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Genetic diagnosis of α_1-antitrypsin deficiency using DNA from buccal swab and serum samples

DOI 10.1515/cclm-2016-0842
Received September 19, 2016; accepted December 16, 2016

Abstract

Background: α_1-Antitrypsin deficiency (AATD) is associated with a high risk of developing lung and liver disease. Despite being one of the most common hereditary disorders worldwide, AATD remains under-diagnosed and prolonged delays in diagnosis are usual. The aim of this study was to validate the use of buccal swab samples and serum circulating DNA for the complete laboratory study of AATD.

Methods: Sixteen buccal swab samples from previously characterized AATD patients were analyzed using an allele-specific genotyping assay and sequencing method. In addition, 19 patients were characterized by quantification, phenotyping and genotyping using only serum samples.

Results: The 16 buccal swab samples were correctly characterized by genotyping. Definitive results were obtained in the 19 serum samples analyzed by quantification, phenotyping and genotyping, thereby performing the complete AATD diagnostic algorithm.

Conclusions: Buccal swab samples may be useful to expand AATD screening programs and family studies. Genotyping using DNA from serum samples permits the application of the complete diagnostic algorithm without delay. These two methods will be useful for obtaining more in depth knowledge of the real prevalence of patients with AATD.

Keywords: α_1-antitrypsin deficiency; α_1-antitrypsin genotyping; buccal swab sample; serum sample.

Introduction

α_1-Antitrypsin deficiency (AATD) is one of the most common hereditary disorders worldwide and is a well recognized genetic risk factor for pulmonary and liver disease [1, 2]. In rare cases, skin diseases such as panniculitis and vasculitis may occur [3, 4]. α_1-antitrypsin (AAT) is encoded by the protease inhibitor (PI) gene *SERPINA1*, which is organized into four encoding (II, III, IV and V) and three noncoding (Ia, Ib and Ic) exons [5]. AAT is a highly polymorphic protein with over 120 alleles. The normal allele is designated PI M and the most common deficient alleles are PI S (p.Glu264Val) and PI Z (p.Glu342Lys) [6]. These deficient variants are associated with AAT serum levels of 40% and 10%–20% of the normal range level, respectively. Therefore, the PI Z allele is characterized as a severe deficiency variant. PI Z homozygosity is also the phenotype most often associated with both lung disease, attributable to the low serum concentration, and liver disease, attributable to hepatocyte endoplasmic reticulum retention of the polymerized variant protein [7, 8]. Nevertheless, there are at least 30 other AAT alleles, called “rare”, which are associated with significantly reduced or absent plasma AAT levels [9].

Although AAT deficiency is the most common hereditary disease diagnosed in adults, there is a generalized lack of knowledge in the medical community about this disorder [10, 11]. Many physicians are not aware that pulmonary conditions, such as chronic obstructive pulmonary disease or liver disease may actually be due to AATD [11]. Panniculitis is another clinical manifestation of AATD and may also be significantly underdiagnosed [4]. In addition, many subjects with severe AATD may have no clinical symptoms [12].

The diagnostic algorithm of AATD used in our laboratory and reported previously [13], is based on the

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quantitative measurement of AAT levels followed by AAT phenotyping and genotyping. AAT levels are mainly measured by nephelometry, and phenotype analysis is performed by isoelectric focusing (IEF) on agarose gels. This technique separates the various isoforms of AAT based on their migration in a pH gradient of 4.2–4.9 [14]. Serum or plasma samples are needed for these techniques [15]. Molecular analysis of the AAT gene (genotyping) is performed using methods such as allele-specific genotyping or exonic sequencing of the *SERPINA1* gene. The allele-specific genotyping assay is used for detecting the more prevalent PI S and PI Z deficiency alleles and the rare variant PI Mmalton (p.Phe52del). Direct sequencing of *SERPINA1* encoding regions is carried out when the allele-specific genotyping assay is unable to provide complete identification of both AAT alleles. Additional DNA extraction from whole blood or dried blood spot (DBS) is usually required for AAT genotyping, causing a significant delay in AATD diagnosis. Serum samples are required to perform the first steps of AAT diagnosis, and thus, the use of DNA obtained from this kind of sample would reduce the time to obtain definitive results. Moreover, a previous study on AATD analysis showed that polymerase chain reaction (PCR) products obtained from serum are of the same quality as those obtained from whole blood [16].

Our laboratory has recently focused on AATD population screening and rapid diagnosis, including some rare variants. We have developed allele-specific genotyping and exonic sequencing of *SERPINA1* protocols using DNA from buccal swabs and residual genomic DNA present in serum samples. The aim of this study was to validate the use of this kind of samples for the complete laboratory study of AATD.

Materials and methods

AAT buccal swab samples

Catch-All Sample Collection Swabs (Epicentre, Madison, WI, USA) were used. Patients were asked to gently brush the inside surface of both cheeks with the brush, approximately 15 times on each side. Samples were then packed and taken to the laboratory where the samples were stored at room temperature. Buccal swab samples were obtained from 16 individuals previously characterized by IEF and exonic sequencing of *SERPINA1*: 2 MM, 2 MS, 2 MZ, 3 SZ, 3 ZZ, 1 IS, 1 Z/Plowell, 1 M/Mmalton and 1 S/Mmalton. Both PI I (p.Arg39Cys) and PI Plowell (p.Asp256Val) alleles are rare variants. The genotypes were unknown to the technician who was blinded.

The study was approved by the Research and Ethics Committee of the Vall d'Hebron Hospital (Barcelona, Spain) and written informed consent was obtained from all the patients for the genetic analyses.

DNA extraction from buccal swab samples

DNA was obtained from buccal swab samples using the Quick Extract DNA Extraction Solution 1.0 (Epicentre, Madison, WI, USA). Each brush was placed into a 1.5 mL plastic tube containing 250 µL of the DNA extraction solution and rotated five times. Then, it was pressed against the side of the tube and removed from the tube to ensure that most of the liquid remained in the tube. The sample was mixed by vortex for 10 s and incubated at 65 °C for 1 min. The sample was resuspended by vortex mixing and incubated at 98 °C for 2 min. DNA samples were stored at – 20 °C.

Allele-specific genotyping assay from buccal swab samples

Genotyping was performed using a LightCycler 480 analyzer (Roche Diagnostic, Mannheim, Germany) which achieves rapid real-time PCR. We used a commercially available kit with specific primers and probes designed for PI S and PI Z mutation detection (Tib Molbiol, Berlin, Germany). The protocol for PI Mmalton allele-specific genotyping has been previously described in detail [17]. All samples (DNA extraction from buccal swab samples) were undiluted and diluted 1 : 2 and 1 : 10, and a commercial positive control was used.

Exonic sequencing of the *SERPINA1* gene from buccal swab samples

This method consists of the amplification of DNA extracted by PCR followed by Sanger automated sequencing of the PCR products. The four exons that code the AAT protein were amplified by PCR using DNA from buccal swab samples. We used the same primers for both PCR and the sequencing methods, and these are shown in Table 1 (GeneBank accession no. K02212). PCR amplification of each encoding exon was carried out utilizing 2.5 µL of Buffer 10× with 18 mM $MgCl_2$, 0.2 mM of dNTPs (each), 400 mM of forward and reverse primers, 0.25 µL of Fast Start High Fidelity Enzyme Blend (Roche

Table 1: Primer sequences for PCR and genome sequencing of *SERPINA1*.

Exon	Sequence	Annealing temperature, °C
II	A Fw: 5′-GATCACTGGGAGTCATCATGTGC-3′	54
	A Rv: 5′-GGTTGAGGGTACGGAGGAGT-3′	
	B Fw: 5′-CCAAGGCTGACACTCACGAT-3′	
	B Rv: 5′-AGGAGAGTTCAAGAACTGATGGTT-3′	
III	Fw: 5′-TTCCAAACCTTCACTCACCCCTGGT-3′	60
	Rv: 5′-CGAGACCTTTACCTCCTCACCCTGG-3′	
IV	Fw: 5′-CCCAGAAGAACAAGAGGAATGCTGT-3′	54
	Rv: 5′-CATTCTTCCCTACAGATACCATGGT-3′	
V	Fw: 5′-TGTCCACGTGAGCCTTGCTCGAGGC-3′	54
	Rv: 5′-GACCAGCTCAACCCTTCTTTAATGT-3′	

Diagnostic, Mannheim, Germany), 1 µL of genomic DNA and PCR grade water for a total volume of 25 µL. The PCR program is shown in Table 2. The PCR product was purified with 2 µL of ExoSAP-IT (Affimetrix, Santa Clara, CA, USA) and subjected to 15 min at 37 °C and 15 min at 80 °C. PCR products were directly sequenced using the Big Dye X-Terminator 3.1 Cycle Sequencing Kit (Applied Biosystems, Austin, TX, USA).

AAT serum samples

For the design of the techniques, serum samples were obtained from two individuals previously characterized by IEF and exonic sequencing of *SERPINA1* as MM and MS. The gold standard for diagnosing AATD in our laboratory is the algorithm described in detail previously [13]. We use the combination of AAT levels, IEF and genotyping (when required) to obtain the definitive diagnosis. Therefore, allele-specific genotyping and exonic sequencing of *SERPINA1* protocols using DNA from serum sample were included in this diagnostic algorithm. Nineteen individuals were processed due to discrepancies between AAT nephelometric levels and IEF phenotyping and their serum samples were incorporated in our diagnostic algorithm. Patients with a phenotype showing one or two M alleles, deficient AAT serum levels, and without other deficient alleles observed by phenotyping were analyzed by the Mmalton genotyping assay. In addition, S and Z genotypes were determined in order to test possible S and Z variants not correctly characterized by phenotyping. When these variants were not detected, exonic sequencing of the *SERPINA1* gene was performed.

DNA extraction from serum samples

DNA was extracted from 200 µL of serum samples using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) based on the spin column technique. The manufacturer's protocol was followed with one modification: the final elution was made using 50 µL of water. DNA extractions were stored at – 20 °C.

Allele-specific genotyping assay from serum samples

Genotyping was performed using the same instrument and protocols for PI S, PI Z and PI Mmalton detection described for buccal swab samples. In this case, the samples were not diluted.

Exonic sequencing of the *SERPINA1* gene from serum samples

Serum samples generally yield far lower amounts of DNA than whole blood samples, making PCR amplification and sequencing difficult. Therefore, two rounds of PCR were necessary to amplify the *SERPINA1* encoding exons. PCR amplification and sequencing were performed using the same primers used in buccal swab sample amplification (Table 1). The reaction was conducted at a final volume of 12.5 µL containing 1.25 µL of Buffer 10× with 18 mM $MgCl_2$, 0.2 mM of dNTPs (each), 400 mM of each primer, 0.25 µL of Fast Start High Fidelity Enzyme Blend and 7.5 µL of genomic DNA. The thermocycling conditions are described above (Table 2). The second PCR round was performed using PCR product from the first round: 2 µL of Buffer 10× with 18 mM $MgCl_2$, 0.2 mM of dNTPs (each), 400 mM of forward and reverse primers, 0.2 µL of Fast Start High Fidelity Enzyme Blend, 1 µL of genomic DNA and PCR grade water for a total volume of 20 µL. Table 3 shows the PCR conditions. The use of two rounds of PCR raises the risk of contamination. We therefore followed security measures to avoid this event, including the use of four different rooms for DNA extraction, mix preparation, sample addition and post amplification steps. In addition, a blank was used in each amplification to check for the absence of contamination.

Final PCR product purification and direct sequencing are described above.

Results

Buccal swab samples

A total of 16 buccal swab samples from previously characterized individuals were analyzed using the allele-specific genotyping assay and sequencing method. All the samples assessed gave concordant results with the previous characterization. Melting peaks in PI S, PI Z and PI Mmalton specific genotyping were clear in all the undiluted and diluted DNA samples (1 : 2 and 1 : 10). We chose the dilution of 1 : 10 because of its similarity to the positive control used. The PI S and PI Z genotyping results from a SZ sample are shown in Figure 1. Buccal swab samples recovered a large amount of DNA, resulting in easy PCR

Table 2: PCR program for *SERPINA1* amplification.

Step	Temperature, °C	Time, hh:mm:ss	Cycles
Denaturation	95	00:02:00	1
Amplification	95	00:00:30	35
	Primer annealing	00:00:30	
	72	00:00:30	
Final extension	72	00:05:00	1
Cooling	4	∞	1

Table 3: PCR program for second PCR round for *SERPINA1* amplification using serum samples.

Step	Temperature, °C	Time, hh:mm:ss	Cycles
Denaturation	95	00:02:00	1
Amplification	95	00:00:30	20
	Primer annealing	00:00:30	
	72	00:00:40	
Final extension	72	00:05:00	1
Cooling	4	∞	1

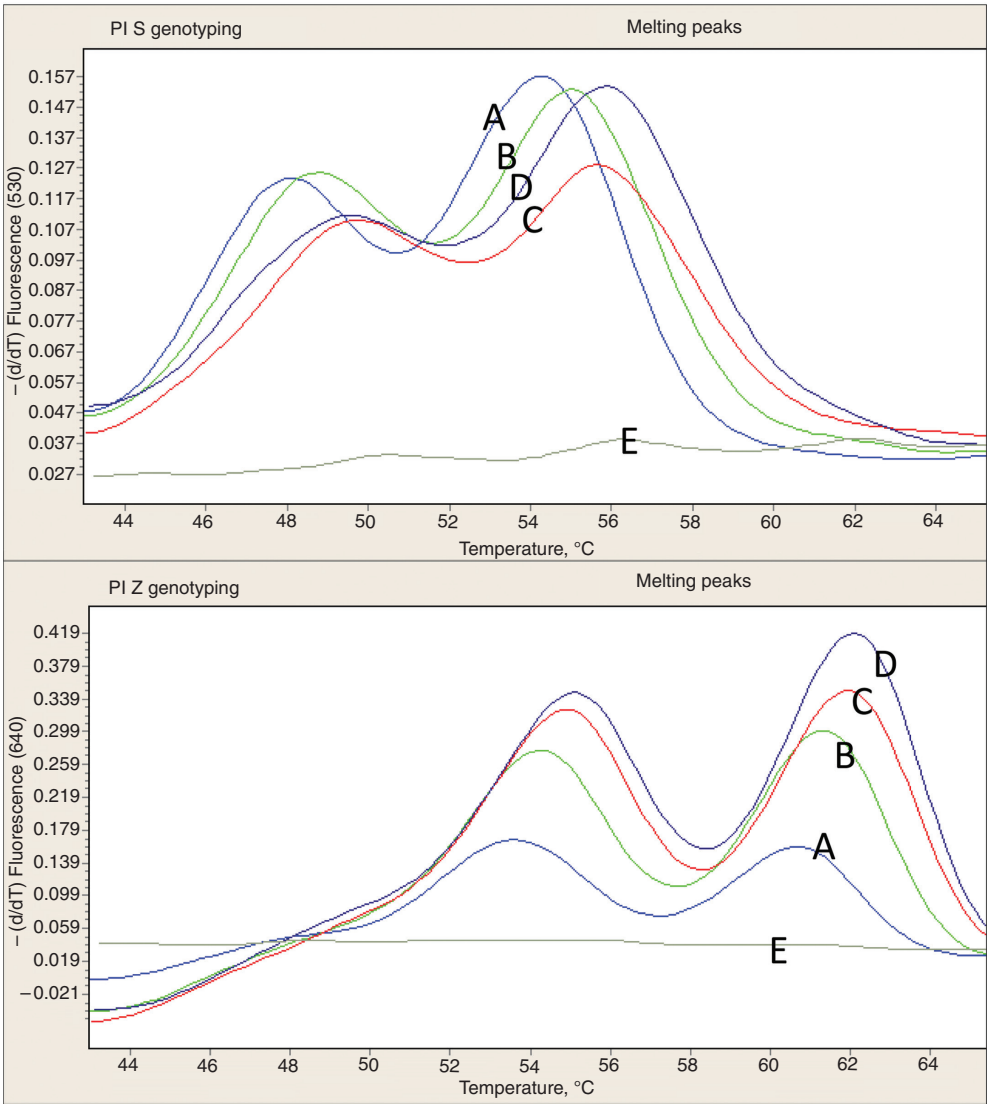

Figure 1: Melting peaks of PI S and PI Z variant detection using DNA from buccal swab samples.
(A) Undiluted sample, (B) 1 : 2 diluted sample, (C) 1 : 10 diluted sample, (D) heterozygote positive control, and (E) blank control.

amplification and sequencing, and providing clear, easy to interpret results (Figure 2).

Serum samples

For the design of the techniques two samples characterized as MM and MS were used. Once the techniques were set up, the results obtained by allele-specific genotyping and sequencing assays using DNA from serum samples were totally concordant with the previous characterization of the samples. The AATD diagnostic algorithm included PI S, PI Z and PI Mmalton specific genotyping and direct sequencing methods using serum samples. A total of 19 serum samples were processed by these techniques because the AAT levels and phenotype were not concordant and whole blood or DBS samples were not available. Fourteen of the 19 samples were analyzed by an allele-specific genotyping assay, achieving definitive results: 4 MZ, 1 MS, 1 SZ, 3 ZZ and 5 M/Mmalton. Three samples were sequenced because the results were not conclusive after the allele-specific genotyping assay: 2 M/QOMattawa (p.Leu353Phe) and 1 MM. The remaining two samples were directly sequenced to confirm the P allele detected by IEF: 1 M/Plowell and 1 S/Plowell. Thus, we characterized the 19 samples following the complete AATD diagnostic algorithm using only serum samples. One case deserves special mention: case no. 4 showed discrepant

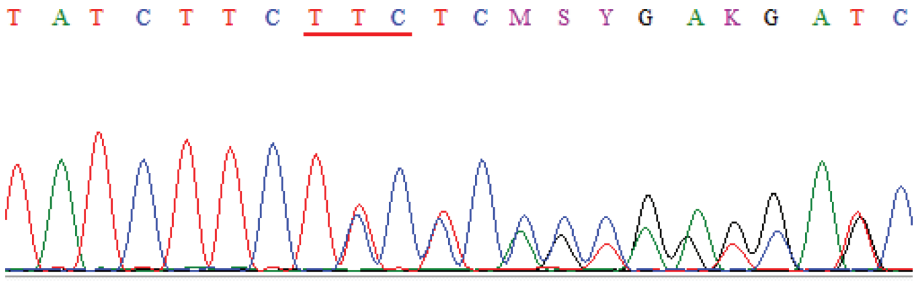

Figure 2: Sequence fragment from one M/Mmalton (Phe52del) patient obtained by exonic sequencing of the *SERPINA1* gene using DNA from a buccal swab sample.

AAT levels (0.64 g/L) and genotype (MM). This patient was studied in depth and no exonic or intronic mutations were detected. At present, we have no explanation for these discordant results, and more analyses are ongoing. Table 4 shows the definitive AAT levels, and the phenotyping and genotyping results. Figure 3 shows a sequence fragment from one M/QOMattawa patient.

Discussion

Delay in obtaining results is one of the most important problems in AATD diagnosis. Early detection of AATD would enable individuals to make changes in their lifestyle mainly with respect to smoking [18, 19] or environmental or occupational pollution. The objective of our study was to increase the number of individuals diagnosed with AATD. Once an index case is identified it becomes necessary to screen siblings and other family members to detect additional severely deficient subjects and initiate preventive or even therapeutic measures. Currently, the use of DBS samples has significantly facilitated the laboratory diagnosis of AATD in specialized centers [20, 21]. The use of DBS allows easier sample preservation and shipping [22, 23]. However, screening may be difficult in families with widely scattered members, disabled individuals or in children and adults with needle phobia.

Table 4: AAT levels, phenotyping and genotyping of the cases analyzed using serum samples.

Case no.	AAT levels, g/L	Phenotype	PI S and PI Z genotyping		Mmalton genotyping	*SERPINA1* sequencing	Definitive results
1	0.87	MM	–	–	Mmalton/Non Mmalton	–	M/Mmalton
2	0.83	MZ	Non S/Non S	Z/Non Z	–	–	MZ
3	0.72	MZ	Non S/Non S	Z/Non Z	–	–	MZ
4[a]	0.64	MM	–	–	Non Mmalton/Non Mmalton	MM	MM
5	0.6	MM	–	–	Mmalton/Non Mmalton	–	M/Mmalton
6	0.81	MP	–	–	–	M/Plowell	M/Plowell
7	0.84	MZ	Non S/Non S	Z/Non Z	–	–	MZ
8	0.88	MS	S/Non S	Non Z/Non Z	–	–	MS
9	0.71	MM	–	–	Mmalton/Non Mmalton	–	M/Mmalton
10	0.49	ZZ	Non S/Non S	Z/Z	–	–	ZZ
11	0.68	MM	–	–	Non Mmalton/Non Mmalton	M/QOMattawa	M/QOMattawa
12	0.94	MM	–	–	Non Mmalton/Non Mmalton	M/QOMattawa	M/QOMattawa
13	0.85	PS	–	–	–	Plowell/S	Plowell/S
14	0.81	MM	–	–	Mmalton/Non Mmalton	–	M/Mmalton
15	0.36	ZZ	Non S/Non S	Z/Z	–	–	ZZ
16[b]	0.53	MZ	Non S/Non S	Z/Z	–	–	ZZ
17	0.92	SZ	S/Non S	Z/Non Z	–	–	SZ
18	0.74	MZ	Non S/Non S	Z/Non Z	–	–	MZ
19	0.72	MM	–	–	Mmalton/Non Mmalton	–	M/Mmalton

[a]This patient was studied in depth and no exonic or intronic mutations were detected. [b]This patient was receiving AAT augmentation therapy. Therefore, phenotype reveals both the patient's own AAT phenotype, as well as the normal phenotype of the protein included in the treatment. –, Following our diagnostic algorithm of AATD, a definitive result had already been obtained, and therefore this technique was unnecessary.

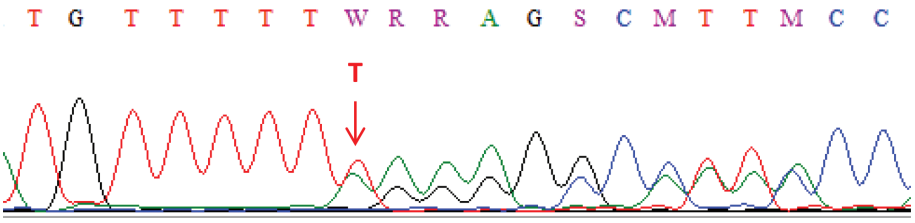

Figure 3: Sequence fragment from one M/QOMattawa (Leu353Phe) patient obtained by exonic sequencing of the *SERPINA1* gene using DNA from a serum sample.

The current study describes a fast, simple means for collecting, delivering and preparing genomic DNA using buccal swab brushes. This kind of sample can be collected by individuals without training, minimizes exposure to blood borne pathogens and avoids patient distress, potentially facilitating the participation of family members in genetic studies [24]. In addition, samples are stable for up 1 month at room temperature, are inexpensive to ship and require no special storage conditions. DNA extraction requires only heat treatment and centrifugation without the use of toxic organic solvents, allowing a large number of samples to be processed in <1 h. In this study, buccal swab samples were assessed as an alternative to DBS samples for AATD family studies. In this sense, Carroll et al. [25] reported the use of buccal swab DNA samples in a study of the prevalence of AATD in Ireland. In this case, samples were screened to detect PI S and PI Z variants by real-time PCR. In our study, we performed PI S, PI Z and PI Mmalton detection using real-time PCR as well as exonic sequencing of *SERPINA1*, which has not been described previously.

The AATD diagnostic algorithm consists of four fundamental steps: AAT serum levels determination, IEF phenotyping, allele-specific genotyping and direct sequencing. The latter technique requires EDTA whole blood or DBS samples, which are usually not available in the laboratory, and most of these patients do not return to the examination room for sample extraction. In this sense, it should be kept in mind that routine AATD studies (AAT levels and IEF phenotyping) are performed in serum samples. In this study a total of 19 serum samples were genotyped by the allele-specific genotyping assay or direct sequencing. Although serum samples generally result in the recovery of a much smaller amount of DNA than whole blood samples, the quantity of DNA obtained by two rounds of amplification was adequate for sequencing and achieving clear results. In our case, we performed exonic sequencing because most of the mutations described of the *SERPINA1* gene are located in exons [26]. However, there are some null variants such as QOMadrid (c.-5+2dupT), QOporto (c.-5+1G>A), QOwest (c.-4+1G>T) and QOBonny Blue (c.-4+1Gdel) that cause mutations located in the regulatory regions of the gene (introns) that affect the mechanisms of transcription and alternative splicing of the *SERPINA1* gene [27, 28]. Intronic sequencing is necessary in these cases, and serum samples could also be an adequate source of DNA to perform this technique. Therefore, the implementation of this protocol in the diagnostic algorithm may contribute to the detection of PI S and PI Z variants as well as rare alleles while also performing routine studies.

This represents an important improvement of the algorithm for “in vitro” AATD studies at a clinical level. Similarly, Bornhorst et al. [29] reported the first protocol of AAT sequencing from serum samples that incorporates a commercial DNA extraction method. Our proposed methodology is similar but uses different primers and amplification protocols. Moreover, we have incorporated this method in our AATD diagnostic algorithm and have used it with routine samples. Our method yields adequate DNA for allele-specific genotyping and direct sequencing of all serum samples.

Despite the accuracy of the results, one possible shortcoming of this study is the small sample size. Therefore, future studies involving a larger number of samples are necessary to reassert the utility of these kinds of samples in the diagnosis of AATD. Nonetheless, this can be considered a preliminary validation study showing proof of principle but requiring further validation.

Conclusions

Widespread use of the methodologies described above could optimize the accuracy of AATD diagnosis and promote the expansion of screening programs. We have developed two methods for the early diagnosis of AATD which are simple and easy to perform by physicians and lab technicians and provide early results for patients.

Acknowledgments: This study was supported in part by a grant from Fundación Catalana de Pneumología (FUCAP 2015) and through funding from Grifols to the Catalan Center for Research in Alpha-1 antitrypsin deficiency of the Vall d'Hebron Research Institute in the Vall d'Hebron University Hospital. Barcelona, Spain.

Author contributions: All the authors have accepted responsibility for the entire content of this submitted manuscript and approved submission.

Research funding: None declared.

Employment or leadership: None declared.

Honorarium: None declared.

Competing interests: The funding organization(s) played no role in the study design; in the collection, analysis, and interpretation of data; in the writing of the report; or in the decision to submit the report for publication.

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