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Genomic DNA extraction from small amounts of serum to be used for α_1-antitrypsin genotype analysis

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ABSTRACT: If laboratory diagnosis of α_1-antitrypsin (α_1-AT) deficiency is usually based on its phenotype identification by isoelectric focusing, α_1-antiprotease inhibitor (Pi)S and PiZ genotypes can also be determined by deoxyribonucleic acid (DNA)-based methods. Recently, several methods have been described for preparing genomic DNA from serum. The aim of the current study was to determine the Pi allele from serum extracted DNA by polymerase chain reaction (PCR) and to compare these results with those obtained with whole blood extracted DNA.

Serum α_1-AT concentration and phenotypic identification were systematically performed in 43 hospitalised patients. Genomic DNA was simultaneously purified from whole blood and from serum. The mutation detection was found using a PCR-mediated site-directed mutagenesis method.

Concerning phenotypic identification, 29 patients were MM homozygotes, 11 were heterozygotes for S (MS=7) or for Z (MZ=4) and three showed a ZZ phenotype. Genotyping analyses gave identical results with serum and whole blood extracted DNA and all the results were in agreement with the phenotyping results.

The authors found that the deoxyribonucleic acid-based test proved to be a reliable tool for α_1-antitrypsin deficiency diagnosis and appears to be an alternative for the labour intensive α_1-antitrypsin determination by isoelectric focusing. The authors also concluded that this method yields good quality deoxyribonucleic acid from serum, equal to that extracted from whole blood and is helpful in retrospective studies of multiple genetic markers.

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The α_1-antiprotease inhibitor (Pi), or α_1-antitrypsin (α_1-AT), is the principal serum inhibitor of lysosomal proteases, such as neutrophil elastase [1]. The αl-AT is a polymorphic single chain glycoprotein of 52 kDa and 394 amino acids, synthesised in the liver and normally present in serum at 150–350 $mg \cdot dL^{-1}$ [2]. It displays >90 different genetically determined phenotypes [3]: phenotype M is the normal variant (90% of the population) and phenotypes S and Z are the two most frequent abnormal variants [3]. Calculated values of PI ZZ prevalence are approximately: 1:1,000–1:145,000 in Western and Northern Europe; 1:45,000–1:10,000 in Central Europe; and 1:10,000–1:90,000 in Eastern Europe and in the southernmost and northernmost areas of the continent. In the white population of USA, Canada, Australian and New Zealand, PI ZZ phenotype prevalence ranges from 1:2,000–1:7,000 individuals. In nonwhite populations α_1-AT deficiency is thought to be a rare or nonexistent disease [4, 5]. Homozygosity for the Z phenotype is the principal cause of α_1-AT deficiency. It typically leads to the development of diverse liver diseases in children and adults and to early adult onset emphysema, with plasma level of α_1-AT in homozygous PiZ individuals reaching only 10–15% of α_1-AT concentration observed in PiM individuals [6, 7]. Although individuals MS or SS are unaffected, SZ subjects may be symptomatic. More recently, α_1-AT deficiency has been associated with asthma, bronchiectasis, vasculitis and panniculitis [8, 9].

The α_1-AT gene comprises seven exons dispersed over 12 kb of the chromosomal segment 14q 31–32.3 and is expressed in hepatocytes and mononuclear phagocytes [2]. The mutation in the PiZ allele consists of a single base substitution (guanine to adenine) in exon V, which results in a change at amino acid 342 (from glutamic acid (GLU) to lysine) [10]. The PiS allele is characterised by the substitution of adenine with thymine, in exon III, which results in the amino acid valine at position 264 instead of GLU [11].

Laboratory tests are absolutely necessary for diagnosis of α_1-AT deficiency. Routinely, this diagnosis is based on the measurement of serum α_1-AT concentration and the identification of αl-AT phenotype by isoelectric focusing (IEF). IEF sometimes presents equivocal results or discordances compared with serum α_1-AT measurements [2, 12, 13]. Since several years, PiS and PiZ genotypes can also be determined by deoxyribonucleic acid (DNA)-based methods [2, 13, 14, 15].

DNA in plasma or serum was first discovered in 1948 by MANDEL and METAIS [16]. Although it is now evident that DNA circulates freely in blood plasma both in health and in disease, the source of this DNA remains enigmatic. It is presumed that circulating DNA in healthy subjects is derived from lymphocytes or other nucleated cells [17]. Recently, several methods have been described for preparing genomic DNA from serum with some of them requiring very small amounts of serum ranging from 20–250 μL [18–21]. These microextraction procedures allow DNA to be obtained and be used as a template to amplify DNA segments as large as 3,789 base pairs (bp). The amplified polymerase chain reaction (PCR) products are of similar quality than that of DNA prepared from whole blood specimens [19]. The aim of this study was to extract DNA from serum, to determine the Pi allele by the PCR-mediated site-directed mutagenesis previously described [22] and to compare these results with those obtained with control DNA extracted from peripheral blood cells.

Methods

Patients

From March–December 2001, a total of 43 venous blood samples were collected from patients who were hospitalised in the Universitary Hospital of Nancy (18 female and 25 male; mean age 52.2±22.6 yrs (mean±SD), range 0.5–90 yrs). A total of 11 patients were hospitalised in the Respiratory Diseases Department and were known to suffer from a pulmonary emphysema (n=10) or a chronic bronchitis (n=1). A total of three of these patients were already diagnosed and were ZZ homozygotes.

Quantitative determination of α_1-antitrypsin concentration

The α_1-AT measurement was performed in serum samples using a rate immune nephelometric method (Immage Immuno-Chemistry System, Beckman-Coulter, Roissy, France). The immune nephelometer automatically dilutes samples 1:36 to achieve optimum antigen-antibody equilibrium in the assay.

α_1-antitrypsin phenotyping

Identification of the phenotype was systematically carried out on 3 μL of each serum sample by use of an IEF technique on flat bed polyacrylamide gels in a pH gradient of 4.2–4.9 (Phast gel dry IEF; Pharmacia, Uppsala, Sweden), as the major variants M, S, and Z focus between pH 4.5 and 4.7 [23]. After Coomassie R 350 staining according to the manufacturer′s recommendations (PhastGel Blue R, Pharmacia), the α_1-AT bands were compared with the control samples corresponding to the PiM, PiMS, PiMZ or PiZZ and the phenotypes were examined.

α_1-antitrypsin genotyping by polymerase chain reaction

Genomic deoxyribonucleic acid extraction. Genomic DNA was simultaneously purified from peripheral blood cells (Nucleon BACC 3; Amersham Pharmacia Biotech, Orsay, France) and from serum taken for 39 patients. In four of the patients DNA was only extracted from their serum. As described by LIN and FLOROS [19], two genomic DNA extraction methods from small amounts of serum were tested. The present authors found better results with a modified proteinase K/sodium dodecyl sulphate (SDS) lysis method. A total of 250 μL of serum was treated with proteinase K (1.7 $mg \cdot mL^{-1}$; Gibco BRL, Life Technologies, Cergy-Pontoise, France) and 5% SDS (Sigma Aldrich, Saint-Quentin Fallavier, France) at 65°C for 1 h. This solution was then heated at 95°C for 10 min to inactivate the proteinase K. The lysate was then phenol extracted and ethanol precipitated. After a centrifugation at 16000×*g*, 4°C for 15 min, the DNA pellet was dissolved in 25 μL of Tris-HCL 10 mM pH 8.5.

Polymerase chain reaction. The procedure for mutation analysis was modified from the PCR-mediated site-directed mutagenesis method of TAZELAAR *et al.* [22]. All amplifications were started in a 50 μL reaction volume containing 50 μM deoxynucleotide triphosphate (Sigma Aldrich, Saint-Quentin Fallavier, France), 2 mM $MgCl_2$ (Gibco BRL, Life Technologies), 12.5 pM of each primer (synthesised by the Common Molecular Biology Department of the Universitary Hospital of Nancy), 250 ng of DNA, 1.25 U of Taq polymerase (Gibco BRL, Life Technologies). After an initial denaturation step at 94°C for 2 min, a first amplification was carried out for 30 cycles, each cycle consisted of a 30 s denaturation time at 94°C, a 30 s annealing time at 64°C and a 60 s extension time at 72°C, followed by a final step at 72°C for 7 min on a Perkin-Elmer thermal cycler (Perkin Elmer, Norwalk, CT, USA). If this first amplification was not sufficient to obtain PCR products, a second identical 20-cycle PCR program was then performed to optimise the results.

Restriction enzyme digestion and electrophoresis. PCR products were first run on a 2% agarose gel in 89 mM Tris-borate buffer containing 1 mM ethylenediamine tetraacetic acid, pH 8.3 at constant voltage of 130 V for 50 min. A restriction digestion mixture was then prepared as follows: 10 μL of PCR product was added to 40 U of *Taq* I restriction endonuclease (Ozyme, Saint-Quentin, Yvelines, France) and to bovine serum albumin (BSA) and endonuclease buffer, to a final volume of 20 μL. The digestion mixture was incubated at 65°C for 2 h, according to the manufacturer′s recommendations (Ozyme). Finally, the digested PCR products were analysed on a 15% polyacrylamide gel after an electrophoresis performed in the same running buffer as described above at a constant voltage of 150 V for 90 min in accordance with the conclusions of TAZELAAR *et al.* [22].

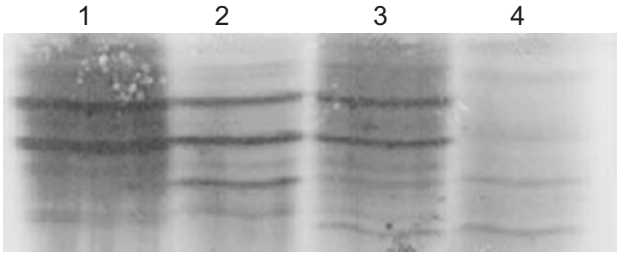

Fig. 1. – Isoelectric focusing gel of α_1-antitrypsin (pH range 4.2–4.9) obtained from serum samples. The anode is at the top. MM homozygote (lane 1), MS heterozygote (lane 2), MZ heterozygote (lane 3) and ZZ homozygote (lane 4).

Results

The blood samples for 43 patients between March–December 2001 were obtained (18 females and 25 males, mean age was 52.2 yrs). A total of 37 serum α_1-AT concentrations were considered as normal or increased and four were diminished, considering the reference values used (91–182 mg·dL^{-1}). Only six serum α_1-AT concentrations were found <91 mg·dL^{-1}.

The identification of the α_1-AT phenotype was obtained by IEF for all of these patients: 29 were MM homozygotes, seven were heterozygotes for S, *i.e.* MS and four patients were MZ heterozygotes (fig. 1). As expected, three patients were ZZ homozygotes (fig. 1). The 6 patients whose serum α1-AT concentration was lower showed respectively MZ (n=3) and ZZ (n=3) phenotypes (fig. 1, table 1). These three ZZ patients were already known to suffer from a pulmonary emphysema.

Table 1. – α_1-antitrypsin serum concentration in the different phenotypic groups

	Phenotype			
	MM	MS	MZ	ZZ
Patients (n)	29	27	4	3
α1-AT serum concentration				
Mean±SD	203±88	164±72	88±10	21±3
Range	105–421	103–313	80–100	18–23

Data are presented as mg·dL^{-1}.

The genotypic analysis was systematically performed with genomic DNA extracted from blood cells and with genomic DNA extracted from serum to validate the protocol for 39 patients. In four patients, genotype was only analysed from DNA extracted from serum.

As described by TAZELAAR *et al.* [22], PCR primers were used to create *Taq* I restriction sites that distinguished between normal and mutant DNA. The primers used to amplify the sequence that included the Z mutation site yielded a product of the correct size (179 bp) in seven cases. Subsequent digestion with *Taq* I gave results in agreement with those obtained by IEF (fig. 2): four patients were heterozygotes (MZ) and 3 patients were homozygotes for Z allele. Similarly, the amplification product that included the S mutation (121 bp) was found in seven cases. All of these patients were heterozygotes (MS).

Serum extracted DNA gave identical results compared with those obtained with whole blood DNA for the 39 patients. The 43 genotyping results were all in agreement with the phenotyping results (fig. 1 and 2).

Discussion

The Pi allele was determined by PCR on DNA extracted from serum in 43 hospitalised patients. It was found by the authors that this procedure yielded good quality DNA equal to that extracted from whole blood, whatever the Pi allele.

DNA circulates freely in blood plasma both in disease and in health but the source of this serum DNA is not completely known [24]. In healthy subjects, it is assumed that circulating DNA is derived from lymphocytes or other nucleated cells. Cancer patients have a greater amount of circulating DNA than healthy subjects [25, 26]. It seems unlikely that this tumoral DNA originates from the lysis of circulating cancer cells because the number of circulating cells usually found in plasma is not high enough to explain the large amount of DNA detected [27]. The role of tumoral necrosis is unclear since DNA plasma levels in cancer patients decreased up to 90% after radiation

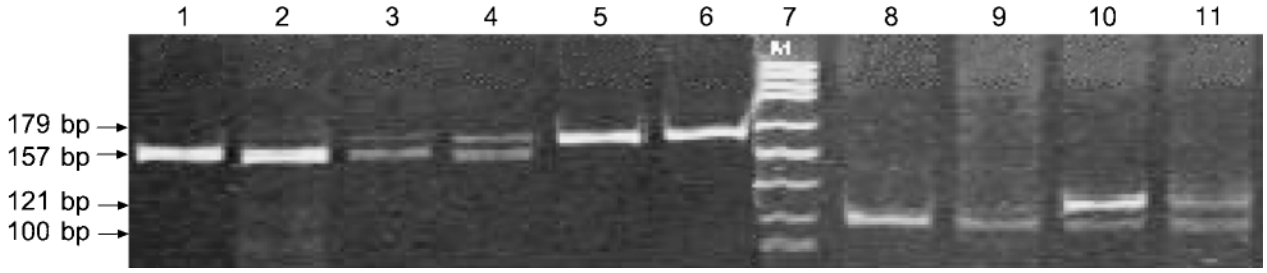

Fig. 2. – Ethidium bromide stained 15% polyacrylamide gel showing results of amplification and digestion of normal and mutant α_1-antitrypsin alleles with *Taq* I restriction enzyme. The genotyping results were in agreement with phenotyping results. Lane 1: MM homozygote deoxyribonucleic acid (DNA) extracted from blood cells; lane 2: MM homozygote DNA extracted from serum; lane 3: MZ heterozygote DNA extracted from blood cells; lane 4: MZ heterozygote DNA extracted from serum; lane 5: ZZ homozygote DNA extracted from blood cells; lane 6: ZZ homozygote DNA extracted from serum; lane 7: size standard (Puc18; Sigma Aldrich, Saint-Quentin Fallavier, France); lane 8: MM homozygote DNA extracted from blood cells; lane 9: MM homozygote DNA extracted from serum; lane 10: MS heterozygote DNA extracted from blood cells; and lane 11: MS heterozygote DNA extracted from serum.

therapy, whereas an increase would be expected if necrosis is a major pathway of DNA release [25]. Apoptosis has been advanced as the origin of circulating DNA [26, 28, 29], however this mechanism is supposedly lost by proliferating cells. A fourth hypothesis is that the tumour actively releases DNA in blood by a mechanism similar to that observed *in vitro* when lymphocytes or whole organs spontaneously release DNA without any cell death [30].

In this study Pi genotyping was compared between DNA extracted from whole blood and DNA extracted from serum. In all cases, the two extraction procedures gave identical Pi genotypes and the results were in accordance with the phenotypic determination by isoelectric focusing. The present authors confirmed that genomic DNA prepared from small amounts of serum could serve as a template to amplify DNA segments in order to detect genetic alterations, as described by LIN and FLOROS [19]. The combination of a micro-extraction procedure from serum and PCR genotyping provide a rapid and costless tool in genetic analysis [19]. A small amount of serum (250 μL) can be very informative, since it yields enough DNA to analyse several genetic markers.

It is generally agreed that PCR methods are useful for the determination of the α_1-AT deficiency variants PiS and PiZ [12]. Recently, COSTA *et al.* [31] have proposed the use of dried blood spot specimens in quantitative α_1-AT detection. Deficiency of α_1-AT was evaluated by combining the results of α_1-AT quantification and phenotyping. In cases in which there was discordance between α_1-AT concentration and phenotype, diagnosis of hereditary α_1-AT deficiency was established by α_1-AT genotyping. In the study by COSTA *et al.* [31], α_1-AT genotype determination was carried out using DNA from dried blood spot specimens and fresh blood samples from four patients with different phenotypes (Pi M, MZ, MS and Z). Identical genotypes were observed with PCR products obtained from dried blood spot specimens or from fresh blood [31].

The present study shows for the first time, to the best of the authors' knowledge, that amplified polymerase chain reaction products can be obtained from serum with the same quality as those obtained from whole blood, when used for α_1-antitrypsin genotype analysis. Whilst further studies are needed to confirm these findings, the initial results are very encouraging. This deoxyribonucleic acid-based method is rapid, convenient, easier to perform and less laborious than the isoelectric focusing technique. The authors believe that genomic deoxyribonucleic acid extraction from small amounts of serum appears to be an alternative to deoxyribonucleic acid extracted from peripheral blood cells. Moreover, it will be a good complement of the isoelectric focusing technique, even though it is unlikely that it will completely replace it, due to the high number of variants that could be detected by this classical method. Also, this deoxyribonucleic acid-based method, allowing genomic deoxyribonucleic acid to be obtained from serum, can be used in genetic analysis with multiple genetic markers others than the α_1-antiprotease inhibitor allele. It should open up possibilities for the analysis of existing specimens, and prove to be useful since serum libraries are more often available in laboratories than deoxyribonucleic acid ones.

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