4. MATERIALS AND METHODS

4.1 Sample selections

The study was conducted with 25 normal persons and 25 allergic persons. 29 persons were from urban area and 21 were from rural area. Among 50 persons, 23 were males and 27 were females. Most of the persons were above 31 years in both males and females. The persons were selected from among the patients visited Asthma Research and Treatment Center, Pollachi.

4.1.1 Inclusion criteria

Those persons who satisfied the following criteria were included in this study

4.1.1.1 For normal patients

1. No history of atopic diseases like bronchial asthma, allergic rhinitis, urticaria, atopic dermatitis, etc.,
2. No family history of atopic diseases.

4.1.1.2 For allergic patients

1. Definite history of wheezing which was diagnosed and treated by a physician with and tablets.
2. Definite history of at least three attacks of wheezing previously.
3. Family history of atopic diseases.
4. Increased total immunoglobulin E level in the serum (>200 IU/ml) estimated by ELISA test.
5. Sometimes history of food hypersensitivity

To estimate the Serum Histamine Binding Capacity in normal and allergic persons for comparison and to establish that Serum Histamine Binding Capacity (SHBC) is more in normal persons than in allergic persons, the following tests were done:

I. ELISA test – to estimate total Immunoglobulin E (IgE) level in the serum – to find out normal and allergic persons
II. Separation of Antihistamine antibody from the serum by ‘Affinity Chromatography’ method.
III. Analysis of the elutes obtained by Affinity Chromatography, method to establish that the elute contains only antihistamine antibody by SDS Page test (Sodium dodecyl sulphate - Polyacrylamide Gel test).

IV. To know the type of antibody – whether it is IgG or IgM or IgA etc., by MALDI-TOF analysis. (Analysis was done by Indian Institute of Science, Bangalore – 560012).

V. Estimation of Serum Histamine Binding Capacity of allergic and normal sera was done as in the case of ELISA test by using Histamine coated micro-wells. This method was a modified method of ELISA test. This method was developed for the first time by us.

4.1.2 Collection of blood sample

Blood samples were collected after explaining to the patients and getting their consent in our clinical laboratory. Patient’s blood pressure was checked and thorough medical examination was carried out. Using 5ml sterile syringe, 3ml of blood was collected by venipuncture under strict aseptic precautions, the blood from the syringe was transferred to sterile 10ml bottle and centrifuged to separate serum. This serum was used for above mentioned tests.

a). to estimate total IgE Immunoglobin E level by ELISA test.

b). to separate antihistamine antibody by Affinity Chromatography method.

c). to estimate Antihistamine antibody level in the elute by Bradford protein assay method.

d). to estimate Serum Histamine Binding Capacity by Histamine ELISA Method.

4.2 TEST 1: ELISA TEST

ELISA test was performed as per instructions from Manufacturer (Omega Diagnostics, UK). Briefly, the number of strips required for the assay was assembled. 100µl of each standard and positive control were dispensed. 20µl of each sample to be tested was dispensed into the respective wells and 80µl of sample diluent was added into each sample well to make a 1/5 dilution. Assay plate was tapped rapidly to mix the well contents and then incubated for 60 minutes, well contents were decanted and the
wells were washed 3 times manually with wash buffer. 100µl of conjugate was then dispensed into each well and incubated for 30 minutes at room temperature. After 30 minutes, the well contents were discarded and washed 4 times carefully. It was ensured that the wells were empty but did not dry out. Then, 100µl of TMB Substrate (3,3’,5,5’ Tetra methyl benzidine) was rapidly added into each well and incubated for 10 minutes after which 100µl of Stop Solution was added to each well. The optical densities of the standards, positive control and samples were measured using a micro-plate reader (Robonic) at 450nm, within 10 minutes. By doing ELISA test, the IgE level was estimated and decided whether the patient was allergic or normal. The list of allergic and normal persons were furnished below

Using the sera of two normal persons NR, NB(NR female with IgE 51 IU/ml, NB 63 year old man with IgE 59 IU/ml) and two allergic sera AJ, AY (AJ 18 year old female with IgE 1785 IU/ml, AY 39 year old man with IgE 343 IU/ml) serum elutes were taken by affinity chromatographic method to know whether the elutes contain antihistamine antibody.

4.3 TEST 2 : AFFINITY CHROMATOGRAPHY

Affinity chromatography is a separation method, based on a specific binding interaction between an immobilized ligand and its binding partner. Examples include antigen/antibody, enzyme/substrate and enzyme/inhibitor interactions.

A commonly used metaphor to illustrate affinity binding is the lock and key analogy. A unique structure present on the surface of a protein is the key that will only bind to the corresponding lock, a specific ligand on a chromatographic support.

In our study, the antihistamine antibodies have to be separated from the serum of normal and allergic persons.

4.3.1 Principle

If serum is known to contain antibodies against a specific antigen, then it can be used for the affinity purification of that antigen. In this test, antihistamine antibody in the serum has to be removed and estimated. The histamine is the antigen. This procedure is also known as Immunoaffinity Chromotography.
The histamine crystals coupled with CNBr activated sepharose as a ligand acts as a solid phase and the serum of patients, containing antihistamine antibodies as a liquid phase.

4.3.2 Protocol for activating and coupling of CNBr activated sepharose Medium (CNBr activated sepharose) preparation

1. Cyanogen Bromide activated sepharose 4B (CNBr activated sepharose from sigma cat#c9142) treated with low pH buffer (pH 3.0) to remove additives present in it.
2. To 250ml of 1mµ HCL 1G of activated CNBr sepharose lyophilized powder was added and allowed to stand at room temperature for 15 minutes for beads to swell (1g of the powder gives ~3.5ml of medium)
3. This was passed through a sintered glass filter and washed with the 1mM HCL for 15 minutes

4.3.3 Ligand coupling

1. Histamine was dissolved in coupling buffer (0.1m NaHCL3 with0.5m Nacl pH 8.3). totally 30mg of the protein for the 3.5ml medium was used for coupling (5-10mg/ml of medium)
2. Ligand in the coupling buffer was added to the prepared medium in a stoppered tube.
3. The tube with the mix was rotated and to end at 4ºC/overnight.
4. The excess Ligand was washed away with 5 medium volumes of coupling buffer.
5. To block the remaining active groups, the medium was added with 0.1M Tris-HCL pH 8.0 and allowed to stand for 2hours
6. Then the medium was wahed with 3-5 cycles of alternating pH with two different buffers. The buffers are – pH 4.0 buffer -0.1 Macetic acid/Na acetate with 0.5M NaCl; pH 8.0 buffer -0.1M tris –HCL with 0.5M NaCl.
7. The medium was wahed with 5 medium volumes of the above buffers and they were used alternatively.
Finally the medium was washed and packed to a column with 20mM Na phosphate buffer with 0.5M NaCl and 0.02% Na azide and stored at 4°C.

4.3.4 Instruments and reagents

1. Chromatographic column
2. Activated Sepharose conjugated with histamine crystals as ligand
3. Sodium phosphate buffer containing various chemicals like sodium phosphate, sodium chloride and sodium azide (pH 7.4), 2 molecular sodium chloride (2M NaCl), Glycine with hydrochloric acid (pH 2.2).
4. Pipette with accessories
5. Collecting Vials and beaker.
6. Spectrophotometer
7. Sera of the individuals

4.3.5 Procedure

The immune affinity column preparation was based on Hogasen, K., Mollaner, T.E., and Harboe, M(1992), and Mohan,J., Saini,M., and Joshi,P. (1995). In this method the Histamine was conjugated with activated sepharose and filled in the chromatograph glass tube. This acted as a solid phase. After washing the Histamine + Sepharose complex with buffer solution sodium phosphate and 2M sodium chloride solution, Normal serum of NR was added to the solid phase. The serum eluted through the solid phase Histamine + Sepharose complex. The antibody to histamine got attached to the Histamine + sepharose complex. Serum, containing other proteins passed through the solid phase without any alteration and it was collected in the beaker.

The Histamine + Sepharose complex to which the antibody to histamine attached, was washed with buffer solution sodium phosphate and 2M sodium chloride solutions. Then to the solid phase, Glycine + Hydrochloric acid (pH 2.2) reagent was added. In the acid medium, the bondage between the Histamine and antibody to histamine was broken. The elute containing the antihistamine antibody was collected in test tubes. The elute must be containing only antihistamine antibodies. Likewise the elutes of normal serum NB and allergic sera AJ and AY of allergic persons were taken.
In order to find out whether the contents of the elute was ‘an antibody’, SDS PAGE test (Sodium dodecyl sulphate polyacrylamide gel test) was done.

**4.4 TEST 3 : SDS PAGE TEST**

SDS PAGE Test with elutes obtained by affinity chromatography method to establish that the elutes contained an antibody (antihistamine antibody) SDS PAGE test.

**4.4.1 Instruments**

1. Polyacrylamide Gel.
2. Sodium Dodecyl Sulfate (SDS) a strong denaturing detergent.
3. Electrophoresis apparatus.

**4.4.2 Procedure**

To the elute added Sodium Dodecyl Sulphate a detergent. The antibody (antihistamine antibody) was digested into its components of heavy and light chains by the detergent. The SDS treated antibody mixture (ELUTE) was added to the wells of polyacrylamide gel. The heavy chain and light chain were separated by electrophoresis according to their molecular weight – molecules of light chain with lower molecular weight migrated farther than molecules of heavy chain with higher molecular weight.

Two distinct bands corresponding to heavy chain (~ 50 KDa) and light chain (~25 KDa) were obtained. To find out the type of antibody whether it was IgG or IgM or IgA, the bands from heavy chain and light chain were sliced and sent for mass spectrometric analysis.

**4.5 TEST 4: MALDI TOF ANALYSIS OF HEAVY AND LIGHT CHAIN (MBU, INDIAN INSTITUTE OF SCIENCE (IISC), BANGALORE, INDIA )**

The sliced heavy and light chain bands of SDS PAGE were sent to Indian Institute of Science, Bangalore for MALDI MS Analysis to find out the type of antibody and it’s molecular weight etc.,
4.5.1 The report from MBU, Indian Institute of Science Bangalore is furnished below:

- Histamine Immuno affinity samples from allergic and normal persons, run on 12% SDS PAGE Gel and bands corresponding to Heavy Chain (~50KDa) and light chain (~25KDa) were sliced from gel and taken for tryptic digestion and Mass Spectrometry analysis. The results were furnished below:

From SDS PAGE test it was ascertained that the elute from Affinity Chromatography contained an antibody; and from biophysical characterization by MALDI MS, using ultrafleXtreme MALDI TOF/TOF (Bruker Daltonics) it was confirmed that the antibody was IgG type since the molecular weight of isolated immunoglobulin was estimated to be 148868 daltons.

From the MALDI TOF analysis by IISc Bangalore, it was ascertained that the molecular weight of the antibody in the elute was 148868 daltons which matched with molecular weight of IgG. The concentration of antihistamine antibodies in the elute was estimated by Bradford Protein Assay Method.

4.6 TEST 5: ESTIMATION OF ANTIHISTAMINE ANTIBODY LEVEL IN THE ELUTES OF NORMAL SERUM (ID NR AND NB) AND ALLERGIC SERA (AJ AND AY) BY BRADFORD PROTEIN ASSAY METHOD

Bradford Reagent is formulated for rapid and accurate quantitative estimation of protein samples by following the Bradford Assay Method which was developed by Marion M. Bradford in 1976.

In order to ascertain whether there is any marked difference in the concentration of antihistamine antibody in normal and allergic sera, we selected 2 samples from normal sera NR and NB (NR- 9 year old female with IgE 51 I.U/ml and NB-63 years old male with IgE 59 I.U/ml) and 2 samples AJ and AY from allergic sera (AJ-18 year old female with IgE 1785 I.U/ml and AY- 39 year old male with IgE 343 I.U/ml) for protein analysis.
4.6.1 Principle

Bradford Reagent (Product code ML106-500ML) is mainly used for quantitative estimation of protein samples by the Bradford Assay Method. Bradford reagent contains a dye, Coomassie Brilliant Blue G-250. The Bradford assay is based upon the formation of complexes between Coomassie Brilliant Blue G-250 and the protein samples in solution. When the protein sample binds to the dye, the colour of the solution turns blue from brown and there is a shift in the absorption maximum of the dye from 465nm to 595nm. This dye binding procedure is completed within 5 minutes and the blue colored complex formed will be stable for 1 hour.

4.6.2 Application

Bradford reagent is mainly used for the quantitative estimation of protein samples by the Bradford Protein Assay method.

4.6.3 Instruments and reagents

1. Nine test tubes
2. Standard protein (BSA) stock solution (1mg/ml)
3. Bradford Reagent
4. Cuvettes
5. Spectrophotometer
6. Pipette with accessories
7. Graph paper
8. Sera of normal person NR and NB and allergic person AJ and AY.

4.6.4 Procedure

Nine test tubes were taken and labeled them as blank and 1 to 8. Made dilutions of standard protein (BSA stock solution 1mg/ml) with concentration of 0.5µg, 1.0µg, 2.0µg, 3.0µg, 4.0µg, 5.0µg, and 6.0µg. Added 200µl of elute of serum (ID NR) to the eighth test tube. Added 200µl of distilled water in ‘Blank’ test tube. Added 1ml of Bradford Reagent to all the tubes. Mixed the contents thoroughly by vortexing the tubes and incubated at room temperature for 10 minutes. Transferred the contents of the tubes to cuvettes and measured the absorbance at 595nm wavelength.
Using the O.D. values plotted a standard curve of absorbance at 595nm on ‘Y’ axis versus concentration of protein µg/200µl on ‘X’ axis.

The OD value of elute ID NR was found to be (1.228-0.622) 0.606. The value of the protein- Antihistamine antibody in the elute of the serum was found to be 3.59µg of protein (in 200µl). Likewise, the concentration of antihistamine antibody in the elutes of sera NB of normal person and AJ and AY of allergic persons in 200µl were determined and found that 3.68, 3.60 and 3.49µg in 200µl respectively. The average concentration of antihistamine antibody was 3.59µg protein in 200 µl. Since the antihistamine antibody in normal serum NR was 3.59µg in 200µl and the average protein concentration for 4 samples was also 3.59µg in 200µl, the serum NR was taken as a standard serum for further reference and analysis.

4.7 TEST 6: ESTIMATION OF ANTIHISTAMINE ANTIBODY IN DIFFERENT DILUTIONS USING THE SERUM (ID NR)

The serum NR was used to find out the concentration of anti-histamine antibodies in different dilution of 1 in 5000, 1 in 10000, 1 in 25000, 1 in 50000, 1 in 750000 and 1 in 100000.

4.7.1 Instruments and reagents

- Histamine coated micro wells (EIA1 X8 strip well plate).
- Serum ID NR in various dilution (1 in 5000, 1 in 10000, 1 in 25000, 1 in 50000, 1 in 750000 and 1 in 100000)
- 1 x wash buffer
- 1 x Assay buffer
- Secondary Antibody conjugate 1:50000 dilution
- Absorbance plate reader
- Microtiter tubes
- Pipette with other accessories
- Incubator
4.7.2 Procedure

Using the normal serum various dilutions of 1 in 5000, 1 in 10000, 1 in 25000, 1 in 50000, 1 in 750000 and 1 in 100000 were prepared with distilled water. Added 100µl of test samples to each histamine coated well and incubated at 37°C for 60 minutes, covering the plate with a [late sealer to avoid variations due to evaporation. Discarded the liquid from the wells and washed each well with 1x wash buffer 3 times and dried the plate on absorbent paper. Diluted the required amount of secondary antibody conjugate (1 in 50000 dilution) with 1x Assay buffer and dispensed 100µl to each well and incubated for 1 hour at 37°C discarded the liquid from the wells and washed each well with wash buffer 3 times and dried the plate on absorbent paper. Added 100µl of chromogen substrate to each well for colour development and incubated at room temperature for 30 minutes. By adding 100µl of 1x stop reagent, stopped the colour (Bluish colour) development. The colour of the solution turned yellow completely. Read the plate in an absorbance plate reader at 450nm wavelength.

The O.D. values of different serum concentrations and corresponding protein (Anti-histamine antibody) concentrations in various dilutions were estimated and furnished below.

From the O.D values, a standard curve for antihistamine antibody was plotted.

Using the above standard curve, the serum histamine binding capacity of various normal and allergic sera may be calculated.

4.8 TEST 7: ESTIMATION OF SERUM HISTAMINE BINDING CAPACITY OF ALLERGIC AND NORMAL PERSONS’ SERA

4.8.1 Instruments and reagents

- Histamine coated micro wells (EIA1x8 strip well plate).
- Sera of normal and allergic persons (Dilution 1 in 50,000).
- 1 x wash buffer.
- 1 x Assay buffer.
- Secondary Antibody conjugate 1:50,000 dilution.
- Absorbance plate reader.
- Microtiter tubes.
- Pipette and other accessories
- Incubator.

### 4.8.2 Procedure

Prepared 1 in 5000 diluted sera. Added 100µl of test samples (1 in 50,000 diluted sera) to each Histamine coated well, and incubated at 37°C for 60 minutes, covering the plate with a plate sealer to avoid variations due to evaporation. Discarded the liquid from the wells and washed each well with 1x wash buffer 3 times and dried the plate on absorbent paper. Diluted the required amount of secondary antibody conjugate (1 in 50,000 dilution) with 1x Assay buffer and dispensed 100µl to each well and incubated for 1 hour at 37°C. Discarded the liquid from the wells and washed each with wash buffer 3 times and dried the plate on absorbent paper. Added 100µl of chromogen substrate to each well for colour development and incubated at room temperature for 30 minutes. By adding 100µl of 1x stop reagent, stopped the color (Bluish colour) development. The colour of the solution turned yellow completely. By comparing the O.D values with the standard curve of antihistamine antibody prepared by using the serum ID NR in various dilutions, the antihistamine antibody bound to histamine (SHBC) was found out. The results were tabulated.