 160 bp and 200 bp. The estimation of the amplicons was based on the DNA ladders. The gel electrophoresis peak profiles for representative samples 1–4 are presented in figure 3.

Using capillary electrophoresis, the fluorescent PCR products (as electrophoresed in figure 2) were visually analysed using Applied Biosystem Genetic Analyzer. The results indicated its potential to allelic discrimination even in case of difference between two pairs of bases between the alleles (Figure 4). This standardised method

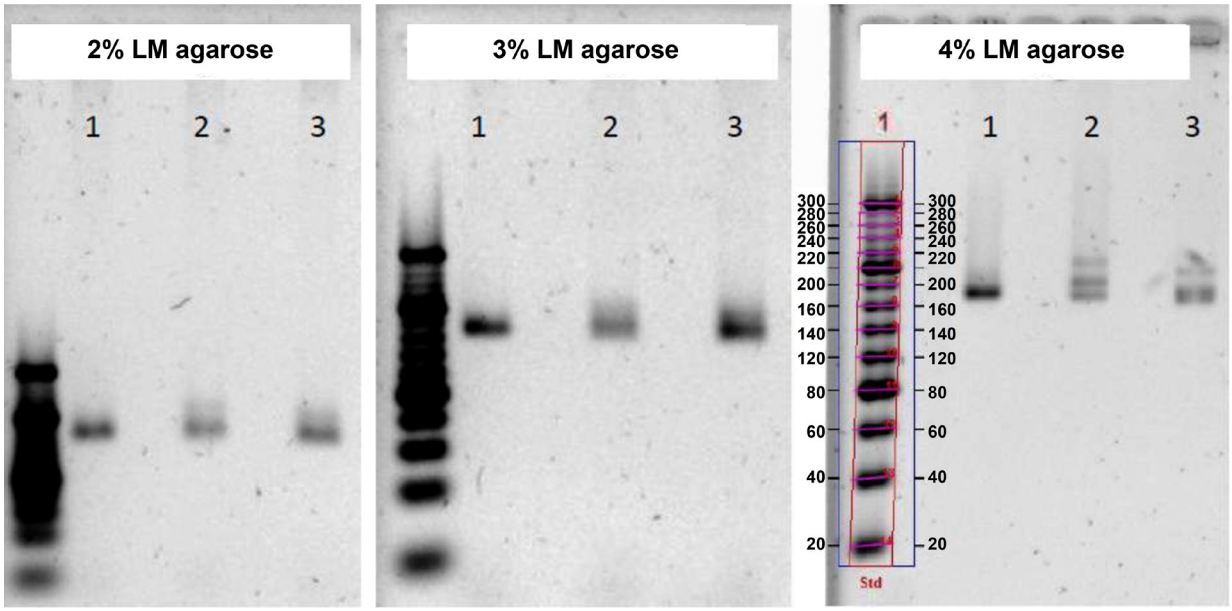

Figure 1: PCR products and 20 bp DNA ladder run in 2%, 3%, and 4% LMA in 1x TBE at 100 V at 90 min.

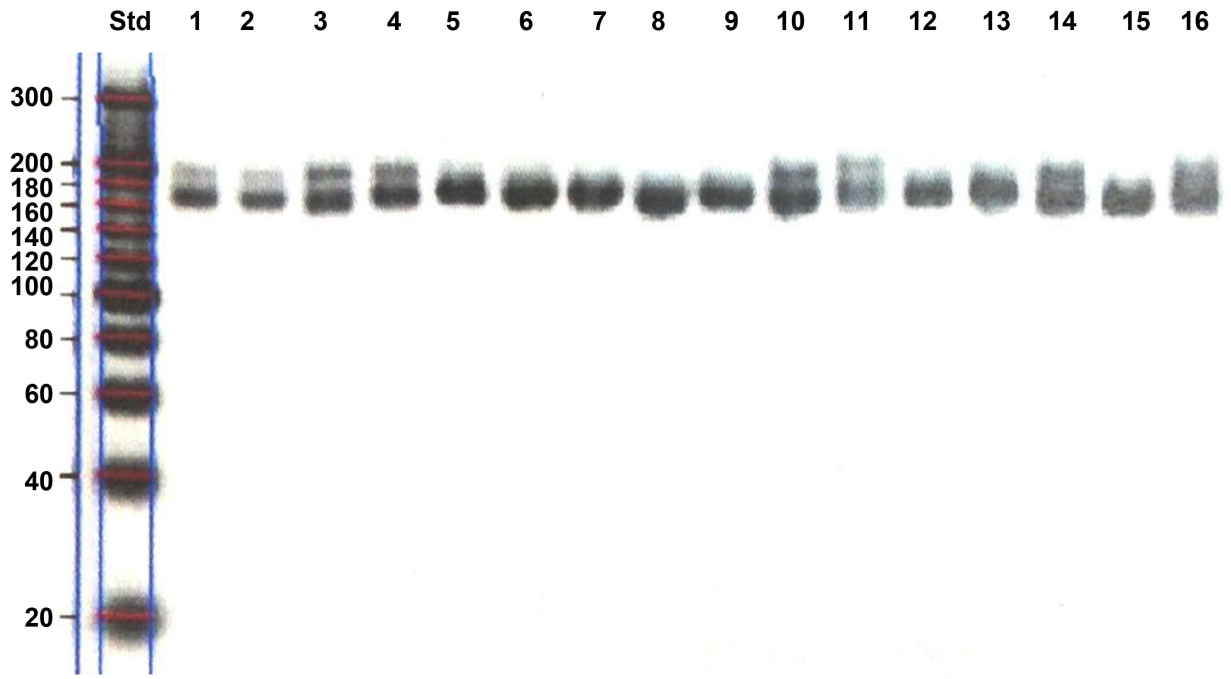

Figure 2: Gel electrophoresis of PCR products in 4% (w/v) LMA gel in 1x TBE buffer at 100 V for 1 hour 25 minutes.

was reproducible and precise.

Multiple alleles have been found at the D19S884 locus. Their amplified sizes are presented in table 2. The number of CA-repetitions is based on a previous study by Urbanek et al. (2009) and summarised in table 3. Because only four samples were used as representative profiles, there were differences between observed allele size obtained by LMA gel electrophoresis and fragment analysis (Table 4).

Discussion

In this work, we employed electrophoresis and signal visualisation techniques in order to determine the reproducible and precise protocols for analysing existing polymorphism in the D19S884 of the FBN3 gene from DNA samples in peripheral blood.

We proved that the primers design in the study by Prodoehl et al. (15) worked for the PCR amplification. The primers were used instead of amplimer AFMa299zc5 as listed in Genbank (National Institute of Health genetic sequence database) (Table 3). The PCR products visualisation using the GelRed™ Gel Stain is also proven to be safer than that using ethidium bromide (16). Although the banding patterns were observed and measured using gel electrophoresis, which suggests that the banding sequence of this microsatellite (CA)n exhibits allelic dimorphism, it is still not the best technique to determine the amplicon size; therefore, the size of the CA-repeats. Therefore, fragment analysis using capillary electrophoresis is needed for more precise analysis.

In our analysis, capillary electrophoresis has also been used to determine the length of

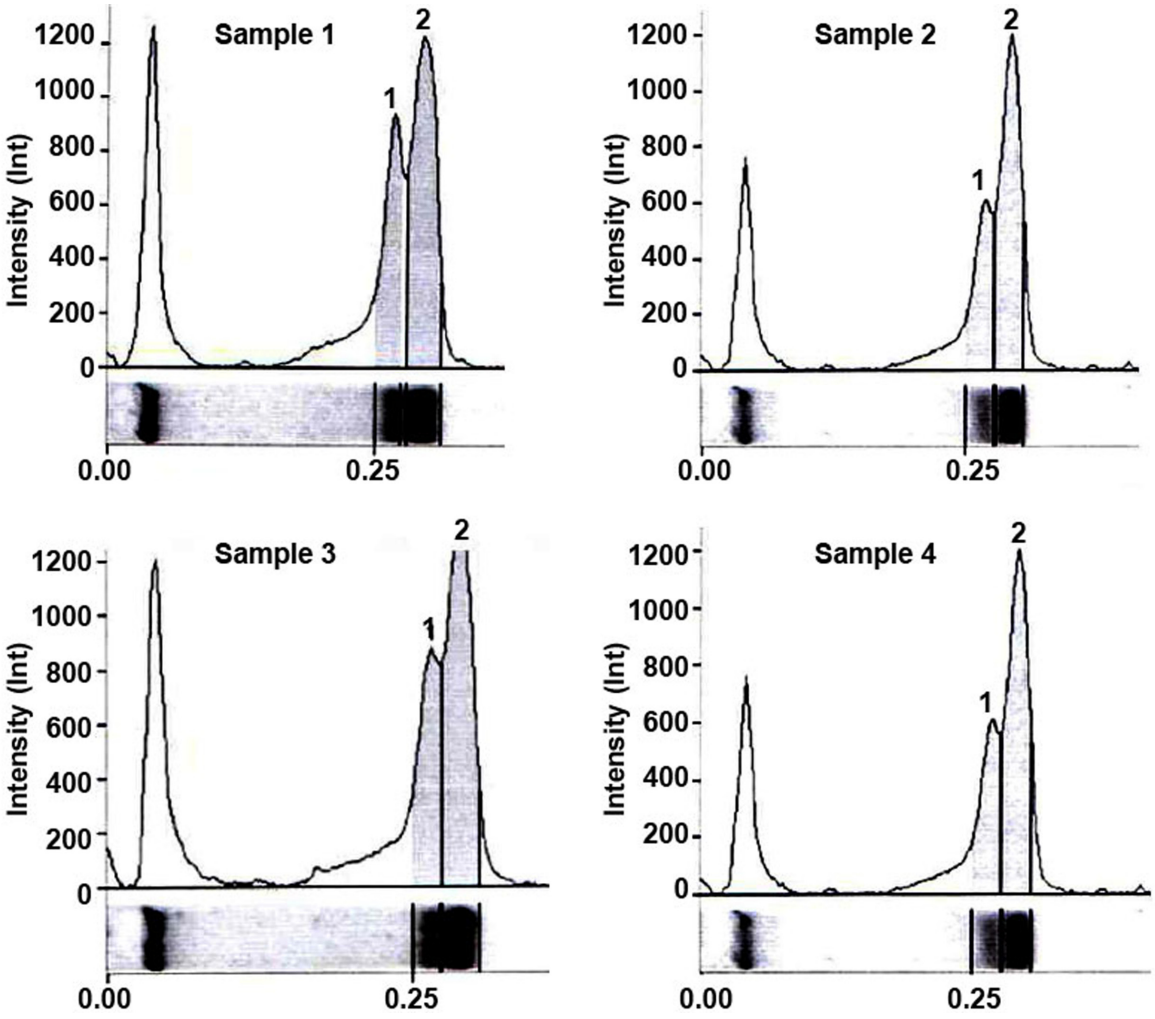

Figure 3: Gel electrophoresis peak profile for sample 1–4 analysed using Gel Doc EZ Imager and Image Lab Software 3.0 (Bio-Rad Laboratories Inc.).

polymorphisms of microsatellite markers (17). This method requires highly sophisticated instruments and fluorescently tagged primers, which are quite expensive. The results are confirmed to be precise and reliable.

Even though there were size deviations between LMA gel electrophoresis and fragment analysis (Table 3), gel electrophoresis is highly relevant because the technique is simpler for band confirmation before proceeding to the higher-cost technique of capillary electrophoresis.

Capillary electrophoresis is an analytical technique that separates ions based on their electrophoretic mobility with the use of an applied voltage, and it gives faster results and provides high-resolution separation (18).

Combining the methods of low-melting agarose gel electrophoresis preliminarily to confirm the success of PCR and fragment analysis by capillary electrophoresis described here, we were able to analyse the microsatellite D19S884 polymorphism of the CA repetition in FBN3. Previous studies in India (19) and China (20) have been able to determine the prevalence of PCOS amongst females in those countries. However, as yet there is no published data on the prevalence of this disorder in Malaysia. In order to investigate whether the (CA)n polymorphism in FBN3 also increases susceptibility to PCOS among Malaysian women, frequencies of these alleles have to be determined and a further association study should be performed using these techniques.

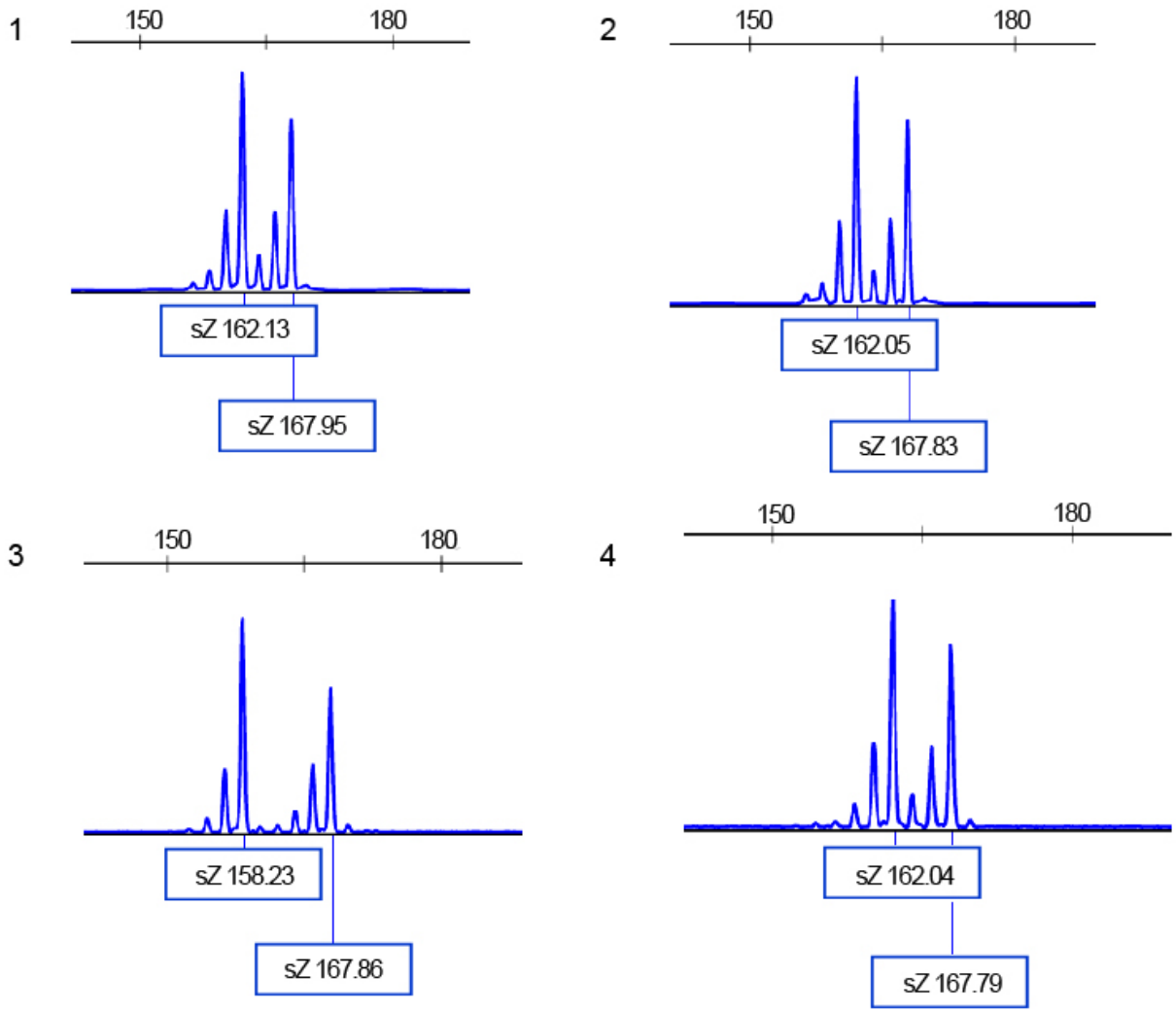

Figure 4: Electropherograms for D19S884 microsatellites in sample 1–4 (as run in gel electrophoresis in figure 3). At each peak, amplified fragment sizes are given in base pairs. Please see table 3 for allele size range; only a representative profile is shown here.

Table 2: D19S884 (CA)n microsatellite polymorphism allele detected in samples (Sample 1–16) analysed using capillary electrophoresis. (The number of CA-repeat is based on Urbanek et. al. (2009)

Sample	No of CA repeat	Amplicon size	Allele identification no.
1	15	162	A6
	18	168	A9
2	15	162	A6
	18	168	A9
3	14	160	A4
	18	168	A9
4	15	162	A6
	18	168	A9
5	18	168	A9
6	16	164	A7
7	18	168	A9
8	14	160	A5
9	16	164	A7
10	14	160	A5
	18	168	A9
11	15	162	A6
	22	176	A13
12	17	166	A8
13	18	168	A9
14	14	160	A5
	14	168	A5
15	14	160	A5
16	15	162	A6
	20	172	A11

Table 3: For each allele in microsatellite D19S884, this list provides information on the sizes of the amplicons using the current primers. The numbers of CA repeats and the identification numbers of alleles are based on previous study (Urbanek et al., 2005)

Amplicon sizes	Number of CA repeats	Allele identification no.
160	14	A5
162	15	A6
164	16	A7
166	17	A8
168	18	A9
170	19	A10
172	20	A11
174	21	A12
176	22	A13
178	23	A14

Table 4: Allele size (bp) comparison between LMA gel electrophoresis and fragment analysis. Only 4 representative samples shown here

Samples	Gel electrophoresis		Fragment analysis	
	Band 1	Band 2	Band 1	Band 2
1	191	169	168	162
2	186	167	168	163
3	193	167	168	158
4	193	174	168	162

Acknowledgement

The authors would like to thank the Director General of Health, Ministry of Health, Malaysia for supporting the work described in this paper and permission to publish this paper. We are also grateful to Assoc. Prof. Dr. Hanifullah Khan of Cyberjaya University College of Medical Sciences, for providing blood samples for this study.

Conflict of Interest

None.

Funds

This work was supported by grant from the Ministry of Health, Malaysia (Grant no. NMRR-13-206-15132; JPP-IMR: 12-042).

Authors' Contributions

Conception and design, drafting of the article: FM
Analysis and interpretation of the data: FM, LAZ, SSH
Administrative, technical or logistic support, collection and assembly of data: LAZ, SSH

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