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Research paper

Analysis of multiple single nucleotide polymorphisms (SNP) on DNA traces from plasma and dried blood samples

Arnold Catsburg [a], Wil C. van der Zwet [a,b], Servaas A. Morré [c,d], Sander Ouburg [c], Christina M.J.E. Vandenbroucke-Grauls [a,e], Paul H.M. Savelkoul [a,*]

[a] *Department of Medical Microbiology and Infection Control, VU University Medical Center, Amsterdam, The Netherlands*
[b] *Department of Medical Microbiology and Infectious Diseases, Deventer Ziekenhuis, Deventer, The Netherlands*
[c] *Laboratory of Immunogenetics, Section Immunogenetics of Infectious Diseases, Department of Pathology and Department of Internal Medicine, Section Infectious diseases, VU University Medical Centre, Amsterdam, The Netherlands*
[d] *Department of Medical Microbiology, Academical Hospital Maastricht, Maastricht, The Netherlands*
[e] *Department of Medical Microbiology, Academic Medical Centre, Amsterdam, The Netherlands*

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Abstract

Reliable analysis of single nucleotide polymorphisms (SNPs) in DNA derived from samples containing low numbers of cells or from suboptimal sources can be difficult. A new procedure to characterize multiple SNPs in traces of DNA from plasma and old dried blood samples was developed. Six SNPs in the Mannose Binding Lectin 2 (*MBL2*) gene were chosen as targets for analysis.

DNA was extracted from plasma obtained from mothers ($n=49$) and their neonates ($n=49$) and from old dried blood samples ($n=204$). Multiple Real-Time SNP analyses in the *MBL2* gene were carried out on all samples. Because of very low DNA concentrations in most of the samples, a pre-amplification step was utilized.

It was possible to analyze all plasma samples ($n=98$), including those with very low cell numbers ($n=21$) and 93% of the old dried blood samples ($n=189$). Results obtained from pre-amplified samples were in full agreement with neat samples. All possible SNP alleles were present in our population. The frequencies of the different alleles from both plasma and dried blood samples ($n=287$) were in agreement with earlier studies of the Caucasian population.

In conclusion, amplification prior to Real-Time PCR SNP analysis is a convenient, cost effective and useful method to significantly improve the reliable SNP detection in specimens containing very low concentrations or poor quality DNA from suboptimal sources.

Keywords: Genotyping; MBL; SNP; Traces of DNA

1. Introduction

Single nucleotide polymorphisms (SNPs) are the most frequent occurring manifestation of genetic variation in the human genome. Approximately one out of every 1000 nucleotides in the human genome is expected to be a SNP site, accounting for more than

Abbreviations: MBL, Mannose Binding Lectin; MGB, Minor Groove Binding; SNP, Single Nucleotide Polymorphism; WBC, White Blood Cells.

* Corresponding author. Department of Medical Microbiology and Infection Control, VU University Medical Center, Postbox 7057, 1007 MB Amsterdam, The Netherlands. Tel.: +31 20 4440552; fax: +31 20 4440473.
E-mail address: p.savelkoul@vumc.nl (P.H.M. Savelkoul).

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90% of all differences between humans. SNPs contribute to the variation in human phenotypes, such as disease susceptibility, responses to drugs and environmental chemicals, and susceptibility to infection (Landegren et al., 1998; Twyman, 2004; Zhou et al., 2005). SNP identification and functional assessment is becoming an increasingly more important tool in molecular diagnostics and biology. Several different genotyping approaches are in rapid development, such as fluorescence homogenous assays, pyrosequencing, Real-Time PCR and mass spectrometry (Alderborn et al., 2000; Griffin and Smith, 2000; Livak, 1999).

At present, Real-Time PCR is the most used technology for detection of SNPs (Kwok, 2002). This assay requires only a small amount of purified DNA. In general, the amount of human DNA in clinical samples is more than sufficient. However, under some circumstances, e.g. when the sources of DNA are samples with very small cell numbers or older samples that have not been stored under optimal conditions for DNA preservation, the amount of DNA may be too low to yield reliable SNP results.

We evaluated a new procedure to characterize multiple SNPs in traces of DNA from plasma and old dried blood samples, using the *MBL2* gene as proof of principle. This gene encodes the Mannose Binding Lectin 2, a C-type lectin that plays a role in the innate immune response to infections. MBL has a bouquet-like structure and it binds repeating oligosaccharides present in a wide variety of bacteria and other microbes. This is followed by a conformation change of Mannose Associated Serine Protease which results in the activation of the lectin pathway of the complement system (Kilpatrick, 2002). As a consequence, the microorganism is neutralized. MBL deficiency and low levels of serum MBL are strongly associated with the presence of six SNPs in the *MBL2* gene (Madsen et al., 1995).

In this study, a new procedure that utilizes a pre-amplification step to improve the reliable identification of multiple SNPs in traces of DNA from plasma and old dried blood samples was validated using the *MBL2* gene as target.

2. Materials and methods

2.1. Blood samples

The samples consisted of plasma specimens collected from 49 mothers and their neonates, and 204, 3–5 year-old, blood spots on Guthrie cards from newborn children. All persons were of Dutch Caucasian ethnicity. The study was approved by the local ethics committee. Parents of eligible neonates were asked for permission (informed consent) to use the Guthrie card of their child.

2.2. DNA extraction

DNA was isolated from plasma samples with the QIAamp Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions using 100 μl of elution buffer. Dried blood was eluted from the Guthrie cards by soaking three discs (3 mm in diameter) punched from each card in 200 μl of TE buffer (10 mM Tris–Cl, pH 8.3, 1 mM EDTA) at 56 °C for 10 minutes with shaking. Samples were centrifuged at 13000 rpm for 10 min; the supernatant fractions and the DNA extracted from plasma samples were stored at −20 °C until use.

2.3. Construction of allelic controls

DNA samples derived from four subjects with known homozygous haplotypes (designated as LXPA (GenBank accession no. Y16580), HYPD (GenBank accession no. Y16581), LYPB (GenBank accession no. Y16579) and LYQC (GenBank accession no. Y16578) (Kilpatrick, 2002) {kindly provided by Dr. A. Roos, Leiden University Medical Center}) were used as controls. Two primers (FMBL2 5′CTG CCA GGG CCA ACG TAG T 3′ and RMBL2 5′GCC CAA CAC GTA CCT GGT TC 3′) were designed to amplify an 876-bp segment flanking the *MBL2* gene including the six SNP sites. Amplification was performed in 50 μl reaction mixtures containing 5 μl DNA template, 1×PCR buffer,1.5 mM $MgCl_2$, 200 μM dNTP, 2 U Taq polymerase (Roche Diagnostics, Mannheim, Germany), 0.5% (w/v) dimethyl sulfoxide (DMSO, Sigma Chemical Co. (St. Louis, MO, USA) and 900 nM of each primer. PCR products were purified with the QIAquick Gel Extraction Kit (Qiagen) after separation in 1% agarose. Purified products were cloned in a pGEM-T-Easy vector (Promega Benelux b.v., Leiden, the Netherlands). Recombinant vectors were transformed in *E. coli* DH5α. After extraction the plasmids were verified for the correct haplotypes by sequencing the recombinant vectors with the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol. The sequence analysis was performed using an ABI PRISM 3100 genetic analyzer (Applied Biosystems).

Serial dilutions in TE buffer of four plasmids (pLXPA, pHYPD, pLYPB and pLYQC) were quantified

by Real-Time PCR with SYBR® Green (Abgene, Westburg, Leusden, the Netherlands) and with Real-Time PCR of the *MBL2* gene on serial dilutions of DNA derived from known numbers of white blood cells (WBC) as standard. Real-Time PCR conditions were: 1× ABsolute™ QPCR SYBR® Green ROX Mix, 300 nM of each primer (FLH and RLH) and 5 µl template DNA in a total volume of 25 µl. To assess the sensitivity of the TaqMan assays, a series of 10-fold dilutions containing 200,000 – 2 plasmid copies of homozygous wild type, homozygous mutant and heterozygous standards of all six genotypes were amplified. Heterozygous controls were obtained by mixing two plasmids in equal amounts.

2.4. MBL2 SNP detection

SNP analysis was performed with six different Real-Time PCR allelic discrimination TaqMan assays. The assay included nonlabeled forward and reverse primers together with two fluorescent TaqMan oligonucleotide probes (wild type-specific probe labeled with VIC™ fluorophore, mutation-specific probe labeled with FAM 6[-carboxy-fluorescein fluorophore]) (Livak, 1999). Primers and probes for the six SNPs in the *MBL2* gene were designed with Primer Express (version 2.0.0, Applied Biosystems) to produce amplicons of 63–109 bp in length, with minimal secondary structure formed between primers and probes. Four different primer pairs and 6 probe pairs were used to detect the six different mutations. One primer pair and three different probe pairs were used for the detection of mutations spanning the nucleotides 154, 161 and 170 (D, B and C) (Fig. 1).

TaqMan probes corresponding to the wild type genotype were labeled with VIC™ (alleles L, Y, P and A) and probes corresponding to the mutation were labeled with 6-carboxy-fluorescein (FAM, alleles H, X, Q, D, B and C) (Table 1). Real-Time PCR amplification mixtures contained 5 µl DNA, 1× TaqMan Universal PCR Master mix (Applied Biosystems), 400 nM primers and 100 nM probes with the exception of the probe used for allele D, which was 50 nM in a final volume of 15 µl. Real-Time PCR amplification of the pre-amplified samples (see section Pre-amplification) was performed on a ABI Prism 7000 instrument (Applied Biosystems), direct SNP amplifications were performed on a 9700 thermal cycler (Applied Biosystems) followed by an endpoint detection on the ABI7000 system. The PCR profile was 2 min at 50 °C, 10 min at 95 °C, and 40 cycles of 15 s at 95 °C and 1 min 60 °C. Each run included two standard plasmid controls for each different genotype and two negative controls with distilled water.

The lower detection limit of the direct allelic discrimination assays was determined by testing limiting dilutions of quantified WBCs and the cloned plasmid controls.

2.5. Quantification of human cells

Whole blood was collected in Vacutainer CPT tubes (Becton Dickinson, Franklin Lakes N.J.) and centrifuged

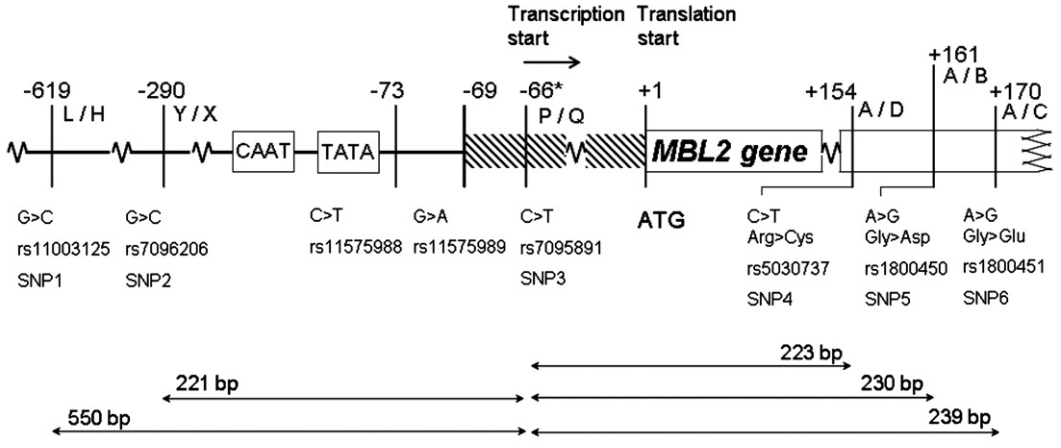

Fig. 1. Promotor region and first part of the coding sequence of the *MBL2* gene. Depicted are the promotor region, untranslated leader sequence, the coding region, transcription and translation start sites, and the six SNPs described in this manuscript. The rs numbers are shown beneath each SNP. All nucleotide positions are relative to the translation start site, according to den Dunnen(den Dunnen and Antonarakis, 2000). *This SNP has previously been positioned at position +4 by Madsen et al. (Madsen et al., 1998). Their nucleotide positions are based on the transcription start site. The current convention is to calculate nucleotide positions relative to the translation start site (den Dunnen and Antonarakis, 2000). Nucleotide distances are given between the SNPs and the transcription start site for easy reference (double arrows).

Table 1
Oligonucleotides used in the MBL2 SNP assay

Oligo	sequence (5′–3′)	Location 5′ Def I	Location 5′ Def II	SNP WT/Mutation
FLH	CCA GAG AAA ATG CTT ACC CAG G	−650		L/H
RLH	CAA CCT CAG ATC AAC CTC AAC CT	−542		L/H
PL	^{VIC} AGC CTG TCT AAA ACA ^{MGB}	−627		L/H
PH	^{FAM} AGC CTG TGT AAA ACA ^{MGB}	−627		L/H
FYX	ATG CAC GGT CCC ATT TGT TC	−320		Y/X
RYX	CGT TGC TGC TGG AAG ACT ATA AAC	−258		Y/X
PY	^{VIC} CTG CCA CGG AAA G ^{MGB}	−297		Y/X
PX	^{FAM} CTG CCA CCG AAA G ^{MGB}	−297		Y/X
FPQ	TGC ACC CAG ATT GTA GGA CAG A	−97		P/Q
RPQ	TCA GGG AAG GTT AAT CTC AGT TAA TGA	−25		P/Q
PP	^{VIC} TTT ACC GAG CAT GC ^{MGB}	13		P/Q
PQ	^{FAM} TTT ACC AAG CAT GCC ^{MGB}	13		P/Q
FABCD	CAG GCA TCA ACG GCT TCC	122		A/D/B/C
RABCD	GCC CAA CAC GTA CCT GGT TC	199		A/D/B/C
PA_{223}	^{VIC} CAT CAC GCC CAT CTT T ^{MGB}	160		A/D
PD_{223}	^{FAM} CAT CAC ACC CAT CTT TG ^{MGB}	160		A/D
PA_{230}	^{VIC} CTT GGT GCC ATC AC ^{MGB}	167		A/B
PB_{230}	^{FAM} CTT GGT GTC ATC AC ^{MGB}	167		A/B
PA_{239}	^{VIC} CAC CAA GGG AGA AA ^{MGB}	162		A/C
PC_{239}	^{FAM} ACC AAG GAA GAA AA ^{MGB}	163		A/C

at 1500 ×*g* for 30 min. The cells were washed in 5 ml phosphate-buffered saline (PBS), centrifuged at 1500 ×*g* for 20 min and the pellet was stored in 500 µl PBS. The number of WBCs was determined with a Sysmex K1000 (TOA, Tokyo, Japan) and DNA was extracted with the QIAamp mini kit (Qiagen) according to the manufacturer's instructions. Serial dilutions in Tris–EDTA buffer were used to quantify the DNA isolated from the WBCs as previously described (Mohammadi et al., 2004). Samples with DNA levels near the lower detection limit for genotyping were pre-amplified before SNP typing as described below.

2.6. Pre-amplification

To increase the amount of DNA for SNP analysis, a pre-amplification was performed on samples with low amounts of DNA. Pre-amplification was carried out with forward primer FMBL2 (5′-CTG CCA GGG CCA ACG TAG T-3′) and reverse primer RMBL2 (5′-CCC TGG ATT TCC TGG AGG C-3′), amplifying a fragment of 1593 bp of the MBL gene in a final volume of 50 µl with 30 µl DNA, 1× PCR buffer,1.5 mM $MgCl_2$, 200 µM dNTP, 2 U Taq polymerase (Roche Diagnostics), 0.5% (w/v) DMSO (Sigma) and 900 nM of each primer. Pre-amplification was performed in an ABI 9700 thermocycler (Applied Biosystems) and involved denaturation at 95 °C for 5 min followed by 30 cycles of 30 s at 95 °C, 1 min at 60 °C and 1 min at 72 °C, with a final incubation of 72 °C for 10 min. The pre-amplification was validated with a series of WBCs diluted down to 1 cell per 10 µl. A sample with a low number of WBCs (~1 cell/10 µl) and two water controls were included in each series as positive and negative controls, respectively. Pre-amplified samples were diluted 5-fold in distilled water and used directly in the Real-Time SNP assay or stored at −20 °C.

All haplotypes were used to determine the Mendelian inheritance by calculating the Hardy–Weinberg equilibrium.

3. Results

3.1. Control based genotype definition

To obtain all possible SNP controls, four different haplotypes were cloned in pGEM vectors: pLXPA, pHYPD, pLYPB and pLYQC. DNA derived from homozygous wild type samples was detected as a VIC fluorescence signal on the *X*-axis, whereas homozygous mutant samples were detected as a FAM fluorescence signal on the *Y*-axis. Heterozygous samples gave an intermediate fluorescence. For example, equal amounts of pLXPA and pHYPD represented a heterozygous H/L control.

3.2. SNP detection limit

For most of the alleles a lower detection limit of ~10 WBCs (20 plasmid copies) was determined, except for

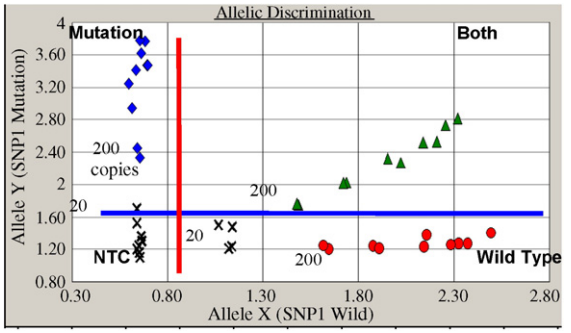

Fig. 2. TaqMan SNP assay for determining sensitivity on controls and WBC. Example shown is SNP1 on the *MBL2* gene on position −619 (L/H). Lowest absolute fluorescence points correspond with an input of 20 plasmid copies/µl DNA. Circles = homozygous wild type, triangles = heterozygous, diamond = homozygous mutation and square = no template control.

the MBL P/Q combination which required 100 cells (200 plasmid copies) to obtain reliable results (Fig. 2).

3.3. Quantification of DNA

Real-Time quantification of the DNA isolated from all neat plasma samples (N=98) and Guthrie cards (N=204) was performed to determine whether pre-amplification was necessary. Twenty-one out of 98 plasma samples and all old dried blood samples of neonates contained insufficient DNA for direct SNP analysis. SNP analysis of these samples was carried out on pre-amplificated DNA. The remaining plasma samples (N=77) contained sufficient DNA for a direct assay, i.e. all six alleles were successfully determined. The pre-amplification step permitted analysis of all plasma samples with small numbers of cells (N=21) and was also successful for 93% of the Guthrie cards (N=189).

3.4. SNP analysis

The genotype distribution was in Hardy–Weinberg Equilibrium and all possible combinations of individual coding and promoter genotypes were present in our patient population (Table 2). In addition, allele frequencies in our Dutch population were consistent with those described in previous studies of the Caucasian population (Boldt and Petzl-Erler, 2002; Cedzynski et al., 2004; Madsen et al., 1998; Minchinton et al., 2002; Mullighan et al., 2000; Skalnikova et al., 2004; Steffensen et al., 2000; Villarreal et al., 2001) (Table 3).

4. Discussion

Real-Time PCR is one of the most frequently used techniques for routine SNP genotyping. Usually, whole blood or WBC fractions are used as sources of DNA. Sometimes the only specimen available contains very small amounts of DNA or even only fragmented DNA. In such cases, SNP analysis may be unreliable. In this study we evaluated a new approach for typing multiple SNPs in one gene on very low amounts of DNA. As a model, we selected the *MBL2* gene, which encodes the Mannose Binding Lectin 2 protein. Six SNPs have been described in this gene, and the genotypes of all six alleles were determined in tandem.

In order to identify all homozygous and heterozygous genotypes in each of the six Real-Time SNP assays, allele controls were first cloned. Copy number quantified plasmid preparations and DNA extracted from known numbers of WBCs were used to determine the lowest detection limit of each assay individually. We found a full log scale (200–20 target copy numbers in PCR) variation in lowest detection limit among the six SNPs within the *MBL2* gene. The occurrence of this phenomenon is not without precedence, and was recently described in a published study where a four-fold difference in detection limits within a single SNP assay for MetHc and MetHt (Mattarucchi et al., 2005) was observed. Although always important, this result establishes the need to determine the lowest detection limit of every SNP assay, even those that are used in

Table 2
Mannose-binding lectin 2 (MBL2) allele frequencies in Dutch population

Promoter	N (%)*	Exon 1	
L/L	111 (41.1)	Codon 52	
L/H	119 (44,1)	A/A	232 (85.9)
H/H	40 (14,8)	A/D	37 (13.7)
Y/Y	161 (59.6)	D/D	1 (0.4)
Y/X	93 (34.4)	Codon 54	
X/X	16 (5.9)	A/A	217 (80.4)
P/P	162 (60.0)	A/B	47 (17.4)
P/Q	85 (31.5)	B/B	6 (2.2)
Q/Q	23 (8.5)	Codon 57	
		A/A	257 (95.2)
		A/C	11 (4.1)
		C/C	2 (0.7)
		Total	
		A/A	175 (64.8)
		A/O	
		O/O**	18 (6.7)

*Total subjects = 270.
**Includes genotypes B/B, C/C, D/D, B/C, B/D and C/D.

Table 3
MBL2 haplotype frequencies in the Dutch population compared with other Caucasian population studies

	Dutch [a]	Australian [b]	British [c]	Czech [d]	Danish [e]	Danish [f]	Polish [g]	Spanish [h]	Euro-Brazilian [i]
HYA	0.296	0.265	0.353	0.325	0.310	0.285	0.513	0.329	0.342
LYA	0.272	0.267	0.211	0.270	0.230	0.280	0.256	0.322	0.257
LXA	0.226	0.218	0.232	0.228	0.260	0.195	0.128	0.185	0.220
LYB	0.102	0.144	0.132	0.116	0.110	0.135	0.090	0.091	0.114
LYC	0.028	0.030	0.016	0.008	0.030	0.020	0.006	0.011	0.002
HYD	0.074	0.076	0.058	0.042	0.060	0.085	0.006	0.062	0.062
LYD	0.002	0.000	0.000	0.011	0.000	0.000	0.000	0.000	0.002
P/P	0.600	Nd	Nd	Nd	Nd	0.610	0.744	Nd	0.668
P/Q	0.315	Nd	Nd	Nd	Nd	0.340	0.141	Nd	0.297
Q/Q	0.085	Nd	Nd	Nd	Nd	0.050	0.115	Nd	0.035
A/A	0.648	0.576	Nd	0.680	0.800	0.560	0.795	Nd	0.653
A/O	0.285	0.348	Nd	0.287	0.200	0.400	0.205	Nd	0.332
O/O	0.067	0.076	Nd	0.033	0.030	0.040	0.000	Nd	0.015
N	270	236	100	359	250	100	78	137	202

n=Total number of subjects analyzed. Nd=no data.
[a] This study.
[b] Minchinton et al., 2002.
[c] Mullighan et al., 2000.
[d] Skalnikova et al., 2004.
[e] Madsen et al., 1998.
[f] Steffensen et al., 2000.
[g] Cedzynski et al., 2004.
[h] Villareal et al., 2001.
[i] Boldt and Petzl-Erler, 2002.

tandem to assess multiple SNPs that are in close proximity within the same gene.

When DNA concentrations are too low to perform reliable SNP detections, a pre-amplification PCR can be used to increase the DNA input prior to performing a Real-Time allelic discrimination assay. Dean et al have described a procedure for whole-genome amplification (Dean et al., 2002). In this procedure the whole genome is amplified with random hexamer primers. However when the SNPs of interest are in close proximity, or only a few SNPs are being determined, our approach provides a more convenient, rapid and less expensive method for testing suboptimal samples.

To decrease the risk of contamination, pre-amplification was only used for those samples that were first shown to contain DNA amounts below the detection limit of the Real-Time SNP assay. We successfully applied the pre-amplification on all plasma samples that contained very low concentrations of DNA and on 93% of the dried blood samples. Results of plasma samples collected from 49 mothers were compared with the results of the plasma samples of their neonates and no discrepancies in maternal genotypes were found, showing the reliability of our method.

Pre-amplification of degraded or fragmented DNA could in theory result in preferential amplification of specific alleles. We have tested this possibility by comparing results obtained using 4 samples from specimens with high quality DNA as compared to the corresponding dried blood samples from the same individual. The SNPs and haplotypes observed in the 4 high quality DNA samples were identical (data not shown) to those obtained in the dried blood samples, showing the reliability of our pre-amplification procedure for the type of samples used in our study.

The MBL2 genotype frequencies that we detected in our samples of plasma and old dried blood were compared to MBL2 frequencies described in eight other studies performed on whole blood or peripheral mononuclear cells (Boldt and Petzl-Erler, 2002; Cedzynski et al., 2004; Madsen et al., 1998; Minchinton et al., 2002; Mullighan et al., 2000; Skalnikova et al., 2004; Steffensen et al., 2000; Villarreal et al., 2001). Our data correlated well with the results found for the Caucasian populations described in these studies, and provides additional support data that the approach described can be used to detect reliably SNPs in old blood samples that have been stored for many years.

In conclusion, we have:(1) confirmed that the detection limit of SNP assays varies, even among assays on multiple SNPs that are in close proximity within the same gene;(2) demonstrated that with pre-amplification it was possible to detect SNPs in samples containing small amounts of DNA even when multiple SNPs are

located in the same gene and,(3) established pre-amplification prior to Real-Time PCR SNP analysis as a convenient, cost effective and useful method to significantly improve the reliable SNP detection in specimens containing very low concentrations or poor quality DNA from suboptimal sources.

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