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Real-time PCR detection of *PI*S* and *PI*Z* alleles of *SERPINA1* gene using SYBR green

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ABSTRACT

Background: Alpha-1 antitrypsin deficiency is an underdiagnosed genetic condition that predisposes to pulmonary complications and is mainly caused by rs28929474 (*PI*Z* allele) and rs17580 (*PI*S* allele) mutations in the *SERPINA1* gene.
Objective: Development of a homogeneous genotyping test for detection of *PI*S* and *PI*Z* alleles based on the principles of allele-specific PCR and amplicon melting analysis with a fluorescent dye.
Methods: Sixty individuals, which included all possible genotypes that result from combinations of rs28929474 and rs17580 single nucleotide variants, were assayed with tailed allele-specific primers and SYBR Green dye in a real-time PCR machine.
Results: A clear discrimination of mutant and wild-type variants was achieved in the genetic loci that define *PI*S* and *PI*Z* alleles. Specific amplicons showed a difference of 2.0 °C in melting temperature for *non-S* and *S* variants and of 2.9 °C for *non*-Z and *Z* variants.
Conclusions: The developed genotyping method is robust, fast, and easily scalable on a standard real-time PCR platform. While it overcomes the handicaps of non-homogeneous approaches, it greatly reduces genotyping costs compared with other homogeneous approaches.

1. Introduction

Alpha-1 antitrypsin (AAT) is the main protease inhibitor in the human serum, which also exhibits anti-inflammatory and immunomodulatory properties. The *SERPINA1* gene encodes AAT protein and shows extraordinary complex regulation of its expression, which allows AAT to function as an acute phase reactant that controls elastin degradation mediated by the neutrophilic elastase, and also balances the immune response (Strnad et al., 2020). *PI*Z* (rs28929474) and *PI*S* (rs17580) alleles represent almost all cases of diagnosed AAT deficiency (AATD), an inherited condition in which AAT serum level and/or activity are reduced and that predisposes patients to develop liver or lung diseases, such as COPD or pulmonary emphysema (Strnad et al., 2020).

*PI*Z* and *PI*S* alleles are enriched in the European population, with estimated frequencies of about 1.6 % and 3.7 %, respectively (according to the ALFA database). Nevertheless, AATD is an underdiagnosed disease, as only about 10–15 % of cases have been estimated to be properly diagnosed (Strnad et al., 2020; Hernández-Pérez et al., 2023); and increasing rates of AATD screening and diagnosis is currently on demand (Craig et al., 2023). Therefore, accurate and cost-effective detection methods for *PI*S* and *PI*Z* alleles are necessary, and the present

Abbreviations: SERPINA1, *Serpin family A member 1*; AAT, Alpha-1 antitrypsin; AATD, AAT deficiency; ALFA, Allele frequency aggregator; COPD, Chronic obstructive pulmonary disease; Tm, , Melting temperature; RFLP, Restriction Fragment Length Polymorphism.

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work aims to satisfy this demand.

2. Methods

A set of 60 individuals, previously genotyped by real-time PCR with fluorescent probes (Hernández-Pérez et al., 2023), were selected for the present study. This set included ten individuals within each one of the six commonly observed genotypes that result from the combination of genetic variants at rs17580 (non-S and S) and rs28929474 (non-Z and Z) loci: *PI*MM* (non-S/Non-S; non-Z/non-Z), *PI*ZZ* (non-S/Non-S; Z/Z), *PI*SS* (S/S; non-Z/non-Z), *PI*MZ* (non-S/Non-S; non-Z/Z), *PI*MS* (non-S/S; non-Z/non-Z), and *PI*SZ* (non-S/S; non-Z/Z). Informed consents were obtained, as well as Ethics Committee approval (Hospital General de La Palma; #HGLaPalma_2010_7).

Genomic DNA was extracted in alkaline conditions from dried blood samples (Ramos-Díaz et al., 2015). The rs17580 and rs28929474 variants were detected in separate PCRs. Amplification reactions contained 2–10 ng of DNA (4 μl of the alkaline extract), 1 × FastStart Essential DNA Green Master Mix (Roche), two forward allele-specific primers, and one reverse conserved primer (Fig. 1A-B), at the concentration indicated in Table 1. PCRs were incubated in a LightCycler 480 instrument (Roche). The thermal profile included a preincubation step (98 °C for 10 min),

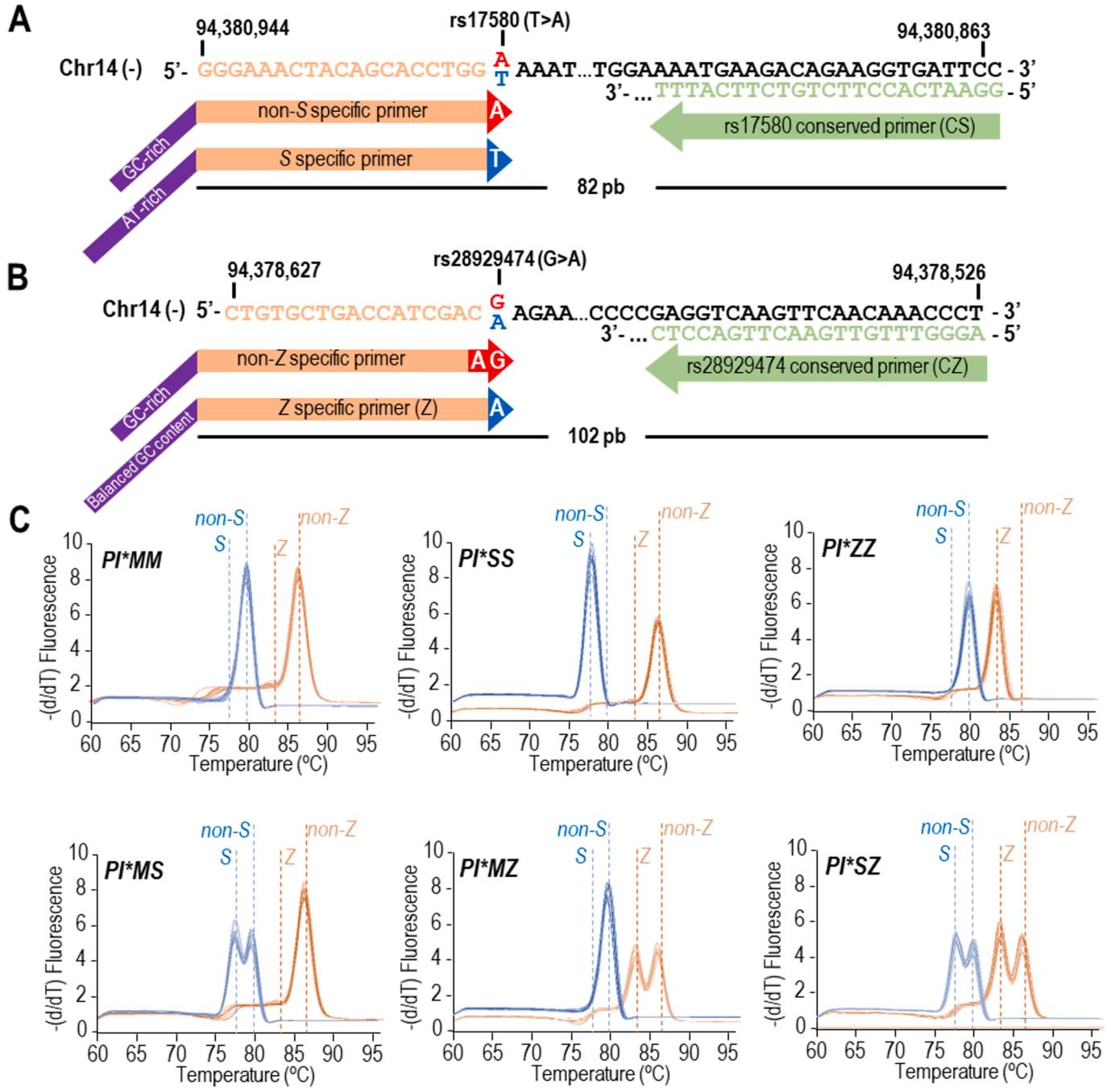

Fig. 1. Overview of the genotyping method and the results obtained with it. A) Genotyping strategy for rs17580 locus (*PI*S* allele). B) Genotyping strategy for rs28929474 locus (*PI*Z* allele). Both for A) and B), allele-specific primers (orange) contains one allele-specific nucleotide at their 3′-end (red/blue arrow head) and a 5′-tail (purple), which allows Tm-based identification of the corresponding amplicon. The conserved primer for each assay is shown in green. Genome coordinates are shown according to GRCh38.p14 human genome assembly. C) Melting peaks obtained with 10 individuals for each *SERPINA1* genotype included in the study. Note that two peaks with similar height are observed in the case of heterozygous individuals, since they carry the two targeted genetic variants.

Table 1
Allele-specific and conserved primers designed for the detection of rs17580 (*PI*S* allele) and rs28929474 (*PI*Z* allele) variants in the *SERPINA1* gene.

Locus	Primer	Sequence (5-3′)[a]	Optimized concentration (μM)
rs17580	non-S	GCCGCCTGCGGGAAACTACAGCACCTGGA	0.2
	S	ATATTTATATTTATTTAGGGAAACTACAGCACCTGGT	1.0
	CS	GGAATCACCTTCTGTCTTCATTT	1.0
rs28929474	non-Z	CCGCCCCGCCGCCGCCCCGGGCTGTGCTGACCATCGAAG	1.0
	Z	CGCCGCCCGTATTATTACTGTGCTGACCATCGACA	0.35
	CZ	GCCCGCCCCAGGGTTTGTTGAACTTGACCTC	0.5

[a] Primer tails are underlined. A mismatch position was introduced in the non-Z specific primer (highlighted in bold) for efficient discrimination of the Z variant.

followed by 35 amplification cycles with denaturing (95 °C for 5 s), primer annealing (65 °C for 10 s), and extension (72 °C for 10 s). A single capture of fluorescence was included at the end of each extension step for monitoring amplification in real-time. Finally, a melting-curve program from 60 °C to 95 °C, with 10 fluorescence acquisitions per °C, was used to calculate the melting temperature (Tm) of each amplicon with the software implemented in the thermal cycler.

3. Results

The developed genotyping assays allowed an accurate detection of the genetic variants that define the *PI*S* and *PI*Z* alleles and the corresponding wild-type variants (Fig. 1C). On the one hand, amplicons obtained with the non-S variant showed a mean Tm (79.77 ± 0.16 °C; n = 50; 91 bp) that was 2.0 °C higher than amplicons obtained with the S variant (77.76 ± 0.21 °C; n = 30; 99 bp). On the other hand, the resolution of melting peaks was even better (2.9 °C) for amplicons derived from non-Z (86.22 ± 0.07 °C; n = 50; 132 bp) and Z (83.29 ± 0.14 °C; n = 30; 128 bp) variants.

With regard to DNA samples from heterozygous individuals, the proposed genotyping assays produced melting peaks with balanced heights within an annealing temperature range of 58–66 °C for non-S/S and of 63–66 °C for non-Z/Z. Thus, both assays can work in the same PCR run using an annealing temperature within a range of 63–66 °C, although we recommend using 65 °C for optimal results.

4. Discussion

Amplification primers presented in Table 1 were designed according to the approach described by Papp et al. (Papp et al., 2003), which combines the principles of allele-specific PCR (Newton et al., 1989) with tailed primers that allow Tm-based identification of the different amplicons. Sometimes, the 5′-extension of a tailed primer is necessary to prevent it from being displaced by the competing primer, as is the case with the primer specific for the Z variant, whose 5′-tail has a balanced GC content. Optimization of this genotyping technique has also involved adjusting the concentration of the different primers in the reaction mix (Table 1) to ensure a balanced amplification of the two allele-specific amplicons with DNA templates from heterozygous individuals. It is noteworthy that the actual Tm of an amplicon depends on the particular composition of the PCR mix and, therefore, is affected by the salt concentration in the DNA sample. Since this method can be used with a wide range of DNA preparations (*i.e.* ethanol-salt precipitation, kit-based purification, or alkaline extraction, among others), the Tm values for the four different amplicons should be annotated after running amplification reactions with validated and suitable positive controls.

The combination of our optimized genotyping method with the protocol for alkaline extraction of DNA (Hernández-Pérez et al., 2023) allows an accurate and cost-effective detection of *PI*S* and *PI*Z* alleles in 2–3 h after receiving the samples in the laboratory. In the strategy presented herein, both amplification and genotype determination occur in a single tube and, therefore, it overcomes the drawbacks of non-homogeneous approaches that have been used to genotype these loci, such as allele-specific PCR, RFLP, or Sanger sequencing (Newton et al., 1989; Tazelaar et al., 1992; Costa et al., 2000). In addition, our genotyping assay take advantage of the cheap, stable, and universal dye SYBR Green, thus avoiding expensive fluorescent DNA probes with limited durability that are used in the HybProbe® and TaqMan® assays (von Ahsen et al., 2000; Kaczor et al., 2007). Indeed, we have estimated that about 70 % of reactive costs can be saved with our method compared with TaqMan® assays.

Recently, several genotyping assays based in high resolution melting analysis have been reported for detecting a set of mutations affecting the *SERPINA1* gene (Bidla et al., 2021). However, single-nucleotide variants are classified into four groups according to their effect on amplicon Tm. Mutations from groups I (C/T and G/A substitutions) and II (C/A and G/T), cause changes in the GC content of affected amplicons, which in turn lead to measurable changes in terms of Tm. However, mutations from groups III (G/C) and IV (A/T) keep the GC content unaltered, yielding very small differences (if any) in the amplicon Tm (Herrmann et al., 2006). Taking this into account, an expensive real-time PCR platform with exceptional thermal uniformity is required to detect the genetic variant associated with the *PI*S* allele, because it is a group 4 mutation (A > T transversion), which typically entails a shift < 0.2 °C of the melt curve.

In summary, our novel *PI*S* and *PI*Z* genotyping method can be easily implemented on any typical real-time PCR platform, provides reliable and quick results, and notably reduces genotyping costs when compared with probe-based techniques. Therefore, this test could be used in the future for large screenings of AATD.

Ethics approval

Ethics committee from Hospital General de La Palma (Canary Islands, Spain) approved this study, with ethics approval number #HGLaPalma_2010_7. Ethical principles for medical research involving human subjects were followed as described by Declaration of Helsinki.

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CRediT authorship contribution statement

Ruth Ramos-Díaz: Writing – review & editing, Methodology, Investigation, Formal analysis, Conceptualization. **Ainhoa Escuela-Escobar:** Writing – review & editing, Validation, Formal analysis, Data curation. **Ana Díaz-Usera:** Writing – review & editing, Methodology. **José María Hernández Pérez:** Writing – review & editing, Visualization, Supervision, Resources, Funding acquisition. **Mario Andrés González-Carracedo:** Writing – review & editing, Writing – original draft, Visualization, Resources, Investigation, Formal analysis, Data curation. **José Antonio Pérez-Pérez:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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