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ARTICLE

Quantitation of *DEFA1A3* gene copy number polymorphism by allele specific amplification and real-time PCR

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ABSTRACT Some of the PCR based genotyping methods are faster and less expensive than sequencing in population-wide studies. One of the cost effective solutions is the allele specific amplification (ASA). We applied this method for quantitative analysis of defensin α1 (*DEFA1*) and defensin α3 (*DEFA3*) genes which are known to have copy number polymorphism in the human genome. The proteins encoded by these genes are human alpha defensins / human neutrophil peptides 1 and 3. Their antimicrobial mechanisms have an important role in the function of innate immune system. Our aim was to improve the reproducibility of ASA using 14 different mastermixes (MMX). Unfortunately, not all MMX-s are suitable for ASA investigations due to their different characteristics of polymerase activity. Here we investigated 14 commercial MMX-s whether they are capable for ASA test. **Acta Biol Szeged 57(1):47-50 (2013)**

KEY WORDS

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In allele specific amplification (ASA) the 3' end of the extension primer is perfectly complement to the mutation site of the target sequence (Fig. 1). The advantage of this technique is the low cost and fast detection (Nørby, 1993). One sign of the popularity is that all of the authors and scientific papers give a new name of all ASA variants, as "allele specific PCR" (AS-PCR), "PCR allele-specific amplification" (PASA), "simple allele-discriminating PCR" (SAP), "amplification refractory mutation system" (ARMS) etc. (Gaudet et al. 2009). All methods mentioned above are used agarose gel in order to detect amplification products. The technique can be combined also with real-time PCR, for instance with hybridization probes (Glaab and Skopek 1999) as a TaqMan mismatch amplification mutation assay (TaqMAMA). In a recent study (Baris et al. 2013) a single-tube strategy combined the tetra-primer ARMS PCR with SYBR Green I-based real-time PCR, and melting-point analysis (T-Plex real-time PCR). Since it is a real-time PCR method, this system is suitable for quantitation.

Defensins are small peptides of 12-50 amino acids which are important components of innate immunity (Ganz and Lehrer 1995). These antimicrobial peptides are divided into three groups: alpha, beta and theta defensins depending on the pattern of disulfide-bridge of the protein (Selsted at al. 1985). The genes encoding human neutrophil peptides 1 and 3 are *DEFA1* (MIM125220) and *DEFA3* (MIM604522) which map to 8p23.1 in the human genome and vary in copy number as a 19-kb tandem repeat unit. The numbers of *DEFA1* gene copies vary between 4 and 11 and the average copy number is 6. The *DEFA3* gene copy number mean is 1.5 and 10 to 37% of the tested subjects have been found to be absent for the *DEFA3* gene in the populations were tested. Exon sequences of *DEFA1* and *DEFA3* differ only one nucleotide. This paralogous sequence variant is C3400A that allows discrimination and separate quantitation of the two genes (Linzmeier and Ganz 2005).

The aim of the study was to improve the reproducibility of ASA using different commercially available mastermixes (MMX) suitable for real time PCR method. The investigations were carried out through determining *DEFA1 / DEFA3* gene copy number polymorphism using primers for specific amplification either only *DEFA1* or only *DEFA3* gene. The use of MMX-s is not only a convenient way of investigation but it minimizes the necessary pipetting steps, improves the reproducibility and reduces the standard deviation In this study we tested 14 commercial MMX-s for ASA quantification of *DEFA1 / DEFA3* genes.

Materials and methods

Patients

205 healthy blood donors were investigated. These control subjects were selected from blood donors at the Regional Centre of the Hungarian National Blood Transfusion Service, Szeged Hungary. All cases and controls were of Hungarian ethnic origin and resident in Hungary.

DNA preparation

Genomic DNA purified from peripheral blood was used. The leukocyte DNA was isolated according to the manufac-

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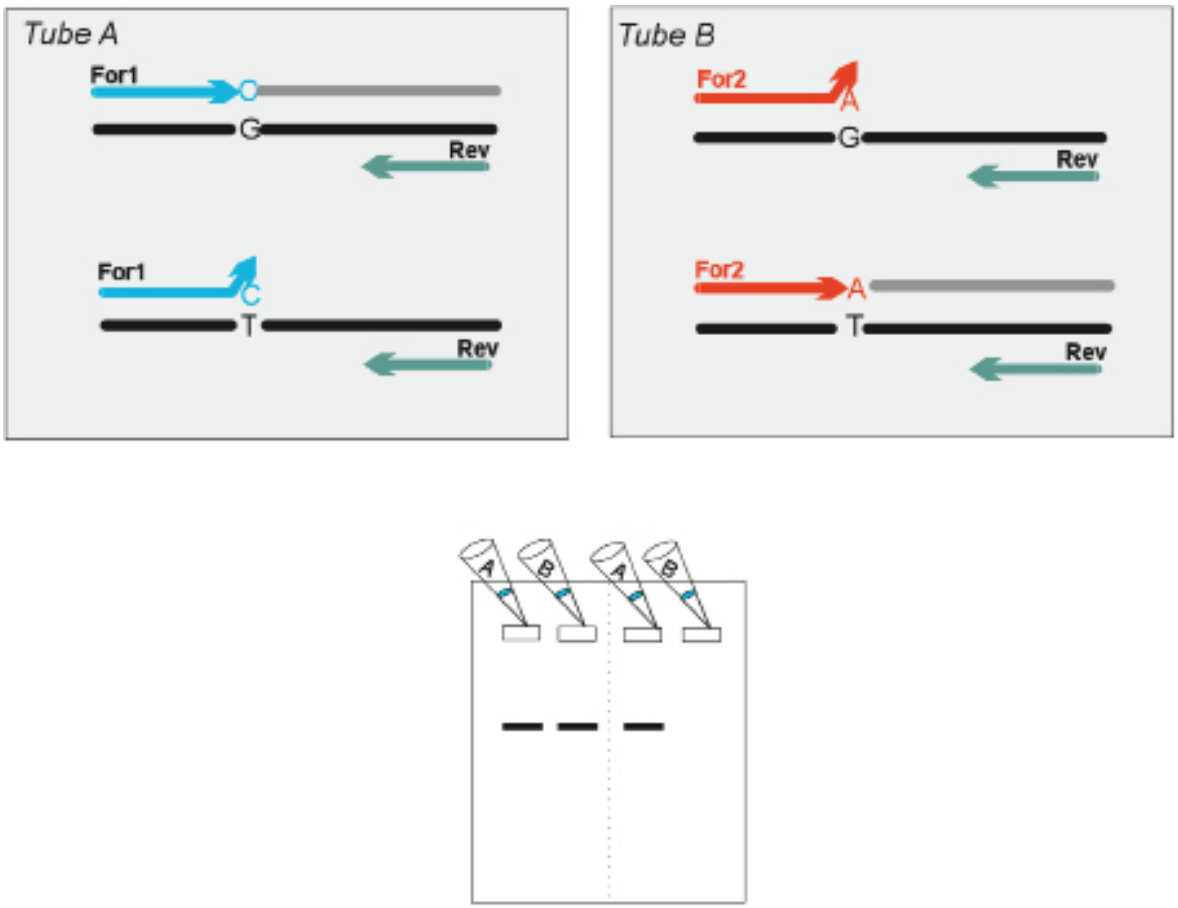

Figure 1. Principle of the allele specific amplification (ASA). The reaction takes place in two tubes. Each tube has one kind of forward extension primer which 3′ end perfectly complement to one of the target sequence. The reverse primer is common. The amplicon indicates the presence of the adequate allele. Investigating heterozygote's, there are amplicons in both tubes, in contrast in case of homozygosis just one of them has.

turer's instructions. (High Pure PCR Template Preparation Kit, Roche Diagnostic GmbH, Mannheim, Germany). DNA samples were stored at –20°C until further use.

Selection of the target DNA

Primarily we needed a suitable sample DNA for the examination of the different MMX-s. At least 10% of the populations investigated earlier (Ballana et al. 2007) had no *DEFA3* gene. For further ASA investigations we chose one of these genomic DNA samples which lacked *DEFA3* gene.

The *DEFA1A3* PCR was carried out as previously described (Linzmeier et al. 2005) with slight modifications. Briefly: BIO-RAD CFX 96 instrument (Bio-Rad, Hercules, CA, USA) was used. The reaction volume was 15 µL, containing 3 µL of DNA, 1 µM each of the primers and 7,5 µL of reaction buffer (Fermentas Probe/ROX qPCR MasterMix, Fermentas, Lithuania). We used the forward primer DEFA1 1 F (5' TAC CCA CTG CTA ACT CCA TAC 3'), reverse primer DEFA1 1 R (5' GAA TGC CCA GAG TCT TCC C 3'). The PCR conditions were as follows: initial denaturation at 95°C for 10 min followed by 40 cycles of denaturation (95°C for 15 s) and extension (54°C for 1 min).

DEFA1 and *DEFA3* genes differ only in a single nucleotide (C3400A) which is in the restriction site of *HaeIII* enzyme. The *DEFA1A3* PCR products were digested overnight at 37°C using 5 U of *HaeIII* izoschizomer *BsuRI* restriction enzyme (Fermentas, Vilnius, Lithuania). The restriction fragments were separated by electrophoresis on 2 % agarose gels containing GelRed Nucleic Acid Stain (Biotium Inc., Hayvard, CA, USA) and visualized by UV illumination. The resulted fragment lengths were 150 bp, 67 bp and 83 bp in case of presence of both alleles (*DEFA1* and *DEFA3*) in the investigated sample.

ASA PCR

All of the investigated MMX-s were used according to the manufacturer's recommendations. The precise amount of previously selected *DEFA1* homozygous DNA (contained

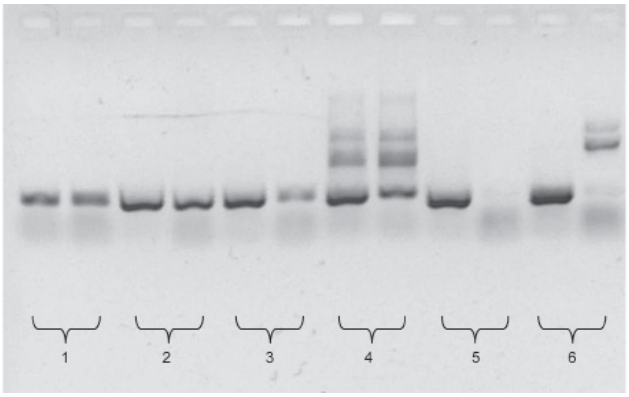

Figure 2. Test of the different mastermixes (MMX).MMX 1, 2 and 3 could not differentiate between the *DEFA1* and *DEFA3* genes because of the proof reading activity of the Taq polymerase. MMX 4 produced aspecific bands as a side effect due to the presence of dsDNA binding protein which stabilizes the polymerase-template complex and able to amplify the target DNA in the presence of inhibitors (BioRad SsoFast EvaGreen Supermix). MMX 5 is suitable for ASA (Fermentas Maxima SybrGreen qPCR Mastermix and BioRad iTaq SybrGreen Supermix). Followed by annealing temperature optimisation MMX 6 also could be used (Promega GoTaq qPCR Mastermix).

only *DEFA1* gene, but no *DEFA3* gene) was added to each reaction mix based on optical density. The reaction volume was 15 μL, containing 3 μL of DNA, 1 μM each of the primers and appropriate amount of MMX. Two separate reactions were carried out for each sample. The first reaction contained the common forward primer CommonF (5' CAG CCA GCA TCA CCT GTC AG 3') and *DEFA1* selective reverse primer 1ADR (5' GCT GGT ATT CTG CAA TAG CGG G 3'), while the second reaction was performed with common forward primer and *DEFA3* selective reverse primer 3ADR (5' GCT GGT ATT CTG CAA TAG CGG T 3'). The PCR conditions were the same as described previously except that the annealing/extension step was carried out at 68°C.

Real time PCR master mixes

We investigated 14 MMX-s from seven manufacturers. In alphabetic order:

Applied Biosystems (TaqMan Universal PCR Master Mix),

BioRad (SsoFast Probes Supermix; SsoFast EvaGreen Supermix; iQ SybrGreen Supermix; iTaq SybrGreen Supermix),

Eppendorf (RealMasterMix Probe)

Fermentas (Maxima SybrGreen qPCR Mastermix),

Promega (GoTaq HotStart Colorless Mastermix; GoTaq qPCR Mastermix),

Roche (LC HRM Mastermix; LC DNA Master SybrGreen; LC FastStart Colorless Mastermix) and

Sigma (JumpStart Taq ReadyMix; SybrGreen JumpStart Taq ReadyMix).

Real-time quantitation using ASA

For quantitation, the above described two tubes ASA PCR have supplemented the third, reference tube/reaction. Reference gene was MPO in quantitation using the primer set of MPO1F (5' CCA GCC CAG AAT ATC CTT GG 3') and MPO1R (5' GGT GAT GCC TGT GTT GTC G 3'). The emitted fluorescence was measured after each extension step. All conditions were the same as previously described with *DEFA1* / *DEFA3* gene amplification. Instead of electrophoresis, real-time quantification was performed by online monitoring for the identification of the exact time point at which the logarithmic linear phase could be distinguished from the background (crossing point). Determination of copy number polymorphism in diploid genome was calculated by ΔΔCt method as described previously (Linzmeier et al. 2005).

Results and Discussion

The *DEFA3* gene was absent in 9.27% (n=19) of the investigated study group (N=205) as determined by *HaeIII* (*BsuRI* izoschizomer) restriction enzyme digestion. This ratio was found to be less than it was in the previously investigated Caucasians (15%) and the same as in the Japanese/Chinese population (10%) (Ballana et al. 2007). The investigated study group was a control of an association study. Patients with diabetes, hypertension or ischemic heart disease were excluded from control group presumably causing the alteration compared to previous results. One of the *DEFA3* gene deficient samples were used for the MMX investigation with ASA PCR.

All of the investigated MMX-s successfully amplified the targeted sequence of the *DEFA1* gene. However, 10 MMX-s could not distinguish between *DEFA1* and *DEFA3* genes (Fig. 2, numbers 1-4). Two of them (*i.e.* Fig 2, number 4) produced aspecific bands (BioRad SsoFast Probes Supermix and SsoFast EvaGreen Supermix). These two MMX-s contain a dsDNA binding protein which stabilizes the polymerase-template complex and thus are able to amplify the target DNA in case of degenerate primers or highly concentrated inhibitors (Horvath et al. 2013). However, in general conditions they amplified aspecific bands.

Two MMX-s produced aspecific products (Promega GoTaq HotStart Colorless Mastermix and Promega GoTaq qPCR Mastermix) and a very week band in the *DEFA3* tube (Fig. 2, number 6). Some of the MMX components diminished the specificity of the reaction. This phenomenon could be fixed by optimization of the reaction which includes the selection of the optimal annealing temperature. Generally the empirical annealing optimum is 2-3°C higher than that calculated with thermodynamic methods. Moreover, the elevated annealing temperature improves the selectivity of the ASA PCR.

Two of the MMX-s (Fermentas Maxima SybrGreen qPCR Mastermix and BioRad iTaq SybrGreen Supermix) was com-

pletely suitable for ASA PCR, they successfully amplified the *DEFA1* alleles and did not amplify the *DEFA3* allele (Fig. 2, number 5). The BioRad iTaq SybrGreen Supermix was used for further investigations with quantitative ASA PCR.

After screening the 205 samples with the real-time ASA PCR the *DEFA1* gene copies varied between one and 13 and the average number were 5.7. The *DEFA3* gene copy mean was 1.7 and the gene copies varied between 0 and 5. These findings are in agreement with the previous data published (Linzmeier et al. 2005).

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References

Ballana E, González JR, Bosch N, Estivill X (2007) Inter-population variability of *DEFA3* gene absence: correlation with haplotype structure and population variability. BMC Genomics 8:14.

Baris I, Etlik O, Koksal V, Ocak Z, Baris ST (2013) SYBR green dye-based probe-free SNP genotyping: Introduction of T-Plex real-time PCR assay. Anal Biochem 441(2):225-231.

Ganz T, Lehrer RI (1995) Defensins. Pharmacol Ther 66:191-205.

Gaudet M, Fara AG, Beritognolo I, Sabatti M (2009) Allele-Specific PCR in SNP Genotyping. in: Komar AA (ed.), Single Nucleotide Polymorphisms, Methods in Molecular Biology, Humana Press, 2009

Glaab WE, Skopek TR (1999) A novel assay for allelic discrimination that combines the fluorogenic 5' nuclease polymerase chain reaction (TaqMan) and mismatch amplification mutation assay. Mutat Res 430(1):1-12.

Horvath A, Santha P, Horváth V, Torok N, Nagy I, Jancsó G, Vágvölgyi C, Somogyvári F (2013) Rapid genotyping of genetically modified laboratory animals from from whole blood samples without DNA preparation. Acta Biol Hung 64(2):262-265.

Linzmeier RM, Ganz T (2005) Human defensin gene copy number polymorphisms: comprehensive analysis of independent variation in alpha- and beta-defensin regions at 8p22-p23. Genomics 86:423-430.

Nørby S (1993) Mutation-specific PCR: a rapid and inexpensive diagnostic method, as exemplified by mitochondrial DNA analysis in Leber's hereditary optic neuropathy. DNA Cell Biol 12(6):549-552.

Selsted ME, Harwig SS, Ganz T, Schilling JW, Lehrer RI (1985) Primary structures of three human neutrophil defensins. J Clin Invest 76:1436-1439.