Most individuals with AAT deficiency have escaped detection by healthcare systems worldwide. The large number of undiagnosed individuals has made it difficult to define the natural history of the clinical disease accurately, and severe ascertainment bias has colored the clinical descriptions of the disease. Most importantly, undetected individuals lose opportunities for important lifestyle changes and preventive therapies. To address this problem, the World Health Organization has recommended that all patients with chronic obstructive lung disease, and all adults and adolescents with asthma, be tested for AAT deficiency. Historically, the AAT Deficiency Detection Center has tested more than 30,000 individuals for the disease, and we have identified more than 1000 cases of AAT deficiency (approximately 30% of the known cases in the United States). Currently, we are implementing methods for determining AAT concentration (level), phenotype, and genotype in specimens of whole blood dried onto filter paper. This full spectrum of robust tests is performed on samples that are easily obtained and shipped to a central laboratory for processing. Wide application of these procedures may help to bring large numbers of presently undiagnosed patients to medical attention.

Key words: phenotyping; genotyping; allele-specific polymerase chain reaction.

Introduction

Despite the fact that more than 25 years have elapsed since the discovery of AAT deficiency, most individuals with the disease worldwide remain undiagnosed. Sweden, Denmark and the Netherlands historically have led the rest of the world in diagnosing AAT deficiency because of medical interest in the disease in those countries, coupled with healthcare systems that facilitate organized diagnostic testing. Other countries have lagged far behind in their efforts to diagnose affected individuals. In the United States, for example, it is thought that fewer than 4000 cases of AAT deficiency have been diagnosed from the estimated 100,000 individuals with the disease. Experience in the United Kingdom has been similar (1).

Individuals who have AAT deficiency, but who have not yet been diagnosed, are losing opportunities for important lifestyle changes and therapies to prevent the onset or progression of lung disease. Further, the large number of undiagnosed individuals has made it difficult to define the natural history of the disease, since the health status of such individuals is largely unknown. The existing clinical reports of AAT deficiency are colored by severe ascertainment bias, since most of the individuals reported have been diagnosed because of extant lung disease (2).

Methods for unequivocal diagnosis of AAT deficiency have been available since the early 1960s. Thus, the limiting factor in detecting undiagnosed patients is simply the ordering and performing of requisite diagnostic tests. Since this is a genetic disease, testing need be performed only once in a lifetime. The World Health Organization has recognized that many individuals with AAT deficiency may be seeing healthcare providers, yet have been given other diagnoses. The WHO addressed this problem with a pivotal recommendation that all individuals with chronic obstructive lung disease, and all adults and adolescents with asthma, be tested for the disease (3). The AAT Deficiency Detection Center has been striving to improve compliance with this recommendation by enhancing the convenience of testing procedures and improving access to testing facilities.

The AAT Deficiency Detection Center

We estimate that the AAT Deficiency Detection Center in Salt Lake City, UT, U.S.A. performs about 25% of the testing for this disease in the United States. In the 9 years of operation between 1 March 1991, and 29 February 2000 we have tested 30,631 blood samples for AAT deficiency, and have diagnosed 1021 individuals with the disease. The
latter represents approximately 30% of the known individuals in the United States. Interestingly, 3.3% of all of the samples that we have received during this period came from individuals with AAT deficiency. Thus, the diagnostic yield of blood samples sent to our laboratory exceeds that for routine screening laboratory tests such as complete blood counts, thyroid tests, chemistry panels and urinalyses (4).

Types of diagnostic tests for AAT deficiency

Diagnostic testing for AAT deficiency can be divided into four stages of tests, which are shown in Table 1. Table 1 shows the diagnostic usefulness of each type of diagnostic test, as well as where each fits into the diagnostic spectrum of testing for the disease. In the past, serum protein electrophoresis was considered to be a useful diagnostic test for the disease, in that visually-determined absence of the α-band was an excellent correlate of AAT deficiency. This test is not shown in Table 1, since its utility has been largely superseded by specific immunoassays. However, AAT deficiency is incidentally detected on occasion when a serum protein electrophoresis is performed for an unrelated reason.

Immunoassay is the stage 1 test for the diagnosis of AAT deficiency. Several are available, and all are useful for diagnosis of the disease. An immunoassay is typically the first test that is performed when an individual is suspected of having AAT deficiency. It measures the concentration (‘level’ of AAT in plasma or serum. In our hands, the reference concentration of AAT in plasma is 32.4 μM; AAT levels of <11 μM are considered to be diagnostic of AAT deficiency. Most individuals with AAT deficiency have plasma levels in the range of 10-15% of normal [mean level in our laboratory, 4.3± (SD) 1.7 μM, n=927]. Heterozygotes (carriers) of AAT deficiency tend to have intermediate AAT levels. However, there is a substantial overlap in AAT levels between normal individuals and heterozygotes for AAT deficiency. The strengths of immunoassay are that it is reliable, inexpensive and subject to automation. Because of the overlap in AAT levels between normals and carriers, immunoassays suffer from the weakness that they cannot reliably detect carriers for the disease, and of course they do not define the genetic makeup of the individual who is being tested.

Phenotyping is the stage 2 test for AAT deficiency. It characterizes the type(s) of AAT protein found in plasma or serum. This is most commonly accomplished by isoelectric focusing of plasma or serum proteins in a polyacrylamide gel over a narrow pH range (in our laboratory, pH 4.2-4.9). In 1979, isoelectric focusing was improved by the addition of a reducing agent, which reduced the background staining of the gels and enhanced our ability to recognize abnormal AAT variants (5). The major strength of phenotyping is that it provides independent confirmation of this critically-important diagnosis. In view of this fact, the WHO has recommended that individuals who have abnormal immunoassay results should subsequently undergo phenotyping (3). This is important because the diagnosis of AAT deficiency makes a lifelong impact on patients’ healthcare. Phenotyping also detects and identifies unusual α1-antitrypsin alleles and identifies heterozygous for α1-antitrypsin deficiency. Phenotyping of the most common AAT variants is illustrated in Fig. 1. Weaknesses of phenotyping are that it is a labor-intensive manual test and considerable experience and expertise are required to interpret the results. Phenotyping results are confusing in patients who are receiving augmentation therapy, since it detects both the infused protein as well as the patients’ native α1-antitrypsin. Further, it cannot detect the presence of ‘null’ AAT genes that do not result in detectable AAT in

| Table 1 |
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| **Stage of testing** | **Name of test** | **Parameter measured** | **Strengths** | **Weaknesses** |
| I | Immunoassay | Concentration (‘level’) of AAT protein in plasma or serum | Relatively inexpensive | Does not reliably detect heterozygotes |
| II | Phenotype | Isoelectric points of AAT isoforms | Independent confirmation of diagnosis | Manual technique |
| | | | Identifies heterozygotes | Requires skill and experience |
| III | Genotype | DNA abnormality, determined by polymerase chain reaction | Definitive | Does not detect null alleles |
| | | | Conclusively identifies specific mutations | Can be expensive |
| | | | Detects null alleles | Requires specific primers for each allele |
| IV | Function | Inhibition of leukocyte elastase | May be useful if stages I-III are normal in suspicious cases | Little clinical experience with its use |
the plasma. Individuals who carry such genes can be identified only through careful family studies or by genotyping. Rare variants may differ minimally in their isoelectric points, leading to difficulty in interpretation of the results and the need for many standards. Standards for isoelectric focusing must be established with certainty, and appropriate standards may or may not be available to all diagnostic laboratories. Finally, the variants may or may not differ in their isoelectric points, as is the case for the PS and PV variants.

We have been interested in trying to improve the objectivity of the interpretation of phenotyping gels. With appropriate standards or a surface pH electrode, the isoelectric points of the major isoforms of AAT can be established with considerable precision. This observation raises the possibility of interpreting isoelectric focusing gels objectively, based on the measured isoelectric points of the AAT protein. If validated by sequencing, new standards for interpretation of phenotyping gels could be established.

Genotyping is the stage 3 method for the diagnosis of AAT deficiency. Genotyping offers a very precise determination of an individual’s genetic makeup at the Pi locus, based upon analysis of the individual’s DNA. We have been involved in a very interesting and productive collaboration with AstraZeneca that has taken advantage of their ARMS technology for allele-specific polymerase chain reaction. This technology amplifies a very large number of copies of a nucleotide of a predetermined size if the complementary DNA is present in an individual’s genome. The process is precise enough that it will detect a single base-pair substitution in the DNA. The ARMS technology is robust and generally applicable to all point mutations and small deletions. One of the strengths of the technology is that it can accurately detect mutations even when only trace amounts of DNA are present, which means that one can use non-invasive samples, such as mouthwash fluid or buccal scrapings as diagnostic specimens. Furthermore, it is possible to detect several mutations simultaneously and cost-effectively. The present/absent nature of the products of the reaction make the technology an ideal candidate for automation.

An example of an ARMS genotyping test is shown in Fig. 2. The procedure shown is a straightforward simultaneous test for the Z mutation (the most common allele.

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**Fig. 1. Phenotyping of α1-antitrypsin.** Five microliters of plasma from individuals carrying the normal (M) and most common deficient (Z) alleles was applied to lanes of a polyacrylamide isoelectric focusing gel. After completion of the isoelectric focusing, the proteins in the gel were stained with Coomassie blue. Left lane: Pi M (normal) individual. Note that variability of glycosylation of the α1-antitrypsin results in isoforms of the M protein having different isoelectric points, which cease migrating at differing positions on the gel. The most prominent (M4 and M6) bands are marked. Center lane: Pi Z individual with α1-antitrypsin deficiency. Note the fainter protein bands that differ in isoelectric points. The corresponding Z4 and Z6 bands are marked. Right lane: Pi MZ heterozygote (carrier) for α1-antitrypsin deficiency. Note the presence of both M and Z protein bands.

**Fig. 2. Genotyping of α1-antitrypsin.** Allele-specific primers, in a polymerase chain reaction, are used to amplify products of specific size that depends upon the alleles that are present. The products are then run on an agarose gel to give the results shown when examined under ultraviolet light. Each panel shows the results from a single individual. Two different amplifications are run for each analysis (A and B). The top and bottom bands in each lane are control amplification products. The second band from the top relates to the Z allele in each lane. If the sequence is normal at the site of the Z mutation, a band of that size amplifies in the A reaction. If the Z mutation is present, a band of that size amplifies in the B reaction. Left panel, Pi*M (only the normal sequence); center panel, Pi*Z (only the abnormal sequence); and right panel, Pi*MZ (both the normal and the abnormal sequence).
associated with AAT deficiency) and for the $S$ mutation (a common abnormal allele associated with much more mildly reduced plasma AAT concentrations). A single ARMS reaction can be designed to amplify products of various sizes depending upon which mutation(s) are present in the individual's genome. We are currently working with AstraZeneca to develop a multiplex test or tests for a variety of other common mutations in the AAT gene. This technology seems to be an ideal platform for expanding the range of mutations in the AAT gene that can be detected with a straightforward test procedure. An important strength of genotyping is that it provides a definitive yes or no answer for each mutation in the test. In addition to the clinical value that this lends to testing, this yes/no answer will lend itself well to automation. Because it is a DNA-based test, genotyping can correctly identify mutations that result in no detectable protein in plasma, and it provides correct diagnoses in individuals who are receiving augmentation therapy. Expense is a weakness of genotyping, depending upon the extent of testing that is necessary to provide a correct diagnosis. It is generally not appropriate as a stand-alone test, although combining the analysis with an immunoassay and phenotyping is appropriate in most cases. Genotypic diagnosis is not complete if the procedure cannot identify both AAT alleles. In selected cases when available primers fail to provide a complete diagnosis, sequencing of exonic DNA is appropriate.

Functional measurement of $Z$-antitrypsin comprises the stage 4 method of diagnostic testing. Rarely, mutations in the $Z$-antitrypsin gene result in a protein with abnormal or even altered function. There has been little experience with large-scale application of these testing methods, which are beyond the scope of this discussion.

The future: full-spectrum testing of dried blood spots

In the 9 years that we have been testing for AAT deficiency, our laboratory has been committed to finding improved methods of testing for the disease. We have found that an important barrier to widespread application of the WHO recommendations for testing has been the inconvenience of obtaining, packaging, and shipping liquid blood to a central laboratory such as ours. Obtaining the blood samples requires disposables and a skilled phlebotomist. Shipping the potentially biohazardous specimens is costly and requires biohazard packaging. We have developed methods for full-spectrum AAT testing (Stages 1-4) that can be performed on a few drops of blood that have been dried onto filter paper.

We have been involved in international collaborative projects with Prof. Stockley in the U.K. and with Marion Wencker in Germany, demonstrating that even international shipment of dried blood spots provides samples that are adequate for full-spectrum testing. We plan to make testing of dried blood spots available in the U.S.A. in the third quarter of 2000.

Summary and conclusions

Although certain diagnostic tests for $Z$-antitrypsin deficiency have been available since the 1960s, most individuals who have $Z$-antitrypsin deficiency worldwide escape diagnosis. Undiagnosed individuals lose potentially life-saving opportunities for lifestyle changes and medical interventions. Identifying this large population of undiagnosed individuals will require improved awareness of $Z$-antitrypsin deficiency and more convenient testing procedures. Full-spectrum diagnostic testing of whole blood dried onto filter paper is technically feasible and convenient, and provides new possibilities for accomplishing the wider testing for the disease that has been recommended by the World Health Organization.

References