

## ISOELECTROFOCUSING AND PCR AMPLIFICATION-REVERSE HYBRIDIZATION ASSAY IN EVALUATION OF ALPHA-1-ANTITRYPSIN DEFICIENCY

### IZOELEKTROFOKUSIRANJE I PCR AMPLIFIKACIJA-REVERZNA HIBRIDIZACIJA U PROCENI NEDOSTATKA ALFA-1-ANTITRIPSINA

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**Summary:** Alpha-1-antitrypsin deficiency is a potentially lethal genetic disorder, which has pulmonary and liver manifestations. The standardized biochemical and molecular diagnostic protocol for detection of clinically relevant alleles is needed. The paper summarizes current concepts about AATD, describes the potentials of isoelectric focusing and PCR amplification-reverse allele specific oligonucleotide hybridization assay in the detection of affected individuals and shortly presents our experiences in the evaluation of AATD. We conclude that the systematic clinical laboratory approach to AATD might be based on the combination of mentioned methods, coordinated by alpha-1-antitrypsin quantification. Additionally, its complete medical implementation is achieved through teamwork between clinical chemists, molecular biologists and clinicians.

**Keywords:** isoelectrofocusing, PCR-reverse hybridization, alpha-1-antitrypsin

### Introduction

Alpha-1-antitrypsin deficiency (AATD) is a potentially lethal genetic disorder, characterized by serum concentrations of alpha-1-antitrypsin (AAT) lower than 0.5 mg/L, which has liver and pulmonary manifestations (1, 2). From a clinical laboratory point

**Kratak sadržaj:** Nedostatak alfa-1-antitripsina je potencijalno smrtonosan genetski poremećaj sa pulmonarnim i hepatičnim manifestacijama. Uočljiva je potreba za standardizovanim protokolom detekcije klinički značajnih alela, koji bi uključivao biohemijske i molekularne metode. Rad prikazuje savremena shvatanja o nedostatku alfa-1-antitripsina, opisuje mogućnosti primene izoelektrofokuseranja i testa zasnovanog na kombinaciji PCR amplifikacije i reverzne hibridizacije sa alel-specifičnim oligonukleotidima i ukratko opisuje naša iskustva u toj oblasti dijagnostike. Može se zaključiti da bi, u kliničkim laboratorijama, kombinacija dveju pomenutih metoda, uz dodatne informacije dobijene kvantitativnom analizom, mogla predstavljati osnovu detekcije genetski uslovljenog nedostatka alfa-1-antitripsina. Neophodno je naglasiti da je za sveobuhvatnu medicinsku primenu takvog laboratorijskog protokola neophodna saradnja medicinskih biohemičara, molekularnih biologa i lekara odgovornih za lečenje bolesnika sa genetski uslovljenim nedostatkom alfa-1-antitripsina.

**Ključne reči:** izoelektrofokuseranje, PCR-reverzna ASO hibridizacija, nedostatak alfa-1-antitripsina

of view, the standardized biochemical and molecular diagnostic protocol for detection of clinically relevant alleles in a timely and cost-effective manner is needed

*Non-standard abbreviations:* AATD – alpha-1-antitrypsin deficiency, AAT – alpha-1-antitrypsin, NE – neutrophil elastase, PR-3 – proteinase, HNP – human neutrophil peptide, eNOS – endothelial nitric oxide synthase, GSTP1 – glutathione S transferase 1 gene, ANCA – anticytoplasmatic antibody, ER – endoplasmatic reticulum, PSM – PCR-mediated site direction polymorphism, RFLP – restriction fragment length polymorphism, ASO – allele specific oligonucleotides, SSCP – single stranded conformation polymorphism, DGGE – denaturing gradient gel electrophoresis, RT-PCR – real time PCR, COPD – chronic obstructive pulmonary disease.

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(3). This paper summarizes the current concepts about AATD, describes the potentials of isoelectric focusing and the PCR amplification-reverse hybridization assay in the detection of affected individuals and shortly presents our experiences in the evaluation of AATD.

### Alpha-1-antitrypsin deficiency

Alpha-1-antitrypsin is the archetype of the serpin superfamily, the members of which control many inflammatory cascades, mainly through tightly grasping and inhibiting serine proteases, such as neutrophil elastase (NE), cathepsin G and proteinase 3 (PR-3) (3, 4). It is a single-chained glycosylated protein with a molecular mass of  $\approx 51$  kDa. The AAT synthesis occurs primarily in the liver (5), although it can be evidenced in the lung, macrophages, renal parenchymal cells and intestinal epithelium (1, 4).

From a physiological viewpoint, AAT represents a major defense against the elastolytic burden in the lower respiratory tract, owing to its ability to inhibit NE. Additional antiinflammatory effects, unrelated to NE inhibition, have also been reported (eg. blockage of the proinflammatory effects of HNP and regulation of cytokine expression) (2). The most important stimulators of AAT synthesis and the consequent increase in plasma concentrations are acute phase response and estrogens. Plasma AAT levels are decreased with genetic deficiency, increased use and urinary or gastrointestinal loss (5).

The AAT is encoded by the gene located on chromosome 14q31, which is 122 kb in length and has seven exons and six introns. The available data suggest that the AATD is inherited in a co-dominant fashion. The gene is highly polymorphic, with more than 120 PI (Protease Inhibitor) identified alleles, which can be classified as: normal ( $M_{\text{(various subtypes)}}$ , X<sub>Christchurch</sub>), deficient (Z, S,  $M_{\text{malton}}$ ,  $S_{\text{iiyama}}$ ,  $M_{\text{heerlen}}$ ,  $M_{\text{procidar}}$ ,  $M_{\text{mineral springs}}$ ), null ( $QO_{\text{granite falls}}$ ,  $QO_{\text{ludwigshafen}}$ ,  $QO_{\text{hongkong}}$ ,  $QO_{\text{isola di procida}}$ ) and dysfunctional alleles (Pittsburgh,  $M_{\text{mineral springs}}$ , Z). The polymorphic features of the AAT gene may lead to the AATD by abnormalities in gene expression, translation and intracellular protein processing (6). The Z and S alleles are the two most frequent and clinically the most important deficient alleles. The Z allele consists of a lysine-to-glutamate substitution at codon 366 (E366K), while the S variation represents a substitution of valine for glutamate at codon 288 (E288V). Normal alleles are related to normal AAT concentration in the serum of 0.8–2.2 g/L (2). Carriers of the Z allele usually have only 15% of normal AAT blood concentration in homozygous and 60–75% in heterozygous state (7). The S allele gives 60% of normal AAT concentration in homozygous and up to 80% in heterozygous carriers (8, 9). The mean gene frequency of Z allele is 0.014, with the highest prevalence in Northern Europe and populations with North European background, while the S has the

mean gene frequency of 0.056, with the peak in the Iberian peninsula (9, 10). Worldwide estimations speculate with the numbers 116 000 000 for the carriers of deficient alleles and 1 100 000 for subjects with severe AATD (11).

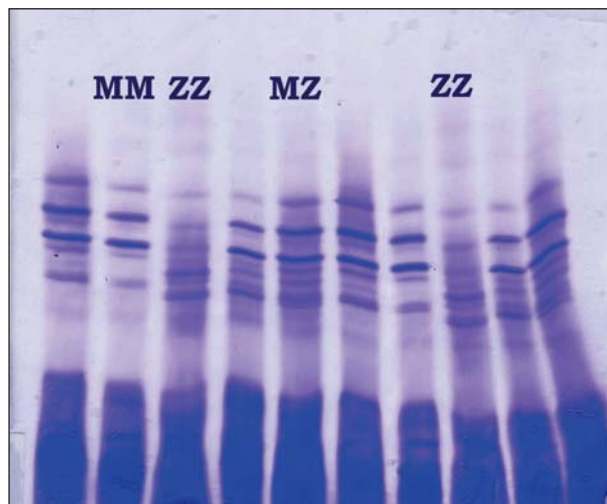
Molecular pathology associated with the Z allele comprises both toxic gain-of- and functional loss-of-function (1). Due to the mutation, the tertiary structure of AAT is distorted and the reactive loop of one molecule interlocks with the  $\beta$  pleated sheet of another, forming fibril-like polymers. Further polymerization leads to the formation of insoluble inclusions, which trigger ER stress and represent hallmarks of AAT liver disease (1, 4, 7, 12). This feature classifies Z-associated AATD as the paradigm of conformational diseases (1). Intracellular accumulation releases only 15% of the synthesized protein into the circulation, so the anti-protease protection on the airway epithelial surface is consequently decreased and an uncontrolled proteolytic attack is allowed (1, 3, 4, 7). Additionally, polymerization of AAT locally produced in the lung is evidenced to engrave pulmonary manifestations and limits local administration of augmentative therapy (4). In the case of S variant, the decreased AAT concentration results in misfolding and increased degradation of molecule within the hepatocytes, while the intracellular accumulation is absent (4). The gene-environmental interactions give a significant contribution to the formation of the »AATD clinical phenotype«. Among them, the most important are: smoking, exposure to dust and inhalants, comorbidities (eg. asthma, sarcoidosis) and other genetic polymorphisms (eg. eNOS, GSTP1) (6, 13).

Concerning its clinical manifestations, AATD can be described as a conformational disease which primarily predisposes to lung (eg. emphysema and bronchiectasis) and liver (eg. chronic hepatitis, cirrhosis, hepatoma) disorders (1, 2, 4). The other well established disease associations include skin disease, such as panniculitis, and ANCA positive vasculitis (1, 4). The connections between AATD and some other diseases (eg. glomerulonephritis, coliac disease, cancer (lung, colorectal and bladder localizations), intracranial and intra-abdominal aneurysms) are postulated, but need further evaluation and clarification (2, 3). The distinctive and suggestive features of emphysema associated with AATD comprise early onset (ie. 30–40 years), panacinar pathology and disproportionate emphysematous involvement of the lung bases (2, 3). Bronchiectasis is recognized in AATD, but data are limited concerning their frequency, type and manifestations (2, 14). The most frequent genotype among individuals with lung disease due to AATD is ZZ (more than 95%), while the second in order of frequency is SZ (2). Hepatic manifestations of AATD usually occur in children (prolonged jaundice, increased liver enzyme activity, hepatosplenomegaly, bleeding tendency) or in the 6<sup>th</sup> decade of life (chronic liver disease, cirrhosis, hepatocellular carcinoma). They

are mostly associated with the homozygous presence of Z or M<sub>malton</sub> alleles or their mutual presence in heterozygous genotypes. It is not common for the same individual to have both the hepatic and pulmonary disease due to the AATD (1, 2, 4).

Therapy of AATD includes environmental measures, primarily smoking cessation, nonspecific medical treatment and specific measures, according to the predilection site of manifestations of AATD (4). The augmentation therapy, administered intravenously or by inhalation, is successful in the treatment of patients with lung manifestations. The potential use of retinoids, hyaluronic acid, antioxidants as well as gene therapy is under investigation. In AATD patients with liver disease liver transplantation is currently the only effective means of intervention, but its efficiency is diminished by a lack of suitable donors and need for immunosuppressive therapy. New therapeutic strategies might include chemical chaperones, synthetic anti-polymerization peptides and gene therapy (1, 2, 4).

Identification of individuals affected by AATD may provide substantial health benefits in the form of opportunities to receive augmentative therapy and to minimize environmental risk factors (smoking, occupational hazards) (15, 16). According to the current recommendations, the AATD should be evaluated in subjects presenting with emphysema with early-onset (age of 45 years or less), absence of a recognized risk factor (smoking, occupational dust exposure, etc.) or with prominent basilar hyperlucency, unexplained liver disease, necrotizing panniculitis, ANCA-positive vasculitis, family history of any of the following: emphysema, bronchiectasis, liver disease, or panniculitis, bronchiectasis without evident etiology (17). Important issues concerning AATD are screening strategies. Neonatal screening is abandoned, due to the presence of serious disadvantages including psychosocial issues and discrimination. A precisely balanced definition of the adult population to be screened is a prerequisite for successful screening, in order to avoid limitations seen both in large population-based screening and in small targeted detection programs in high risk groups (4, 17).

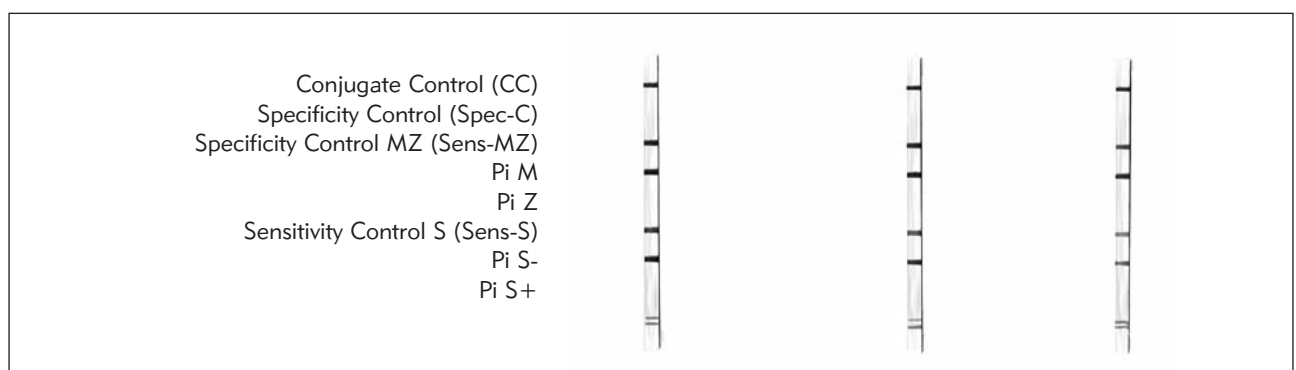


**Figure 1** Isoelectrofocusing of various AAT variants in the pH range 4.2-4.9 after Coomassie Brilliant Blue staining (anode at top).

### Laboratory methods for the evaluation of AATD

The first laboratory approach to AATD combined paper electrophoresis, agar-gel electrophoresis and immunoelectrophoresis, while the multiallelic nature of the PI locus was confirmed using starch-gel electrophoresis (18). The identification of PI phenotypes was further improved by the application of isoelectrofocusing methods (19-21). The information about AAT-coding DNA sequence, provided by cloning and DNA sequencing, enabled detailed characterization of known and new AAT variants, on the protein and gene level (18, 22-24). Comprehensive laboratory testing can be divided into four stages: quantification, phenotyping, genotyping and evaluation of AAT function (3, 17).

Quantitative analysis, which measures the »level« of AAT in serum or plasma, is the first test to



**Figure 2** Genotyping of AAT using PCR amplification and reverse ASO hybridization. The text at the left side is the instruction from the reading card.

be performed when an individual is suspected for AATD. Clinical laboratories almost exclusively perform immunonephelometric methods, which are reliable, inexpensive and subject to automation (3, 5). The main constrain of AAT quantification is that it is not sufficient to confirm the genetic cause of AATD. The obtained concentrations might be elevated due to inflammation or injury, or reduced by causes such as liver damage or protein-losing enteropathies. Nevertheless, the AAT level is an important feature of the protocol for detecting AATD (eg. indicator for reflex tests) and clinical evaluation of AATD (3, 5, 25).

The potentials and constraints of isoelectrofocusing as a laboratory tool for AATD detection will be discussed later.

Molecular approach to AATD evaluation encompasses methods for detection of the Z and S alleles, as clinically the most relevant ones, and DNA sequencing. Unambiguous diagnosis of Z and S variants can be achieved at a molecular level using PCR-PSM, PCR-RFLP, ASO hybridization, SSCP, DGGE and RT-PCR methods. Although expensive and time-consuming, DNA sequencing methods are needed, concerning the diversity of AATD symptoms and the questionable reliability of phenotyping methods in some situations (18, 26–28). Genotyping in routine clinical laboratories may be facilitated by the use of commercially available test kits. Such an approach is offered by the method combining PCR amplification with allele specific oligonucleotide hybridization (29, 30), which will be discussed later.

The inhibitory function of AAT can be evaluated using a semiquantitative assay which measures serum inhibition of trypsin or elastase against small substrates. However, this test is not specific for AATD, concerning the presence of other serpins in serum (3, 5).

A standardized laboratory protocol for AATD detection must be adapted for use of both standard (ie. obtained by venipuncture) and alternative samples (ie. dried blood spots). This issue appears rather important, because many AATD detection programs perform all types of necessary analyses from whole blood drops absorbed on special filter paper (3).

### Isoelectrofocusing in AATD evaluation

Isoelectrofocusing or isoelectric focusing (IEF) is an electrophoretic method for the separation of amphoteric molecules, according to their isoelectric points (pI). The separation is achieved in stabilized and continuous pH gradient, with regular and constant conductivity and buffer capacity. The technique can be carried out on polyacrylamide or agarose gels. The pH gradient is formed in the electric field by the carrier ampholytes (both kinds of gels) or by immobilized pH gradients, in which the buffering groups are part of the medium (only polyacrylamide gels). The main area of IEF application is the identification of genetic variants

and investigation of physico-chemical and biological influences on proteins and hormones, although it can be employed for preparative purposes (31). Analytical IEF can be carried out in capillary electrophoresis equipment, what makes it a very important tool for quality assurance of biopharmaceutics and also for proteome analysis, especially if coupled with mass spectrometers (32).

The phenotyping of AAT in serum by IEF is recommended as a gold standard for laboratory confirmation of AATD. The test is recommended for individuals with a decreased level, detected by immunonephelometry, or in situations when high index of clinical suspicion for AATD persists although AAT are not below the lower reference limit (15, 17). Clinical laboratories perform the test on precasted, narrow-pH-gradient (pH 4-5), thin-layer polyacrylamide gels. A gel pattern with the typical microheterogeneity of some common AAT variants of clinical importance is made visible by Coomassie Brilliant Blue staining. The phenotype associated with the homozygous presence of normal alleles consists of two major and three minor bands, whose presence is due to the various sialic acid contents and the length of chain. If deficient alleles are present, some bands have altered mobility, while some are not visible. In each IEF run, a sample of the MZ heterozygote, confirmed by an alternative independent method, should be applied as a marker of phenotyping and for quality control. In the case of PI phenotyping from stored and frozen-thawed samples, a reduction with cysteine is recommended as the pretreatment. Phenotypes must be identified by two persons independently, and in any case of discordance the test is repeated (33, 34). *Figure 1* demonstrates a gel pattern obtained in our laboratory.

The application of IEF in the evaluation of AATD has several limitations. Concerning technical difficulties, it must be highlighted that IEF is a challenging procedure, which requires highly skilled personnel. Rather often, errors in PI phenotyping are due to poor sample quality or iatrogenic causes (ie. sample taken from patient receiving AAT augmentation therapy). Finally, interpretation of the gels is the most challenging aspect, because of the complex microheterogeneity of AAT and the large number of variants (25).

### PCR amplification-reverse allele specific oligonucleotide hybridization assay in AATD evaluation (PCR-reverse ASO hybridization)

The allele specific oligonucleotide (ASO) hybridization is a very reliable method to detect mutations involving single base changes and small deletions. The method is based upon the ability of the single stranded short oligonucleotide to hybridize with completely complementary single stranded target DNA. A mutation, even in a single nucleotide will abolish hybridization. The ASO hybridization can be



applied as forward and reverse. The principle of the forward ASO hybridization is the binding of target DNA onto solid membranes, and subsequent denaturation and hybridization to labelled oligonucleotide probe. In the reverse ASO hybridization, the tailed single stranded oligonucleotides, with a poly dT tail at the 3' end, are covalently attached to the solid phase and the amplified target DNA is then hybridized with immobilized oligonucleotides. A label is incorporated into PCR reaction to make the bound PCR product visible (29).

The detection of the Z and S AAT alleles, using a commercial kit which combines PCR amplification and reverse ASO hybridization, is achieved through the following steps: DNA isolation from a patient sample, multiplex amplification with biotinylated primers and reverse hybridization. Blood samples for DNA isolation can be collected with EDTA or citrate, while the samples collected with heparin can be used after the additional pretreatment. The hybridization includes the following steps: chemical denaturation of the amplification products, hybridization of the single-stranded, biotin-labelled amplicons to membrane-bound probes, stringent washing, addition of a streptavidin/alkaline phosphatase (AP) conjugate, and an AP mediated staining reaction. A reading card ensures the easy and fast interpretation of the banding pattern obtained (30, 35). *Figure 2* shows banding patterns obtained in our laboratory.

The advantages offered to the clinical laboratory by the above described methodology are numerous. The assay, structured as a multiplex test, provides simultaneous, unambiguous determination of both Z and S alleles. The whole procedure is not time-consuming and requires a minimum of expertise in the molecular biology techniques. The main limitation, shared by other allele-specific assays for the Z or S AAT mutations, is that the assay provides no evidence for the alleles other than M, Z and S (30).

### **The interplay of IEF and PCR-reverse ASO hybridization assay in AATD evaluation**

After consideration of the previously mentioned advantages and disadvantages of both methods, it can be stated that IEF and the PCR-reverse ASO hybridization assay for AAT are complementary methods. Consequently, the laboratory approach to AATD has to be modified to meet the challenges concerning the presentation of obtained data in clinically useful format. The combination of quantification and genotyping by the PCR-reverse ASO hybridization assay are prompted to be used as the first level of testing. The IEF is mandatory in all situations when serum AAT concentrations do not correlate with the determined genotype or clinical findings. Considering the numerous physiologic and pathological conditions influencing AAT concentrations, in the majority of cases phenotyping is expected to confirm the genotype result. The IEF, as the reflex

test in such situations allows for more confidence in the resulting diagnosis. The real importance of IEF as a reflex test is conferred by its ability to suggest the presence of a null allele or rare deficiency variants (eg.  $M_{\text{malton}}$ ,  $S_{\text{iiyama}}$ ,  $M_{\text{mineral springs}}$ ,  $QO_{\text{isola di procida}}$ ), in homozygous state or in combinations with M, Z and S alleles. In such cases the final evaluation of genetic causes of AATD is given by DNA sequencing done in specialized molecular biology laboratory (25, 36). The combination of quantification, detection of the Z and S allele by targeted genotyping and phenotyping is generally accepted by screening programs in the United States and most of the European countries with established AATD National Registers (25, 37, 38).

### **Our recent experiences in AATD evaluation**

The frequency of Z allele in the Serbian population (0.013) is comparable to that in the populations of Central Europe, while the S allele frequency (0.0066) is the lowest among the populations of Europe, except for the Finish (39, 40).

Since January 2007, we evaluated AATD in 42 adults and 3 newborns. The main clinical features which prompted for AATD evaluation in adults were: COPD (22 patients), emphysema (9 patients), pneumothorax (3 patients), pulmonary infiltration (1 patient), asthma (1 patient) and bronchiectasis (1 patient). Familial testing was conducted in four cases. We confirmed 2 cases of severe AATD, associated with the homozygous presence of the Z allele. The presence of MZ phenotype was detected in 7 adults. Surprisingly, in three MZ carriers, the AAT concentration was normal, but this increase was attributed to inflammation, evidenced by an elevated level of CRP. This finding highlights the need for phenotyping despite the normal AAT concentration, in cases which are highly clinically suspicious. The newborns were referred for AATD evaluation due to the presence of prolonged jaundice and cholestasis. Among them, one carrier of the MZ phenotype was identified.

### **Conclusion**

The combination of IEF and PCR-reverse ASO hybridization, coordinated by AAT quantification, offers a systematic clinical laboratory approach to AATD evaluation. Through teamwork between clinical chemists, molecular biologists and clinicians we can best achieve the shift of the AATD medical focus from treatment to prevention.

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