Facilitating the Laboratory Diagnosis of $\alpha_1$-Antitrypsin Deficiency

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Abstract

$\alpha_1$-Antitrypsin (AAT) deficiency leads to deterioration of the lungs that can be prevented with diagnosis and treatment. Isoelectric focusing (IEF) electrophoresis is the current biochemical gold standard for detecting AAT deficiency variants but involves complex interpretation. Variant AAT samples were collected over a 2-year period. Stability of AAT for phenotype determination was assessed in whole blood, dried blood spots, and dried serum spots. A compendium displaying 13 common and 5 rare AAT phenotypes was created, and a detailed methodology describing how to recognize AAT banding patterns and interpret a rare phenotype accompanied these visual data. AAT was stable for IEF phenotype analysis for at least 1 week in whole blood and for 24 hours on dried serum spots. In conclusion, a reference compendium of known AAT phenotypes was established that can serve as a resource for interpreting AAT phenotypes.

The $\alpha_1$-Antitrypsin (AAT) is a member of the serine protease inhibitor (SERPIN) superfamily. The main substrate for AAT is neutrophil elastase, a broad-specificity serine protease secreted by neutrophils during inflammation.1,2 AAT is synthesized and secreted by the liver and functions primarily in the lungs, where it protects pulmonary tissue from degradation by neutrophil elastase. A deficiency of AAT results in promiscuous activity of neutrophil elastase, resulting in early onset emphysema and chronic obstructive pulmonary disease. In addition, specific AAT variants can aggregate in hepatocytes, leading to hepatocellular death and liver disease. Pulmonary symptoms generally present in the fourth or fifth decade of life; hepatic injury can occur at any age, including neonates.3,4

Epidemiologically, AAT deficiency is an autosomal codominant genetic disorder1 that is relatively common (approximately 1/3,000 individuals) yet often goes unrecognized.5,6 It is estimated that in the United States alone, approximately 100,000 people have severe AAT deficiency, but less than 10,000 have been diagnosed and are receiving treatment.7,8 Furthermore, AAT-deficient patients may have symptoms for a number of years and/or see multiple physicians before a diagnosis is made. The gap between prevalence and diagnosis has clear effects on patient care, as proper treatment has been shown to reduce mortality.5,8,9 In addition, national support groups are available to help relieve psychosocial stressors associated with diagnosis.

The SERPINA1 gene that codes for the AAT protein is highly polymorphic, with more than 100 alleles identified in the literature.1 The protein variants produced by these alleles are traditionally named with the prefix Pi (proteinase

Upon completion of this activity you will be able to:
• explain the importance of diagnosing $\alpha_1$-antitrypsin (AAT) deficiency.
• interpret AAT phenotypes using isoelectric focusing electrophoresis.
• define the stability of AAT for isoelectric focusing electrophoresis in whole blood and dried blood spots.
inhibitor) followed by capital Roman letters as identified by protein migration patterns in an isoelectric focusing (IEF) electrophoresis gel. PiB is the most anodally migrating variant, and PiZ is the most cathodally migrating variant. Many of these variants are clinically benign, resulting in normal AAT expression and function. The most common nondeficiency allele codes for the wild-type PiM protein variant. Approximately 97% of the population is homozygous for this allele. Other AAT variants may produce deleterious effects by either decreasing the protective coating of the lungs and/or polymerizing in hepatocytes. The most common AAT deficiency alleles code for the PiS and PiZ protein variants.

Definitive diagnosis of AAT deficiency is most often accomplished in the clinical laboratory using a combination of biochemical and/or genetic tests. These include quantifying AAT protein concentration in serum, determining the AAT phenotype, and/or genotype testing to identify specific AAT alleles. Serum AAT concentration alone has a low sensitivity for detecting an AAT deficiency. AAT phenotype determined by IEF electrophoresis is considered the gold-standard test for identifying AAT variants. Genotype testing, often used to identify individuals with PiS or PiZ alleles, is accomplished using DNA amplification techniques with melt-curve analysis using Förster resonance energy transfer probes specific to the region of the gene where a mutation is located. Algorithms have been proposed that integrate each of these 3 tests.

IEF electrophoresis is the most commonly used method to definitively detect the presence of an AAT phenotype that puts a patient at risk for AAT deficiency. Because of the different AAT variants that may be encountered in the clinical laboratory, there is a need for an objective guide to assist laboratorians in correctly identifying AAT phenotypes. Here, we present a compendium displaying and describing 13 common and 5 rare AAT phenotypes. Furthermore, we describe a process to aid in the recognition of AAT variant patterns observed by IEF electrophoresis. In addition, we evaluate the stability of AAT in whole blood for phenotype analysis and the ability to interpret AAT phenotypes from specimens extracted from dried blood spots (DBS) and serum separator cards. Together, these data will facilitate the biochemical investigation of AAT deficiency.

**Materials and Methods**

**Samples**

For the compendium of AAT phenotypes, we selected 18 residual serum samples sent to ARUP Laboratories (Salt Lake City, UT) for AAT quantitation and phenotype testing between January 2007 and January 2011. The AAT phenotypes of these 18 samples represented approximately 98% of the phenotypes observed in that laboratory, including 13 common and 5 rare AAT phenotypes. All specimens were de-identified before use. This study was approved by the University of Utah Institutional Review Board.

To assess AAT stability, 11 separate tubes of fresh whole blood (approximately 1 mL/tube) were collected from 2 apparently healthy individuals (PiMM phenotype), stored at ambient temperature (21-24°C) or 4°C for 0 to 168 hours, and analyzed as described below. One sample of EDTA-anticoagulated whole blood from an individual (PiMZ phenotype) was stored at 4°C for 12 days prior to using it for AAT stability studies. Forty-four EDTA-anticoagulated whole-blood samples sent to our laboratory for AAT phenotype/genotype testing were selected to evaluate the stability of samples obtained from dried blood spots and serum separator cards.

**AAT Phenotype**

AAT phenotypes were determined by IEF electrophoresis using the only Food and Drug Administration (FDA)–cleared commercially available kit according to the manufacturer’s instructions (Hydragel A1AT Isofocusing; Sebia, Inc, Norcross, GA). Samples were applied to a 0.1% agarose (pH 4.2-4.9) IEF gel, and electrophoresis was performed for 45 minutes at 20°C using 700 to 1,000 V. AAT bands were visualized using peroxidase-conjugated AAT-antiserum that produced a pigment after addition of acidic dimethylformamide in hydrogen peroxide. The AAT phenotype was determined by visual inspection and comparison to known patterns.

**AAT Concentration**

Serum or plasma AAT was quantified using an immunoturbidimetric assay on a Roche Modular Analytics P instrument (Roche Diagnostics, Indianapolis, IN) using the manufacturer’s instructions.

**Stability of AAT in Whole Blood**

One tube of whole blood from each PiMM individual was centrifuged immediately after the sample clotted. The resulting serum was frozen at −20°C to be evaluated as the time zero (T₀) time point. Of the 10 remaining tubes of whole blood, 5 were stored at ambient temperature and 5 were stored at 4°C for 7 days. For 4 consecutive days, 1 tube of whole blood stored at each temperature was centrifuged, and the resulting serum was stored at −20°C; a final sample was processed similarly after 7 days. After all time points were completed, AAT phenotypes were determined for each serum specimen.

The stability of the PiMZ phenotype was assessed using a 12-day-old EDTA-anticoagulated whole-blood specimen that had been stored at 4°C. Plasma was obtained from this specimen, and its AAT phenotype was reevaluated.
Extraction of AAT From Dried Blood Spots and Serum Separator Cards

From individuals with PiMM (n = 10), PiMS (n = 3), and PiMZ (n = 5) we obtained 700 µL EDTA-anticoagulated whole blood and applied it to either Whatman paper (for DBS) or a serum separator card. Serum separator cards are patented technology that uses a proprietary paper to retain red cells at the application point but allows migration of the serum or plasma through the paper. This creates an area of the card that contains dried serum or plasma. The cards remained stationary for 24 to 48 hours before removing a 6-mm paper circle using a standard 1-hole punch. To evaluate stability of phenotype analysis using serum separator cards, we kept the card at ambient temperature until its appropriate time point (up to 21 days) for the punch to be removed. The 6-mm paper circle was placed in a 96-well microtiter plate and incubated with 75 µL distilled water overnight (16-18 hours) at ambient temperature. Depending on the experiment, the AAT phenotype test from the extracted sample was performed immediately or the sample was stored at –20°C until the IEF electrophoresis was performed.

In addition, 26 EDTA-anticoagulated whole-blood samples representing a variety of AAT phenotypes were used to evaluate the ability to interpret various phenotypes using the serum separator cards. Of these, 4 were omitted from analysis because excessive hemolysis made it impossible to determine the AAT phenotype. Samples were applied to the serum separator cards by 1 individual (D.N.G.) who was not blinded to the AAT phenotype. A second individual (M.C.E.-J.) who was blinded to the AAT phenotype completed the extraction, IEF electrophoresis, and AAT phenotype interpretation.

Results

AAT Compendium

The nomenclature of AAT first originated from the common M, S, and Z variants and the rare F variant. F was named for its fast (anodal) migration, followed by M for middle, S for slow, and Z for zeta (last). Additional variants were named alphabetically according to how they migrated relative to these 4 variants. There are some exceptions to this pattern (discussed below).

Each variant has multiple bands associated with its phenotypic pattern. These are commonly referred to as major and minor bands. Major bands stain darker than minor bands. They are referred to systematically and numerically as the letter of the variant followed by 2 (most anodal), 4, 6, 7, or 8 (most cathodal). Bands 2, 7, and 8 are the minor bands, and 4 and 6 are the major bands. Although M2 may appear to be darkly stained in Image 1, the interindividual variability of the intensity of this band is considerable (Image 1, lane 1 vs 5), and thus M2 is considered a minor band.

When evaluating an AAT phenotype, the reviewer should first identify the major bands and their migration relative to the M bands (Image 1, lane 1). The M variant has 3 major subtypes known as M1, M2, and M3 (Image 1, lanes 9 and...
10). Although there is no clinical need to distinguish between these subtypes, it is important to understand their migration pattern. The M1 and M2 major bands migrate identically, and so the minor band must be used to distinguish between them. M3 migrates between M1 and M2 and can only be identified if it is placed in a lane next to an M1M2 sample. The D variant (Image 1, lane 3) migrates similarly to the M variant; however, the D variant has no minor bands, and all bands stagger slightly anodally relative to the M bands. Since the D variant does not correspond to a deficiency allele, misclassification of D as an M should have no clinical ramifications.

Identifying the S and Z migration patterns is also integral to detecting many of the other variants. The S variant is a commonly encountered deficiency variant (Image 1, lane 12). The S major bands migrate anodally to M6 and cathodally to M7. In general, the S major bands will not be resolved from M6 but will be resolved from M7. The S8 minor band can usually be easily visualized at the cathodal region of the gel. The Z allele codes for the most deleterious of the AAT variants. Concentrations of AAT protein in individuals with the Z variant are often so low that the minor bands cannot be visualized. The major bands are visible, albeit sometimes faint, and migrate just cathodally to the M minor bands (Image 1, lane 17). The heterozygous Z patient shown in Image 1 stains characteristic of this phenotype. However, it is important to remember that the reference interval for a homozygous Z sample is less than or equal to 29 to 52 ng/mL, meaning that there could be up to a 2-fold variability in expression levels for these individuals, which can directly influence the intensity of the stain.

Care should be taken to correctly identify variants that can be easily confused as S or Z. The T variant (Image 1, lane 13) migrates almost identically to the S variant. Distinguishing these variants is important because, of the 2 variants, only the S variant is consistently associated with AAT deficiency. The T variant has not been directly associated with AAT deficiency, but 1 study did show that relative to M phenotypes, the mean AAT concentration from T individuals is slightly decreased. T7 and T8 stagger with S7 and S8, with the T bands migrating slightly more cathodally. E variants can also be confused for S variants because their minor bands migrate identically to the S major bands (Image 1; compare lane 4 with lanes 6 and 12). E variants can be distinguished by the major E4 and E6 bands that migrate just cathodally to the M2 and M4 bands. In addition, the S major bands are more distinct relative to the E minor bands. Unlike the S variant, E variants are not associated with an AAT deficiency. Similarly, the N variant can be mistaken for a Z variant because the N8 minor band migrates almost identically to the Z major band (Image 1, lane 16). To discriminate between the Z and N variants, one should locate the N major bands, which will appear as a doublet in heterozygous MN individuals. In general, the N variant has 1 major band that migrates directly between M4 and M6 and a second major band that migrates directly between M6 and M7. Some N variants migrate anodally to the midpoints specified, as shown in Image 1, lane 16. In the case of an NZ individual (not shown), the Z major bands would be slightly resolved from the N minor bands. Evaluation of total AAT concentration is important to evaluate when discriminating between an N and a Z banding pattern; the concentration of total AAT in an N specimen should be higher than that of a Z specimen. In addition, the Z bands migrate almost equivocally to the Y bands (Image 1, lane 15), but the total AAT concentration should resolve any confusion between the 2 bands, as Y carriers should have an AAT concentration within the reference interval (Table 1).

Nomenclature can also cause confusion when identifying variants. For example, The Z Pratt variant is coded by a nondeficiency allele. It migrates most cathodally of all the variants (Image 1, lane 18) but was not discovered until 1980, which was after the conventional nomenclature had already been established and the Z deficiency allele was already named. The migration pattern of the Z Pratt variant is distinct, with bands that migrate more cathodally compared with the Z variant. In contrast to the Z bands, Z Pratt bands are punctate and distinct, corresponding to a normal AAT concentration.

Certain variants will migrate identically but must not be assumed to have similar AAT concentrations. This is the case for the G and I variants, which have identical migration patterns. The AAT reference interval is 100 to 200 mg/dL. Additional reference intervals can be found in Bornhorst et al. and Donato et al. AAT concentrations were not available. The range shown was determined from other samples with identical phenotypes.

<table>
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<tr>
<th>Variant</th>
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<tr>
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</tr>
<tr>
<td>CM</td>
<td>194</td>
</tr>
<tr>
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The AAT reference interval is 100 to 200 mg/dL. Additional reference intervals can be found in Bornhorst et al. and Donato et al. AAT concentrations were not available. The range shown was determined from other samples with identical phenotypes.
patterns and can only be definitively differentiated using genotype analysis. G variant (Image 1, lane 5) major bands G4 and G6 are juxtaposed to the anodal and cathodal sides of the M4 band, respectively, and G minor bands are often absent. I variants (Image 1, lane 6) migrate identically to G variants, but G variants generally stain darker due to higher concentrations of AAT (Table 1). Although difficult, it is important to distinguish G and I variants because the latter is associated with AAT deficiency, whereas the former is not.15,20

Determining AAT concentration is often a fundamental component of diagnosing a deficiency of AAT. However, the F variant is a deficiency allele that is expressed at concentrations within the reference interval but has diminished catalytic activity.21 F variants (Image 1, lane 7) show a distinct doublet pattern. One set of doublets appears between the M2 and M4 bands, and the other set appears between the M4 and M6 bands. In contrast, AAT concentration is particularly important when evaluating a P variant (Image 1, lane 11) because these variants are associated with both normal and deficiency AAT alleles.22 The multiple P variants have slightly different migration patterns, but their defining feature is the P4 band, which migrates just anodally to the M6 band. The P7 band will comigrate with the minor M7 band in an MP heterozygote, and therefore, this band will appear to be a major band. The P variant should not be mistaken for the L variant (Image 1, lane 8). The L and P variants have very similar migration patterns with 2 exceptions. First, the L variant has a distinct major band that migrates just anodally to the M4 band. This band is absent in the P variant. Second, in an LM heterozygote, the L7 band resolves from the M7 band, where they comigrate in the MP heterozygote.

The remaining 2 variants illustrated in this compendium do not result in an AAT deficiency,15 and their migration patterns follow the conventional nomenclature. The C variant (Image 1, lane 2) has major bands C4 and C6 juxtaposed to the anodal and cathodal sides of the M2 band, respectively. The C variant also has a minor band, C2, that migrates anodally to the C4 major band. The bands corresponding to the X variant all migrate cathodally to the M bands, with the major X bands migrating along with the M minor bands (Image 1, lane 14).

**Stability of AAT in Whole Blood**

We evaluated the stability of the AAT PiMM phenotype in whole blood collected without preservatives and stored at ambient (21-24°C) and refrigerated (4°C) temperatures over 7 days Image 2A. There was no change in the migration of AAT from samples stored at either temperature over this time period. To evaluate the stability of a deficiency variant, we performed IEF electrophoresis on a 12-day-old whole-blood sample obtained from a PiMZ heterozygote Image 2B. Similar to what was observed with the PiMM phenotype, the PiMZ pattern remained easily identifiable at this extended time point.

**Image 2** Stability of MM (A) and MZ (B) for phenotype analysis in whole blood. PiMM samples were stored at either room temperature or 4°C for 0, 1, 2, 3, 4, or 7 days before analysis. The PiZZ sample was stored at 4°C for 12 days. The PiMZ control was a fresh serum sample.
Analysis and Stability of AAT Using Dried Blood Spots and Serum Separator Cards

Previous AAT phenotyping methods such as IEF electrophoresis, followed by silver staining or Western blot detection, were adapted to analysis after extraction from DBS. However, AAT phenotyping using DBS has never been reported using IEF electrophoresis with immunochromatographic detection of AAT. Homozygous wild-type individuals (PiMM) were easily identified after extraction from DBS (Image 3, lane 3). In contrast, the phenotype of deficiency carriers (MZ) appeared as PiMM because the Z bands were not detected (Image 3, lane 5). In an attempt to use a similar yet cleaner matrix, we extracted AAT from serum separator cards. Samples obtained from the serum separator cards did allow for correct identification of the Z deficiency variant (Image 3, lane 6). However, the Z phenotype could be correctly interpreted for only 1 day after spotting the whole blood onto the serum separator card. The M variant was stable on the serum separator card for all time periods tested (up to 21 days; data not shown).

Accuracy of Serum Separator Cards for Identifying Various AAT Phenotypes

Twenty-two whole-blood samples with known AAT phenotypes and AAT protein concentrations were spotted onto serum separator cards. An individual (M.C.E.-J.) blinded to the known phenotype extracted the serum, performed IEF electrophoresis, and determined the AAT phenotype. Of the 22 results, only 1 MZ phenotype was interpreted incorrectly as an MM (Table 2). Hemolyzed specimens gave uninterpretable phenotyping results (data not shown).

Discussion

Diagnosis of AAT deficiency relies heavily on the clinical laboratory. AAT phenotype testing by IEF electrophoresis is the biochemical gold standard for distinguishing AAT variants. However, the interpretation of AAT phenotypes is complex. In this work, we have created an interpretive guide to assist laboratorians with the identification of several AAT phenotypes. This compendium illustrates more than 98% of the phenotypes encountered in the laboratory, which includes 13 common and 5 rare variants. This phenotype frequency estimation is supported by 2 recent studies that have reviewed large clinical populations to assess the prevalence and associated serum protein concentration of AAT variant phenotypes. Although we strived to make this compendium comprehensive, it is possible to encounter a variant that we did not describe. Additional phenotypes not illustrated in this compendium, such as the PiV and PiSS, can be found in the original reference documenting the IEF method used in this study. Furthermore, it may be necessary to use SERPINA1 gene sequencing to classify a novel AAT phenotype or to resolve a genotype/phenotype discrepancy.

There are 2 important sources of preanalytical error regarding AAT phenotype testing. First, the treatment for AAT deficiency includes intravenous administration of recombinant AAT (PiMM). This variant will be detected in specimens obtained from individuals receiving therapy and can result in unlikely AAT phenotype patterns (eg, an individual with an apparent phenotype of PiMSZ) or an incorrect phenotype assignment (eg, a PiZZ individual appearing as PiMZ). Although there is usually no clinical need to perform AAT phenotype testing on individuals with known AAT deficiency and who are receiving replacement therapy, such
samples are occasionally submitted to the laboratory for phenotype testing, presumably in error. Similarly, another source of error occurs in individuals who were recently transfused with blood products containing plasma, as the AAT phenotype of the donor may also be detected.

AAT deficiency fits the criteria for disease screening as defined by the World Health Organization. For example, it is a relatively common disease that can be treated, there is usually a latent stage of the disease, and there is a test to detect the condition. Unfortunately, it is estimated that fewer than 10% of individuals with severe deficiency are diagnosed. Accordingly, widespread screening is likely justifiable, and testing might be more widely available if DBS or serum separator card samples could be used. Others have shown the utility of DBS for AAT genotype analysis. DBS also have been used to determine AAT phenotypes using earlier electrophoretic methods. Our work suggests that DBS are an inadequate sample type for AAT phenotype determination using the only FDA-cleared method for AAT phenotype determination (Hydragel A1AT Isofocusing; Sebia). Use of DBS did not permit the detection of the Z variant, even when the card was freshly spotted with blood. In contrast, the use of serum separator cards allowed for the Z bands to be detected when analysis was completed within 24 hours of sample collection, but detection of the Z band decreased considerably at later time points. Given that the utility of DBS or serum separator cards lies in the ability to screen populations, the inability to detect deficiency variants after only 24 hours suggests that this matrix would be inadequate. These results are troubling, as a US pharmaceutical company is currently marketing the use of DBS, free of cost, as a sufficient way to screen for AAT deficiency.

The use of capillary whole-blood samples is an attractive alternative to DBS and serum separator card samples, although the stability of AAT in whole blood has not been reported previously. We show here that both the PiM and PiZ variants are stable in whole blood for at least 14 days, suggesting that capillary samples may be sufficient for population screening. However, it should be noted that using whole blood as the matrix for IEF can result in AAT bands that are less punctate relative to comparative serum samples. Since interpretation of AAT phenotypes is already complex, it may be problematic to implement analysis on whole blood or capillary samples. Further studies regarding AAT variant stability in whole blood and alternative sample types, such as DBS and serum separator cards, are required.

In conclusion, this work provides clinical laboratories with fundamental information for assessing AAT phenotypes. This was accomplished by first creating a reference guide to AAT phenotype interpretation and second by assessing phenotype stability in various sample matrices. Currently, no other publication, in either a textbook or the scientific literature, provides such a comprehensive evaluation of AAT phenotypes. Combined, this work will facilitate the laboratory’s role in the proper diagnosis of AAT deficiency.

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