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Utility of Nephlometry, ELISA and Serum Protein Electrophoresis as Diagnostic Tools for Alpha1-Antitrypsin Deficiency in COPD and Smokers

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Evaluation of analytical techniques used in the detection of alpha1-antitrypsin (AAT) deficiency. The serum AAT was determined by several techniques, namely nephlometry, enzyme-linked immunosorbent assay (ELISA) and serum protein electrophoresis for 3 groups of samples: group (1) compromise of patients with emphysema or COPD, a group (2) consisted of age matched normal control population belonging to both sexes and group (3) comprised of smokers with a minimum of 10 pack years of smoking history. The research was focused on comparing the different quantitative techniques of serum antitrypsin level in the above sample groups. We evaluated three different methods of quantification namely, immune nephlometry, ELISA, and a protein profiling by serum protein electrophoresis. Comparison of AAT quantification, ELISA was the better precision method than nephlometric one. Also, serum protein electrophoresis revealed indications of the deficiency and the heterozygous genetic makeup of the affected individuals. The genotype of AAT deficient samples was investigated using polymerase chain reaction revealed in the presence of the S and Z alleles. PCR results confirmed of s, z or sz genotypes in some of deficient samples. Serum protein electrophoresis can be used as a valuable diagnostic method for early lab diagnosis of risk people.

Keywords: Alpha-1-antitrypsin; Nephlometre; ELISA; Serum protein electrophoresis

1. INTRODUCTION

Alpha1-antitrypsin (AAT) is a serine protease inhibitor secreted by liver cells. This enzyme acts as an inhibitor of a number of proteolytic enzymes, the most prominent of which is elastase.

Elastase is secreted by neutrophils when they disintegrate or are activated, and it degrades the elastin and other host extracellular matrix constituents [1]. A deficiency of this enzyme is a risk factor for the development of pulmonary and liver diseases [2]. The gene responsible for the synthesis of AAT is located on chromosome 14, the highly polymorphic nature of AAT indicates that the gene locus is also highly variable. The variants are indicated codominantly and are classified according to the protease inhibitor (PI) system which depends on the electrophoretic mobility of the different proteins on acrylamide gels. The MM (medium mobility) phenotype is the most common one with serum AAT levels range between 150-350 mg/dL [3].

Low AAT levels occur as a result of the inheritance of two alleles (S and Z) that encode decreased levels of this protein. Homozygons (PI ZZ) give serum AAT levels ranging from 15 - 50 mg/dL which leads to incapacity and death. The S allele is more common than the Z allele, the SZ genotype (PI SZ) gives AAT levels ranging from 45 - 105 mg/ dL, while the SS genotype (PI SS) levels ranging from 100 - 140 mg/ dL [3]. SZ heterozygotes persons are three times more likely to develop COPD [4]. The role of genetic factors in the etiology of COPD in nonsmokers has not been properly studied so far. However, there is evidence of genetic factors influencing the development of COPD in response to cigarette smoking [5]. Case studies have revealed that nonsmoking protease inhibitor (PIZ) subjects are at increases risk for developing COPD, although to a lesser degree compared with PIZ smokers [6].

The deficiency of this enzyme is often reported to be under- recognized or under treated by clinicians because of lack of specific and rapid routine diagnostic tests for detecting the enzyme levels in patient serum. The objective of the present study was to quantitate the serum levels of AAT using several techniques to know the most specific and rapid routine diagnostic tests and a preliminary estimation about the presence of Z and S alleles.

2. MATERIAL AND METHOD

We conducted a case control study in collaboration with University Hospital and pulmonary specialized hospitals in Saudi Arabia. Ethical approval was obtained from the institutional review board of the respective hospitals and informed patient consent was taken. We collected blood samples and medical history of people belonging to 3 groups. A group (1) compromises of patients who were clinically diagnosed as having emphysema and chronic bronchitis (subsets of COPD). Group (2) consisted of age matched normal control population belonging to both sexes. Group (3) comprised of smokers/ex-smokers to clarify the effect of smoking on the enzyme deficiency. As per the targeted objectives of the study, a comparative study was undertaken to compare the sensitivity of the quantitation of AAT using some techniques such as: Nephlometry determination of serum AAT levels was performed using (BN prospec, Siemens, Erlangen, Germany). The normal reference range for this test is from 120 – 200 mg/ dL, Sandwich enzyme linked immune sorbent assay (ELISA), human alpha-1- antitrypsin ELIZA Kit (My Biosource, CA, USA) as used for this experiment, the optical density (OD) of the solution was measured spectrophotometrically in an ELISA plate reader (IRE 96, SFRI reader, France) at 450 nm. A standard curve was plotted using the OD value and the concentration of

the standard sample. Concentration of the protein in the patient samples was calculated from the standard curve using the OD values observed.

Also, serum samples stored at – 20 °C was thawed and diluted 1:1 with 2x sample buffer (Novagen, USA), and heated at 80 °C in water bath for 3 minutes and loaded into the Precasted ready gel (Min- Proteon TGX Stain free, BIORAD inc, CA, USA). Unstained molecular weight marker (invitrogen, USA) was loaded for the first well of the precast gel. Denatured samples of patients and smokers were loaded and sodium dodecyl sulphate polyacrylamide gel electrophoresis were done in mini protean gel assembly (BIORAD Inc, CA,USA) using 1xtris glycine running buffer at a voltage of 170 for 1 hour. The gel was taken out of the cating frame and gel image was captured using Gel DOC EZ image lab software (BIORAD, CA, USA).

According to the standard method [7], genomic DNA was isolated from whole blood and was preserved in sterile distilled water at 4 °C until the PCR assay. PCR – mediated mutagenesis is performed as described in the protocols [8] for PI genotyping.

Genotyping by PCR-DNA sequencing was performed according to the procedure reported by [9]. The amplification of the four coding exons 2,3,4 and 5 of the SERPINA1 gene was done in a thermalcycler (ABI, Gene amp 9700, U.S). The samples that were found deficient by the preliminary screening analysis were screened for the deficiency related alleles. The primer sequence was as follows (Table 1).

Table 1. Sequence of primers used for amplification of s, z alleles

Mutation	Position	Prime sequence (Gene bank, accession No: K02212)
S(Forward)	P9504	5'-CGTTTAGGCATGAATAACTTCCAGC-3'
S(Reverse)	P9629	5'-GATGATATCGTGGGTGAGAACATTT-3'
Z(Forward)	P11916	5'-ATAAGGCTGTGCTGACCATCGTC-3'
Z(Reverse)	P12014	5'-GAACTTGACCTCGAGGGGGATAGAC-3'

3. RESULTS

Estimation of alpha1-antitrypsin enzyme was done using serum samples of patients who were clinically diagnosed with (COPD), smokers who have a smoking history of at least 10 pack years and normal healthy individuals. The quantification of AAT enzyme was done using two methods, Immune Nephlometry (BN Prospec, Siemens, Erlangen, Germany) and sandwich enzyme linked immune sorbent assay (IRE 96 Reader, SFRI, France). The results of both methods were compared. Immune nephlometric readings were higher than that of ELISA (Figure 1). Both techniques are immune complex reactions, ELISA seemed to be more sensitive.

Protein profile of serum samples was analyzed and interpreted. There were five prominent protein bands, Albumin, alpha 1 zone, alpha 2, beta 1, beta 2 and gamma globulin region. Alpha 1 region included the protease inhibitor called alpha 1 antitrypsin which showed upon the gel as a 52 KDa protein band. SDS page was used to analyze serum samples of COPD, we found that the enzyme

deficient samples had a heterozygous genotype as a compared to the normal. This was seen as two separate protein bands in the gel (Figure 2).

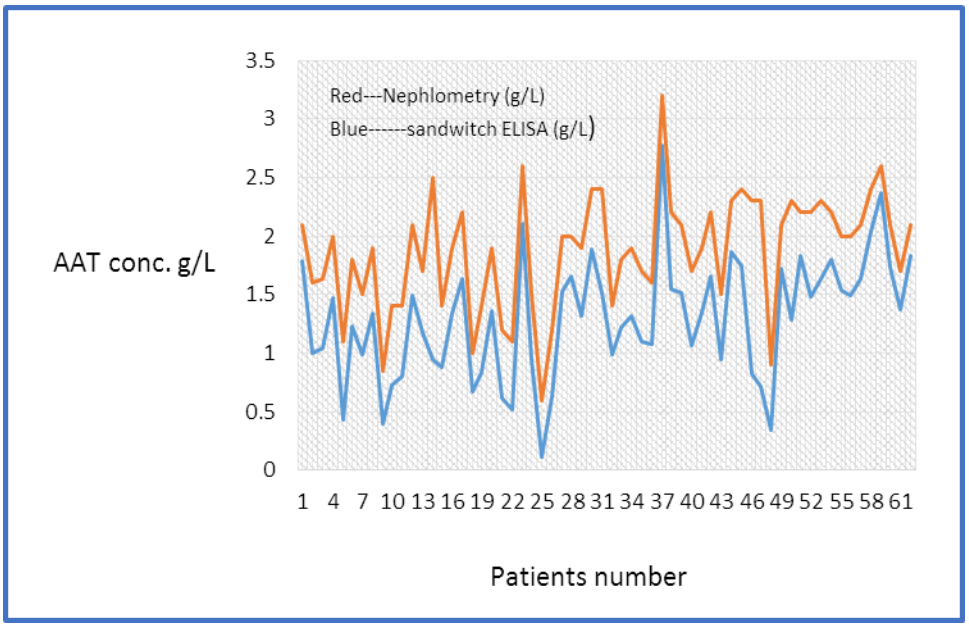

Figure 1. Comparative quantification of alpha1-antitrypsin using Nephlometry and ELISA techniques

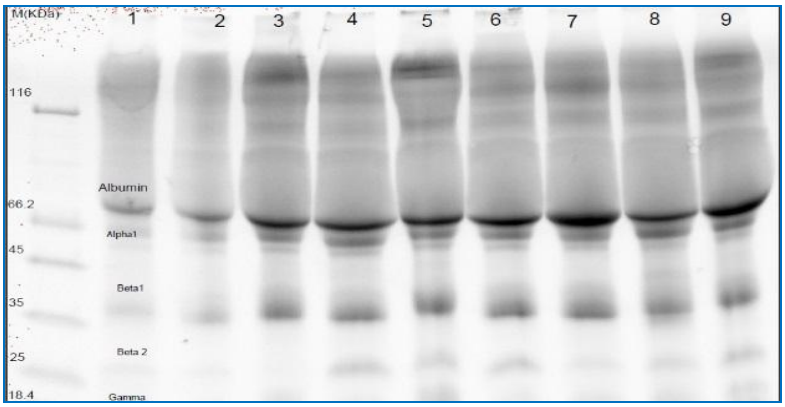

Figure 2. The illustrated bands of alpha 1-antitrypsin deficiency in COPD patients: Lane 1-Protein marker, Lane1-9: COPD patient samples

The smokers with 10 pack years of smoking or more were included in the study. The protein profile was found helpful in identifying deficient phenotypes as a preliminary screening tool when compared to the protein profile of a healthy nonsmoker where it was found that there was a marked reduction in the protein concentration of the AAT band (Figure 3).

In order to identify any change in the exons coding for the protein, abnormal genotypes were not encountered so far in the study (Figure 4), but more of sequencing results are awaited for further exploration.

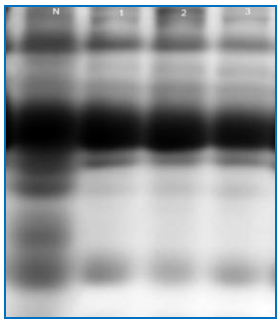

Figure 3. Serum protein profile of Smokers; N-Normal Control Serum profile, Lane 1-3 Smoker serum profile showing changes in protein bands of alpha 1, alpha2, beta1 and beta 2 regions.

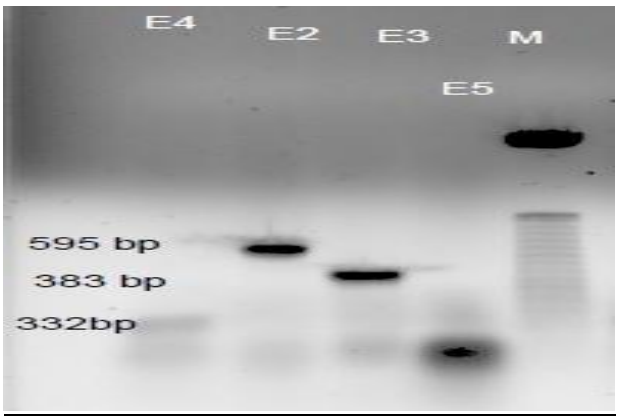

Figure 4. Amplicons of exons of SERPINA 1 gene. Lane1-4:exons4, exon2 (595bp), exon3 (383bp), exon5 (332bp), Lane5:100bpDNA ladder

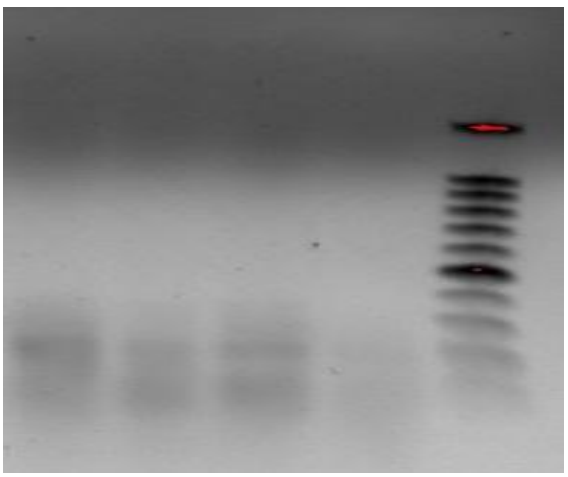

Figure 5. Lane (1-4): PCR amplicons of SZ genes, 50bp DNA ladder

The SZ PCR experiment was done as confirmatory test to identify the heterozygous genotype of the deficient samples identified by preliminary screening using serum protein profiling. PCR results confirmed the presence of s, z, or sz genotypes in some of the deficient samples (Figure 5).

4. DISCUSSION

It is important to determine the AAT deficiency by sensitive and rapid techniques as this deficiency is not rare, but it is rarely diagnosed [10]. The current method of AAT quantification in Saudi Arabia is by immune nephelometry. In the present study quantification of AAT enzyme was done using two methods, immune Nephlometry (BN Prodpec, Siemene, Erlangen, Germany) and Sandwich Enzyme linked immune sorbent assay (IRE 96 Reader, SFRI, France).

When we compare the results of the two methods, immune nephlometric readings were higher than that of sandwich ELISA. Since the serum sample may contain interfering biomolecules such as lipids or hemoglobin in addition to the immune complex, they may add up to the readings [3]. The detection of the limit of the ELISA is lower compared to that of nephlometry, therefore any deficiency will be detected more accurately determined using ELISA than by nephlometry.

The Sandwich ELISA that we used seemed to the detect AAT protein specifically and the optical density taken was the indication of the actual amount of protein in the serum. While, in nephlometry the light scattered by the antigen, antibody complex was measured. However additional components in the serum may also add to the values determined by this technique. Therefore, sandwich ELISA seems to be more sensitive and cheaper than nephlometry. We can consider ELISA as a preferred analytical technique due to its Cost effectiveness even in ordinary clinical setting accessible for the common man.

The serum protein profile was analyzed as per the literature on serum proteins [11]. Serum protein of the COPD patients showed two protein bands in the alpha 1- zone indicating heterozygsity (Figure 2). In the second category of samples, smokers it was found that there was a marked reduction in the protein concentration of AAT band (Figure 3). This preliminary Screening technique can be used as a guide for further analysis and genotyping of the samples.

Deficient samples were analyzed for the presence of S and Z alleles, PCR results confirmed the presence of S, Z or SZ genotypes in some of the deficient samples. Therefore, the preliminary screening by serum protein electrophoresis followed by the implication of SZ genes can give enough information about the severity of enzyme deficiency and thereby helping clinicians in therapeutic decision making, thus availability of serum protein electrophoresis methods give finer details in sub fraction of serum proteins and preliminary diagnosis of deficiency at both phenotypic and genotypic levels. While at this stage typing of genetic variants by electrofocussing would more appropriate [12].

Finally, Serum protein electrophoresis using ready precast SDS PAGE gels were performed for a qualitative analysis of the protein profile of the serum samples. Alpha1-antitrypsin has been the major protein in the alpha1 region of the serum showed up clearly in the gels. Additionally the genotypes hetrozygosity can be visualized from the experiment without waiting for molecular experiments to be completed. Serum protein profiling thus becomes a handy and quick way of

identifying a heterozygous sample. A comparison of the protein concentration of the patient sample with that of a normal control can give a visual clue about the quantity of the alpha1 protein in the serum. Therefore, quantitative and qualitative preliminary screening can be performed with this experiment.

So, serum protein profiling can be used as a valuable diagnostic tool for early lab diagnosis. This technique is faster than that of phenotypic and genotypic identification by isoelectric focusing, PCR and sequencing.

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