Characterisation and Treatment Analysis of Allergens Prevailing in Egg yolk

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1- Egg allergy
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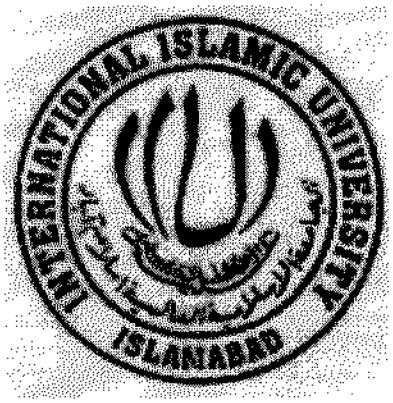

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A thesis submitted to Department of Bioinformatics and Biotechnology, International Islamic University, Islamabad as a partial Fulfillment of requirement for the award of the Degree of MS Biotechnology

DECLARATION

I hereby declare that the work present in the following thesis is my own effort except, where otherwise acknowledged and that the thesis is my own composition. No part of the thesis has been previously presented for any other degree.

Date: 24.08.2015

Sarwat zareen

DEDICATION

I would like to dedicate my efforts to

my beloved Parents

and

all family members

for their countless prayers and support.

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All praises to Almighty Allah, the most Beneficent, and the most merciful who bestowed me with the potential to complete this desire task. All the reverence and esteem for his beloved Prophet Mohammad (Peace be upon him) the most perfect and exalted among and of born on the surface of earth, who enlightened the mankind on the true path of life and is a source of inspiration for all knowledge seekers.

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Last but not least, I also Revere the affected families, without their cooperation research work could not be accomplished.

Sarwat Zareen

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LIST OF ABBREVIATIONS

Acronym	Abbreviation
°C	Degree Celsius
µl	Microliter
3D	Three-dimensional
Ab	Antibody
ACD	Acid Citrate Dextrose
Ag	Antigen
APCs	Antigen-presenting cells
APT	Atopy patch test
ATP	Adenosine Triphosphate
Bp	Base Pair
BSA	Bovine serum albumin
DH_2O	Distilled Water
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribose nucleotides Triphosphate
EAACI	European Academy of Allergy and Clinical Immunology
ELISAs	Enzyme Linked Immuno Absorbent Assay
FALCPA	Food Allergen Labeling & Consumer Protection Act
Fig	Figure
Gal d 5	Gallus domesticus 5
HCl	Hydro Chloric Acid
Ig G	Immunoglobulin G
IgA	Immunoglobulin A
IgE	Immunoglobulin E
IgM	Immunoglobulin M
Kb	Kilo base
KD	Kilo Dalton

KHCO3	Potassium Bicarbonate
KRL	Kahuta Research Laboratories
L	Liter
Mm	Millimeter
MW	Molecular Weight
NaCl	Sodium Chloride
NaoH	Sodium Hydro oxide
NCBI	National Center for Biotechnology Information
NH_4Cl	Ammonium Chloride
nm	Nano Meter
NSB	Non-specific binding
OD	Optical Density
OFC	Oral Food Challenge
OTI	Oral tolerance induction,
OVM	Ovomucoid
PBS	Phosphate Buffer Saline
PBST	Phosphate Buffer Saline Tween
PCR	Polymerase Chain Reaction
p-NPP	p-nitro-phenyl phosphate
SD	Standard Deviation
SDS	Sodium Dodecyl Sulphate
SPSS	Software Package Used For Statistical Analysis
Tris	Tris Amino methane)
U	Unit
UV	Ultra Violet

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ABSTRACT:

Adverse reactions to egg i.e. egg allergy and intolerance have gained sizeable consideration. This study focuses on the particular characterisation and management associated with IgE mediated Egg allergy that is believed to be responsible for most immediate-type food-induced hypersensitivity reactions. Clinically, these reactions are characterised by a variety of signs and symptoms that occur within minutes as well as hours after consumption of egg. Reactions may be constrained and up generalised together with involvement of the skin, nose, eyes, and/or lungs. In more severe and worse cases, anaphylactic shock can occur. The evaluation of egg allergy relies on a careful history, physical examination, appropriate skin testing or in vitro testing with eggs. Avoidance remains the main stay of therapy so labelling should be done. Immunoassay are the method of choice for fast detection of food allergen. The aim of the study is to develop an enzyme-linked immunoabsorbent assay (ELISA) for the prognosis associated with egg allergen present in patient sera. The primary antibody IgE, and conjugate secondary antibody anti-human IgE antibody labelled with alkaline phosphate was developed in laboratory and used to measure the presence of Gal d 5(Antigen). An indirect Elisa was developed for the purpose to check the presence of Gal d 5 which is the major allergen present in egg yolk. A successful Antibody/Antigen reaction was developed and colorimetric change was occurred and observed in all patient's sample indicating the presence of Gal d 5 while not any optical change was occurred in any of our control samples. All patient's samples were then placed in plate reader and check their Absorbance relevance (A.D) through spectrophotometer, all sample showed O.D value > 0.04 which showed the positive results. Positive result was confirmed in all patient's sample indicating the ability of the antibody IgE to bind and detect Gal d 5 (antigen) which is the major egg yolk protein and is responsible for antigenic and allergenic activity in our patients.

INTRODUCTION

1.1 Egg Allergy

Egg allergy is one of the most prevalent IgE-mediated food allergies in infants and young children. Egg allergy develops when the body's immune system becomes sensitized and overreacts to proteins in egg yolk or white. When eggs are eaten, the body sees these protein as a foreign invader and sends out chemical to protect against it. Those chemical substances causes the symptoms of an allergic reaction.

Experts estimate that as many as 2 percent of children are allergic to eggs. Egg allergy has a cumulative prevalence of approximately 2.6% by 2.5 years of age (Eggesbo *et al.*, 2001) luckily, studies show that about 70 percent of children with an egg allergy will outgrow the condition by age 16.Still the risks are very high. Children who are allergic to eggs can have reactions ranging from a mild rash to anaphylaxis, a life-threatening situation that impairs breathing and can send the body into shock.

Egg allergy is the most common food allergy in infants. Food allergy is cause by allergen present in the food. The allergens in foods are almost always naturally occurring proteins. More than 160 foods have been illustrated as causing food allergies, but allergy specialists only consider a limited number of those to be of public health concern (Taylor and Hefle *et al.*, 2001).

There are Eight foods item or food groups that are consider to account for more than 90% of all IgE-mediated food allergies on a Worldwide basis and have come to be known as the "Big Eight" These food groups are Milk, Eggs, Fish ,Shell fish,, Peanuts, Soybeans, Tree nuts and Wheat (Taylor and Hefle *et al.*, 2001). The Food Allergen Labeling & Consumer Protection Act (FALCPA) that was passed by the United States Congress in 2004 and officially took action on January 1, 2006 call for labeling of packaged foods containing any of the eight "major food allergens including egg,"

1.2 Definition

"According to the classification presented by the European Academy of Allergy and Clinical Immunology (EAACI) chicken egg allergy is an adverse reaction with an underlying Immunological pathogenic mechanism produced by the intake or contact with egg and its proteins." (Bruijnzeel, *et al.*, 1995).

1.3 Sign and Symptoms of Egg Allergy

Egg allergy reactions vary from person to person and symptoms usually occur a few minutes to a few hours after eating eggs or foods containing eggs. Signs and symptoms vary from mild to severe and usually occur soon after exposure to egg. It may include skin reaction, nasal congestion and digestive problem and in rare cases it may cause anaphylaxis. Most of the reactions (up to 90%) affect the skin. Egg accounted for 7% of severe anaphylactic reactions in infants and children in a German survey (Mehl A *et al.*, 2005). Fatal reactions to egg are rare, but have been reported (Macdougall CF *et al.*, 2002). The allergic reaction is easily activated in people with egg allergy when they come in contact with the egg either through touching, smelling or eating. Reactions usually occur immediately after food ingestion (Scurlock AM *et al.*, 2005 & Allen JA *et al.,* 2006). Most common clinical features include

- Skin reactions, such as hives or a rash, Pimples ,Itching ,swelling
- Nasal congestion Runny nose and sneezing Red or watery eyes
- Digestive problem that include Stomach pain, nausea, cramps ,vomiting or diarrhea
- Respiratory problems e.g. Asthma signs and symptoms such as coughing, wheeziness, chest tightness or shortness of breath
- Anaphylaxis (less common)

1.4 Mechanism

Normally, when the immune system recognises food proteins as being foreign invader to the host, immunoregulatory mechanisms are established that lead to the attainment of tolerance. Alterations and variations in these regulatory mechanisms alter the induction of tolerance,

resulting in food allergy. It is important to distinguish true food allergies from food intolerance and other non-immunological reactions because determining the specific etiology can allow correct management of these adverse reactions to food (Koppelman & Hefle *et al.*, 2006).

Adverse reactions to foods are either immune mediated or non-immune mediated reactions depending upon whether the immune system is primarily involved in causing the reaction. Reactions to foods, which are not due to immunological mechanisms, are generally regarded as food intolerance. The proteins of eggs usually come into contact with the host immune system through the digestive tract. In the first stages of life the infant can come into contact with food proteins through breast milk, The body has a series of physiological barriers that protect it from the foreign antigens. In the digestive system these barriers comprises of two groups of elements (1) Non immunological (gastric acid, pancreatic enzymes, Intestinal enzymes, mucus, the mucosal layer, the membrane of the microvilli and intestinal peristalsis) and (2) immunological (IgA, IgE, IgM, IgG, lymphocytes, intestinal secretory IgA and secretory IgA in breast milk and macrophages,).

When the composition of the diet is modified as for example when new foods are introduced or when the infant is stopped from breastfeeding, many physiological changes take place .This condition can have a deep effect on response of immune system, not only because the antigens found in the lumen are of different types but also because of the changes in ingestion and digestion. The mucus and proteolytic enzymes present in the digestive secretions, the rate of absorption and intestinal transit, bowel motility all influence the amount of antigen present in a given segment of the intestine.

Most proteins in the diet are broken down to amino acids through the action of proteolytic enzymes during digestion, although 2% of the ingested proteins are absorbed as immunologically recognisable peptides. These proteic structures can be simple linear epitopes or conformational epitopes with a three-dimensional (3D) structure. The immune response of the gastrointestinal mucosa is characterised by a complicate balance between host defense and immune regulation. Normally, when the immune system recognises foreign food proteins elements, immunoregulatory mechanisms are established that lead to the attainment of tolerance. Alterations and changes in these regulatory mechanisms alter the induction of tolerance, which results in food allergy.

Different factors including the age, genetic susceptibility and commensal intestinal flora of the individual, the presence of other proteins, antigen exposure, lipids, vitamins, and especially the type of antigen-presenting cells (APCs) are factors that generate tolerance (Lack G *et al.*, 2008, Worbs *et al.*, 2006). Reactions to foods, which are not because of immunological mechanisms, are generally regarded and considered as food intolerance. Types of food intolerance includes toxins, metabolic disorders, or other undefined reactions (Boyce *et al.*, 2010).

Because adverse food reaction is an expansive term demonstrating a link between the ingestion of a food and an abnormal response, the ability to classify these reactions into 4 clearer categories is a significant step towards understanding the complex issue. The mechanisms underlying the induction of oral tolerance include energy/deletion or active suppression mediated by regulatory T lymphocytes (Treg), which play a key role in the development of peripheral tolerance. These cells suppress the immune response by inhibiting the generation of effector T cells in lymphoid tissue and in the target organs through the production of cytokines (Chehade M, *et al.*, 2005, Strobel, *et al.*, 2006). Furthermore, transcription factor FOXP3 is an essential regulating element of the Treg cell line (Coombes JL, *et al.*, 2007).

1.5 Prevalance

Egg allergy is one of the most prevalent IgE-mediated food allergies in infants and young children (Osborne NJ *et al.*, 2011 & Venter C *et al.*, 2008). Egg allergy accounts for one of the most prevalent food hypersensitivities in industrialized countries. The estimated prevalence of egg allergy varies between 1.6 and 3.2% and, thus, makes it the second most common cause of food allergy in children (Mine *et al.*, 2008).

Egg allergy has a cumulative prevalence of approximately 2.6% by 2.5 years of age. The prevalence of egg allergy is about 35% in food allergic children (Crespo *et al.*, 1995, Resano *et al.*, 1998) Allergy to hen's egg usually presents in the second half of the first year of life, with a median age of presentation of 10 months (Boyano Martinez T, *et al.*, 2001). The prevalence could be up to 73% in children with atopic dermatitis (Sampson & Ho *et al.*, 1997), whereas frequency of egg allergy in food allergic adults is about 12% (Wüthrich *et al.*, 1993).

The severity of reactions of egg allergy can be unpredictable, and is potentially life threatening, and can vary from episode to episode. Anaphylaxis can occur with exposure to egg, and asthmatics, in particular, are at high risk for severe allergic reactions (Colver AF *et al.,* 2005 & Ross MP *et al.,* 2008).Egg accounted for 7% of severe anaphylactic reactions in infants and children in a German survey (Mehl A *et al.,* 2005).Fatal reactions to egg are rare, but have been reported (Macdougall CF, *et al.,* 2002).

All across the world egg allergy effecting 0.3% of children (Rona *et al.,* 2007). In a group of 355 children diagnosed with food allergy in Spain, the prevalence of allergy to egg proteins was 20.1% (Crespo JF *et al.,* 1995).In united states of America overall 0.2% of children's are allergic to eggs (Liu *et al.,* 2010) and about 1.8% of the allergy is observed in the patients having the age lies between 1 to 5 years (Liu *et al.,* 2010). Ingestion of raw or undercooked egg may trigger more severe clinical reactions than well-cooked egg (Eigenmann PA *et al.,* 2000).

In UK egg allergy is more common among infants and accounts for about 2 % at the kids of 3 years (Venter *et al.* 2008).In Korea, egg allergy is known as the most frequent food allergy in children. (Ahn K, *et al.,* 2012) It is also known that the natural history of egg allergy has a good tolerance prognosis(Kim *et al.,* 2009).it is also reported that 41% of children had developed tolerance to egg allergy by 3 years of age and 60% by 5 years of age in Korean toddlers with atopic dermatitis. In three studies carried out in Spain, 50% of the patients with egg allergy reached tolerance at 3-5 years of age and 64-74% at 9 years of age (Boyano *et al.,* 2002, Montesinos E *et al.*, 2010, Alonso Lebrero E *et al.*, 2001).

1.6 Risk Factor

Some people are allergic to the whole egg, while others are allergic to either the yolk or egg white mediated food allergies in infants and young children .To date, risk factors for egg allergy remain largely unknown. Allergy to hen's egg usually presents in the second half of the first year of life, with a median age of presentation of 10 months (Boyano Martinez T, *et al.,* 2001). The positive family history of egg allergy and atopi are the common risk factor of egg allergy.

Egg allergy is closely associated with atopic dermatitis and was found to be present in about 2/3 of children with positive oral food challenges (OFC) performed for allergy evaluation of atopic dermatitis (Niggemann B *et al.,* 1999). Studies shows that there are evidences that this allergy may be frequently occur in those kids that are having there maternal uncle or aunty also suffered from egg allergy.

Environmental factor may also involve. Exposure in the first year of life to siblings and dogs may decrease the risk of subsequent egg allergy (Koplin JJ *et al.,* 2012).The strongest risk factor for egg allergy in this cohort was having one or more parents born in East Asia. Interestingly, parents born in East Asia were less likely to report a history of allergic disease themselves, while their infants are at increased risk of egg allergy and eczema (Martin *et al.,* 2011).

This is consistent with the recent finding that US-born children of immigrants were at highest risk of food sensitisation, while those born and raised outside of the United States have the lowest risk (Keet *et al.,* 2012). This could potentially be explained by a gene–environment interaction, whereby immigrants have particular genetic polymorphisms that protect against intestinal parasite infection but predispose to allergy risk when growing up in a pathogen-poor environment (Keet *et al.,* 2012). This hypothesis is consistent with finding that having a parent born in Europe did not increase the risk of infant egg allergy.

It is also observed that the development of specific IgE against egg during the first year of life is a predictor of the risk of atopic disease. Different studies indicate that immune reactivity to egg may presently be the main and earliest serological marker of the risk of posterior sensitisation to aeroallergens and of the development of respiratory allergic disease (Whan U *et al.,* 2000).Furthermore, When the egg allergy is associated to atopic dermatitis, the risk of other respiratory allergic disease also increases and reaches at 80 % at 4 years of age (Tariq SM *et al.,* 2000). Study illustrates that having siblings and having a dog inside the house are inversely associated with egg allergy, while having a family history of allergy or a parent born in East Asia are associated with an increased risk of egg allergy (Koplin JJ *et al.,* 2012).

1.7 Egg Allergen

Any molecule which elicit or activate the antigenic response in body is called as allergen. Egg is one of the most important allergens in childhood feeding, and egg allergy can pose quality of life concerns. Hen's egg comprises about 8-11% shell, 56-61% white and 27-32% yolk. The white is essentially an aqueous protein solution (10% protein and 88% water), and the yolk is composed of 50% water, 34% lipid and 16% protein, giving it quite different properties (Poulsen LK, *et al.,* 2001)).Ingestion of raw or undercooked egg may trigger more severe clinical reactions than well-cooked egg (Eigenmann PA *et al.,* 2000).

Five major allergenic proteins from the egg of the domestic chicken (Gallus domesticus) have been identified; these are designated Gal d 1-5 (Heine RG *et al.,* 2006). Most of the allergenic egg proteins are found in egg white, including ovomucoid (Gal d 1, 11%), ovalbumin (Gal d 2, 54%), ovotransferrin (Gal d 3, 12%) and lysozyme (Gal d 4, 3.4%) (Bernhise) *et al., 2006.* Although ovalbumin (OVA) is the most abundant protein comprising hen's egg white, ovomucoid (OVM) has been shown to be the dominant allergen in egg (Cooke SK *et al.,* 1997).

In egg yolk, alpha-livetin (Gal d 5) is the major allergen and is involved in the bird-egg syndrome (Quirce S *et al.,* 2001). Several other allergens have been identified in egg yolk, including vitellenin (apovitellenin I) and apoprotein B (apovitellenin VI), although their roles in food allergy remain unclear. Manufactured food products often contain trace amounts of egg Lecithin as emulsifiers, but ingestion of trace amounts of egg lecithin is probably insufficient to elicit allergic reactions (Heine RG et *al.,* 2006).

Gal d 1 belongs to serine protease inhibitor and its molecular weight is 22.6 K D. Gal d 2 belongs to serpins family and its molecular weight is 42.8 KD. Gal d 3 belongs to transferrin family and it is of 77.8 KD. Gal d 4 belongs to glycoside hydrolase family 22 and its molecular weight is 14.3. Gal d 5 is of serum albumin family and its molecular weight is 69.9 KD. Gal D 5 is mostly involve in bird egg syndrome (Quirce S *et al.,* 2001). The detection of allergens present in particular food is of extreme importance.

The manufacturing of foods is a complex multidimensional process, and with each step involved, creates various opportunities for the presence of undeclared allergens to unintentionally or mistakenly appear in a product. Some of the reasons for the occurrence of hidden allergens in processed foods include cross-contact through shared equipment, carry-over from rework material, unknown ingredients in raw material, or contamination from maintenance or cleaning tools (Besler *et al.,* 2002).

Reliable food allergen detection methods are vital for many food manufacturers to avoid expensive food product recalls, and to support the safety of food allergic consumers. Thus, analytical methods become an essential tool for validation of hygiene practices by food processors. These analytical systems are cost effective, specific, & sensitive enough to reliably detect traces of food allergens in a diverse range of food medium(Kerbach *et al.,* 2009).

Currently, several analytical approaches have been developed for the detection and characterisation of allergen traces in food products. The methods typically focus on targeting the allergenic protein for the source of concern or a marker that indicates the presence of the allergenic commodity (Cucu *et al.,* 2013; Poms *et al.,* 2006). The various allergen detection techniques include immunochemical methods such as Enzyme-linked Immunosorbent assays (ELISAs) and lateral flow devices (dipsticks), DNA-based methods such as polymerase chain reaction (PCR), mass spectrometry, and ATP tests are most common. Immunochemical methods depend on antibodies to reliably detect the allergenic protein. They can give both quantitative or qualitative results and are fairly rapid and sensitive.so we use Elisa method in our research work, for this purpose we use Gal d 5 the major egg yolk protein as an antibody in our experimental work.

1.8 Treatment Analysis

1.8.1 Diagnosis

As with all food allergies the diagnosis of egg allergy is based on the following

1. The existence of an Indicative clinical history.
2. A positive allergy study.
3. Allergy Test.

The clinical diagnosis requires a detailed diagnostic workup of egg allergy and should starts with a detailed history and physical examination of the patients. The next step may include in vitro and/or in vivo allergy tests that are used to support the diagnosis of egg allergy. These may include measurement of food-specific IgE antibodies, skin-prick tests, atopy patch test (APT) and oral food challenge (OFC).

1.8.2 Treatment and Management

Treatment is very crucial but management could be follow by following means. Egg avoidance advice is the cornerstone of management.

1.8.3 Avoidance of Egg

The treatment of egg allergy is based on avoidance of egg protein intake. In the case of children with confirmed tolerance of cooked egg only the uncooked egg containing food product is to be avoided (fresh mayonnaise, toppings, omelet, ice cream).Maintaining a strict egg avoidance diet is not easy, the patient family and school environment should receive education and training in the avoidance of egg and in the management of the possible harmful and adverse reactions. Attention should be maximized, carefully check the labelling of processed food. In this context current legislation as reflected by Spanish Royal Decree (SRD)1245/2008, of 18 July, and regulation (EU) 1169/2011 of the European Parliament and of the council of 25 October 2011, which publish the list of allergenic ingredients of obligate declaration, requires such labelling to identify those products which containing eggs or its products.

Many processed products and dishes containing egg are sold in fast food restaurants, and bakeries where the ingredients are sometimes not easy to identify, and where egg cross-contamination is much more possible. Likewise, some proteins of egg origin such as lysozyme are used as bactericidal agents in certain foods. Other products such as sweets, Cold meats, pasta, soup Stocks, beer, coffee drinks and wine can also contain egg protein in the same way as certain Cosmetics product .Websites are available where information can be obtained on the presence of egg in different foods and other commonly consumed products (for example: www.seicap.is/familiares.asp).

Careful reading of ingredient labels is essential and legislation has been passed in the United States instructing clear labeling of food packages to identify the presence of the 8 major food allergens, including egg .Some products in the United States may have advisory labeling, such as "may contain egg". This type of labeling is not currently regulated. Based on a recent study (Ford LS *et al.,* 2010), avoidance of advisory-labeled products should be recommended because they present a small but real risk of allergic reactions, especially products from small companies.

Other countries are also addressing issues of food labeling as well. All children with egg allergy should receive measles, mumps and rubella (MMR) vaccination. Influenza and yellow fever vaccines should only be considered in egg-allergic patients under the guidance of an allergy specialist.

1.8.4 Medication

Different medicines may also be used by egg allergic patients. Antihistamines can be helpful where food allergies cause symptoms such as itchy rashes (urticaria) Adrenaline may be prescribed in an injectable form (such as Emerade, EpiPen or Jext) if a person has a history of a severe allergic reaction (anaphylaxis). It is important that this should be carried on your person at all times, and that you are trained in its use, or how to give it to your child.

1.8.5 Oral Tolerance Induction

Until now, the only practical option for people with food allergies is strict avoidance of allergen-containing food. It is difficult to avoid egg because it is found in many foods. Even with avoidance, the fear of accidental ingestion from mislabelled foods or cross-contamination is an ever-present fear for even the most careful of food-allergic individuals. Accidental consumption of egg-containing foods might cause a life-threatening event. Although there are only a small number of published studies, there is a new type of treatment for egg allergy called 'oral immunotherapy' (also known as 'oral desensitization' or 'vaccination'). This is comprised of daily consumption of a small amount of egg Protein, which is gradually increased over time until a full serving is reached. This method could alter the allergic response to the egg protein by the body's immune system, increasing the amount of egg that can be eaten without inducing an adverse reaction.

In 1908 the first description was published of a child with egg allergy (anaphylaxis) satisfactorily treated with 'oral immunotherapy'' (oral tolerance induction, OTI). (Schofield AT *et al.,* 1908). At present, this treatment can be regarded as a management option for IgE mediated egg allergy. (Nowak-Wegrzyn A *et al.,* 2011).

Traditional subcutaneous immunotherapy, which is effective against certain aeroallergens is unsafe for the treatment of food allergy (Nelson *et al.,* 1997). Oral immunotherapy appears to be safer than subcutaneous immunotherapy for food allergens and induces desensitization. Oral immunotherapy has been successful in desensitizing patients to several food allergens in small clinical trials, most of which were not controlled (Meglio P *et al.,* 2004)

1.8.6 Future Treatments

Currently, there are no treatments that can completely cure or provide long-term relief from egg allergy. However, several treatment strategies are under investigation. These approaches are either allergen-specific or aimed at reducing the overall allergic response.

Oral tolerance induction studies to food allergens are still experimental (Beyer and Burks, *et al.,* 2008) and a few studies show very promising results (Patriarca G *et al.,* 2003, Staden U *et al .,* 2007 and Buchanan AD *et al.,* 2007).However, the adverse reactions are common (Beyer and Burks *et al.,* 2008).There is still uncertainty of whether oral immunotherapy (OIT) achieves true tolerance or transient desensitization (with reappearance of symptoms after discontinuation of therapy).

In view of a high probability of spontaneous tolerance development to egg it is unclear whether OIT changes the time course to the development of tolerance. With recent reports indicating that extensively heated egg is tolerated by a majority of egg allergic patients and that the associated immunologic changes with continued ingestion of extensively heated egg appear favorable, incorporation of extensively heated egg in the diet may present a more natural form of immunotherapy. At this point, OIT is still considered investigational, and therefore is not recommended in routine clinical practice.

Aims and objectives of the study

Egg allergy is the most common food allergy in infants. Food allergy is cause by allergen present in the food. The allergens in foods are almost always naturally occurring proteins. Egg is one of the most important allergens in childhood feeding, and egg allergy can pose quality of life concerns. Egg allergy can occur as early as infancy. Most children, but not all, outgrow their egg allergy before adolescence. Five major allergenic proteins from the egg of the domestic chicken (Gallus domesticus) have been identified; these are designated Gal d 1-5 (Heine RG *et al.*, 2006), including ovomucoid (Gal d 1), ovalbumin (Gal d 2), ovotransferrin (Gal d 3) and lysozyme (Gal d 4) and alpha-livetin (Gal d 5).

In egg yolk, alpha-livetin (Gal d 5) is the major allergen and is involved in the bird-egg syndrome (Quirce S *et al.*, 2001). Several many other allergens, including the vitellenin (apovitellenin I) & apoprotein B (apovitellenin VI), have been identified in egg yolk .The main focus of this thesis was characterisation of the allergens prevailing in egg yolk for this purpose an indirect Elisa was developed for detection of Gal d 5. ELISA provides relatively simple, rapid, sensitive, accurate, specific, robust, and cost effective analytical methods to the food industry for food allergen detection. ELISA can detect allergenic residues even at very low level including micrograms per gram (µg/g) or parts per million (ppm). Additional aims were to investigate the clinical manifestation and treatment analysis of Allergic patients. Finally, this thesis aims to analyse circumstances surrounding the reactions.

1. Development of an indirect Elisa in which Antigen/Antibody reaction will perform for detection of Gal d 5 antigen which is the major egg yolk allergen.
2. The Clinical manifestation and treatment analysis of Allergic patients.
3. To analyse circumstances surrounding the reaction.

MATERIALS AND METHODS

2.1 Subjects

The present study was designed for characterisation and treatment analysis of the allergens prevailing in Egg yolk. The patients having allergic symptoms after eating egg were included in the present investigation. The blood samples were collected from those patients and the other relevant information was gathered through questionnaire developed for the study.

2.2 Study Protocol and Study Approval

The present study basically consist of two main and fundamental parts

- The first part is based on collection of egg allergic samples.
- The second part is laboratory work (wet Lab).

This study was conducted to check out the allergen present in egg yolk and it was approved by committee of faculty members of IIUI.

2.3 Human subjects

For this study Egg allergic patients were collected from different areas of the country. The patients were evaluated by personal information, family history, clinical and laboratory investigation, all information was included in a consent form.

2.4 Blood sample collection

5 ml of blood was drawn of each participant from the median cubital vein at the elbow joints using a sterile 5 ml sterile syringe. The blood was immediately transferred to evacuated 8.5 ml sterile BD vacutainer glass blood collection tube containing Acid Citrate dextrose (ACD)

(BD Franklin Lakes NJ, USA).ACD in vacutainer serves as anticoagulant and preservative for the fresh blood sample. The vacutainer tubes were inverted few items immediately and stored at 4°C till further processing.

2.5 Identify number allotment

All the samples were assigned specific identity numbers (ID), e.g. Egg A 01 Egg A 02 and so on and stored at 2-8°C till DNA extraction.

2.6 Extraction of Genomic DNA

Aseptic technique was followed in all step of this procedure. Composition of reagents used in DNA extraction are shown in table 2.1

Experiments

2.6.1 DNA extraction

DNA was extracted from blood samples of Egg allergic patients from peripheral blood lymphocytes according to Phenol-chloroform Extracted method (Sambrook and Russell, 2001).DNA extraction procedure was completed in three days. The detail of the method is described as below:

Day 1

1. Firstly the blood samples were vortex in order to make them homogenize and transferred from ACD Vacutainers to the labeled 50ml centrifuge tubes.

2. The cell lysis buffer-I (KHCO3, NH4Cl and 0.5M EDTA)of 3X volume was added to the blood samples to lyses the red blood cells and kept the tubes at -20°C for 30 min.

3. Falcon tubes were balanced and centrifuged at 1200 rpm for 10min at 4°C in refrigerated centrifuge (Eppendorf Refrigerated Centrifuge 5130).

4. The Supernatant was discarded and pellet was re-suspended, if the pellet was found to be reddish then 10 ml of cell lysis buffer was added. The sample tubes were again spun at 1200rpm at 4°C for 10 min.

5. Step 2 was repeated until all red blood cells were lysed and the supernatant were clear but not more than 2 times.

6. 5 ml of STE buffer was added to falcon tubes.

7. While gentle vortex was done 250µL SDS was added drop wise and 10 µL of proteinase K (10mg-20mg/ml) (Fermentas, Lithuania) using separate tips.

8. Samples were placed at 55°C for overnight in shaking water bath (Orbit Shaker Bath, Lab-Line, USA).

Day 2

9. Samples were extracted with 5ml (equal quantity) of equilibrated phenol (pH=8) and were shaken for 10 minutes and kept on ice for 10 minutes.

10. Samples were then centrifuged at 3200rpm for 30 min at 4in refrigerated centrifuge (Eppendorf Refrigerated Centrifuge 5130).

11. The supernatant was removed with the help of sterile cut tip into the separate labeled 15ml falcon tubes.

12. 5ml of chilled chloroform isoamyl alcohol (24:1) was added in 15 ml falcon tubes, Shaken well for 10 minutes and kept on ice or -20°C for 10 minutes.

13. Centrifuged at 3200rpm for 30 minutes at 4°C in refrigerated centrifuge (Eppendorf Refrigerated Centrifuge 5130).

14. The supernatant was collected in separate 15ml of falcon tubes with the help of sterile cut tip.

15. 500 µL of 10 M ammonium acetate and 5 ml of chilled Isopropanol was added in to the of falcon tubes and tubes were shaken and inverted few times until DNA precipitates are visible as white threads.

16. Samples in falcon tubes were placed overnight at -20°C (or for 15 minutes at -70°C) .

Day 3

17. Samples in falcon tubes were spun at 3200 rpm for 60 minutes at 4°C in refrigerated centrifuge (Eppendorf Refrigerated Centrifuge 5130).

18. Supernatant was discarded and the pellet were resuspended.

19. 5ml of chilled 70% ethanol were added to wash the DNA pellet.

20. Centrifuged at 3200rpm for 40 minutes at 4°C in refrigerated centrifuge (Eppendorf Refrigerated Centrifuge 5130).

21. Supernatant was discarded and falcon tubes were inverted overnight on tissue paper in order to dry the pellet.

22. on next day pellet was stored in 500 µL 10 Mm Tris-HCl (pH=8, volume of the buffer added according to the size of the pellet).

23. The DNA sample were then placed in shaking water bath at 55 °C (Orbit Shaker Bath, Lab-Line, USA) approximately for 3-4 days to completely dissolve the DNA.

24. DNA samples were transferred to (1.5ml) eppendorf tube labeled with ID number and stock concentration and store at -20 ^{0}C.

Reagents	**Chemicals With Their Compositions**
Cell Lysis Buffer	1. KHCO3 (Potassium Bicarbonate) =1 gm/L 2. NH_4Cl (Ammonium Chloride) =8.29gm/L 3. 0.5M EDTA (Ethylene Diamine Tetra Acetate) =200µL Volume was measured accurately and made up to 1L using dH_2O. Stir it on magnetic stirrer and then stored at room temperature.
STE (Saline Tris EDTA)	1. 3M NaCl (Sodium chloride)= 33.3ml 2. 1M Tris –HCl (pH=8)=4.0ml 3. 0.5MEDTA (pH=8)= 2.0ml. Volume was measured accurately and made up to 1L using dH_2O. Stored at room temperature
SDS(Sodium Dodecyl Sulphate)Solution	1. SDS = 10gm (10%) Reagent was mixed and volume was made up to 100 ml with dH_2Ofiltered with 0.4mm filter paper. Stored at room temperature.
Proteinase k (20mg/mL)	Stored at -20°C till further use.
Chloroform-Isoamyl Alcohol (24:1)	1. Chloroform=480ml 2. Isoamylalcohol=20ml Above two solutions were mixed in a glasscylinder under the hood and stored at 4°C.
Isopropanol and Ethanol	Stored at room temperature and was ready to use.
1M Tris	Trisma base=121.1g For 2 litres = 121.1×2=242.2g

Table 2.1-Reagents used in DNA Extraction

2.7 Working solution of DNA

DNA dilutions were made according to following ratio. Dilutions were kept at 4°C.

DNA stock: dH2O (de ionized water).

60 µL:140 µL

2.8 Quantitative and qualitative assessment of DNA

Quantity and quality of DNA was assessed by measuring optical density. All the samples of DNA were diluted to 50 folds by adding 6 µL of DNA and 29 µL of dH2O and then quantified at 260nm and 280nm wavelength on UV spectrometer (U-3210, Hitachi, Japan).

Optical density (OD) ratio for each sample was calculated as:

OD =Absorbance at 260nm/ Absorbance at 280nm

The ratio of good quality DNA lies between 1.7-1.9.

The concentration of DNA samples were calculated by using the following formula.

DNA concentration (µg/ml) = Absorbance at 260nm × dilution factor × correction factor

Where dilution factor = 50 and correction factor = 50

All the extracted DNA samples were stored in lab for process of gene screening in near future to investigate the gene that might be responsible for cause of allergy.

2.9 Elisa (Enzyme-linked Immunosorbent Assay)

Technical approaches designed to detect the presence of food allergens have been available for a number of years but ELISA based methods remain the most commonly used by the food industry and official food control agencies (Abbot *et al.*, 2012 & Hen gel *et al.*, 2007). As proteins are the causative agent in food allergy, ELISAs have an advantage over other methods by detecting the actual allergen protein molecule instead of a surrogate marker such as DNA or ATP. ELISAs are relatively quick, simple and low cost technique to carry out.

The ELISA is an immunochemistry format that is based on specific binding between an antigen and an antibody, In the ELISA method, an enzyme is covalently linked to a specific antibody and when the antibody recognizes a target antigen, the complex binds to it. The enzyme component of the antibody-enzyme complex catalyzes a reaction with the addition of a suitable substrate that ultimately produces a colored product. Thus, the presence of the colored product indicates the presence of the antigen and the extent of reaction permits the measurement of small quantities of antigen (Berg *et al.*, 2002).

ELISAs are relatively quick and simple to carry out and can handle a large numbers of samples in parallel. All ELISA protocols include five common steps beginning with the coating of antibody or antigen on a solid phase followed by the addition of a blocking buffer containing a nonspecific protein which occupies the remaining uncoated surface on the solid phase. The blocking step minimizes nonspecific reactions and also protects the adsorbed antigen or antibody from surface denaturation (Nielsen *et al.*, 2010).

Different immunoassay reagents are then incubated at a specified temperature and time followed by washing. The last step involves a color reaction that can be visualized and measured rapidly and give the confirmation. Immunoassay techniques provide complementary and alternate approaches in reducing the use of costly, sophisticated equipment and analysis time while still maintaining reliability and remarkable sensitivity. Immunoassay techniques in their most simple forms provide excellent screening tools to detect contaminations.

2.10 ELISA Development

The following are the main steps that are essentially required for the development of a successful Elisa.

2.10.1 Antibody Production

The first step in the development of an immunoassay is the preparation of suitable Antibodies (Bonwick & Smith *et al.*, 2004). ELISAs, like other immunoassays, based on the ability of antibodies to interact and bind antigen as a means of generating a measureable result. Therefore, the choice of antibodies is of extreme importance and lays the foundation for a successful assay. Antibody was produced from a single cell line using hybridoma technology. Hybridomas are antibody-producing tumor cells that produce many copies of the same antibody and grow easily in laboratory cell culture.

2.10.2 Blocking the Plates

The next critical step in creating an ELISA is the blocking of the plate. The blocking step stabilizes proteins bound to the surface and prevents non-specific binding (NSB) of other proteins or biomolecules to uninhabited spaces on the surface, which can be detrimental to the specificity and sensitivity of the assay results. The blocking method can depend on the type of surface, the type of biomolecule immobilized to the surface, and the type of detection system employed. Generally, there are two major classes of blocking reagent, proteins and detergents. Non-ionic detergents such as Tween 20 are considered temporary blockers because they do not provide a permanent barrier to biomolecule attachment to the surface and can be washed away. Instead, non-ionic detergents can be beneficial included in the wash buffer to further decrease nonspecific binding.

2.10.3 Addition of an Enzyme Substrate

ELISAs requires the use of an appropriate enzyme label and a matching substrate that is suitable for the detection system being employed to produce a measurable signal. For colorimetric assays, horseradish peroxidase & alkaline phosphatase are common enzymes used

as labels (Gibbs *et al.*, 2001). These enzymes are typically used because they produce a sensitive, inexpensive, and easily performed assay. Alkaline phosphatase was used as an enzyme in this experiment. The criteria include stability at typical temperatures (4, 25, and 37°C). The enzyme was stored at 4°C. It is commercially available, capable of being conjugated to an antigen or antibody, inexpensive, easily measurable activity, and unaffected by biological components of the assay (Raskhit *et al.*, 2006).

For all enzyme-linked immunoassays, the final stage is the addition of the enzyme substrate. The substrate is chosen for its quantitative yield of a colored reaction product. Choosing the best substrate for any type of assay depends on the sensitivity desire, the timing requirements, and the detection device to be used. The most widely used substrate for alkaline phosphatase is p-NPP (p-nitro-phenyl phosphate).

By following all these steps keenly we developed a successful Elisa for determination of antigenic and allergenic properties of Gal d 5 which is the major Egg yolk allergen

2.11 Methodology

2.10.1 Coating the Plate with Antigen

1. Samples was diluted to a final concentration of 1-20 µg/ml using PBS or carbonate/bicarbonate buffer (pH 9.6).
2. 10 ug/ml of the Primary antibody IgE were added and the derivatives solutions were coated to a 96-well micro titer plate in 0.1 M sodium carbonate buffer (pH 9.6) at a concentration of 10 mg/ml.
3. The plate was sealed and incubated over night at 4°C.
4. The plates were washed three times with phosphate buffer saline containing 0.05% Tween 20 (PBST).

2.11.2 Blocking the Plates

5. The remaining protein-binding sites in the well were blocked by adding 200 uL blocking buffer (3% bovine serum albumin (BSA) in PBS).
6. The plate was sealed and incubated for 2 hours at 37°C (room temperature) or overnight at 4°C.
7. It was washed three times with PBST.

2.11.3 Incubate with Primary and Secondary Anti body:

8. 100 ml of diluted sera, 1/25 for IgE detection, were added in PBS containing 1% BSA (Sigma) to each well.
9. The plate was sealed and incubated for 1 hour at 37°C or 2 hours at room temperature.
10. It was washed three times with PBST and blotted on paper towels after last wash.

2.11.4 Addition of an enzyme:

11. 100 ml of monoclonal anti-human IgE conjugated alkaline phosphatase (1/1000) (Sigma) for IgE detection were added.
12. Plate was washed three times with PBST.

2.11.5 Detection:

13. 100 ml of p-nitrophenol phosphate (Sigma) were added in 0.1 M diethanolamine buffer (pH 9.8) for 60 min at room temperature.
14. The reaction was terminated by 25 ml of 3 N sodium hydroxide.
15. Absorbance at 405 nm was read by the microplate reader (Model 550, Bio-Rad Laboratories, Hercules, CA).

16. The IgE binding activity was expressed as a percentage of IgE binding to intact Gal d 5.

2.11.6 Solutions:

Sterile the filter and store at 4°C for up to 1 week.

Results:

The antibody IgE utilized in this ELISA are robust enough to detect the specificity and displayed adequate specificity to egg yolk Gal d 5 Protein.

Elisa Results:

The ELISA was based on specific binding between the allergen Gal d 5 and antibody IgE. In the ELISA method, an enzyme is covalently linked to a specific antibody and when the antibody recognizes a target antigen, the complex binds to it. The effectiveness with which the antibodies used in the ELISA to detect the antigen of interest, along with the efficiency with which these antigens are extracted from sample, are the most important parameters that can affect the overall performance of an ELISA-based method. ELISA may be run in a qualitative or quantitative format. Qualitative results provide a simple positive or negative result (yes or no) for a sample. We develop Elisa for qualitative results to check out the presence of Gal d 5 antigen in sera of egg allergic patients.

The median age of patients was 25 years old (2-70 years) and 33.3% were female. A majority of the patients don't undergo skin testing (75.6%).People mostly uses social mean to treat themselves.45 Patients who are allergic to eggs and 45 normal control were requested to undergone this Elisa test. The qualitative test for Gal d 5 antigen was done by using a commercial Elisa kit PF4 Enhanced; GTI, Waukesha, WI,USA).Primary antibody IgE and Secondary antibody anti Human IgE conjugate were specifically designed for this purpose. ELISA is a sensitive technique for accurately determining the amount of protein or other antigen in a given sample by means of an enzyme-catalysed colour change.

Qualitative Results

Blood Sera of 90 people (among which 45 are allergic to egg while 45 are normal control) were analysed for the presence of antigen Gal d 5 using Elisa kit. Samples were added in 96-well plates which permits high throughput results. Antibody IgE specific to the test protein Gal d 5 were adsorbed onto a solid substrate, and a measured amount of the blood sample was added all molecules of the test protein in the sample were bound by the antibody.

A second antibody anti Human IgE conjugate was added this was conjugated with an enzyme Alkaline Phosphatase, which catalyses a colour change in the fourth reagent, added

finally to the sheet. The colour change give the indication of positive results. It was measured photo- metrically and then determine the absorbance value for each sample.

Observance of Colour development

Colour development were observed in first 45 samples of the microliter plate while remaining samples were not prone to colour development. Colorimetric change in these samples indicating the presence of targeted antigen Gal d 5 in all patient samples (Fig 3.0) .The substrate p-NPP (p-nitro phenyl phosphate) was used in our experiment. It produces an intense yellow color measurable at 405 to 410 nm. An advantage of this substrate is that it can be allowed to develop for extended periods to obtain a corresponding increase in sensitivity.

Normally p-NPP has a slow reaction rate which should be allowed 30 to 60 minutes to reach optimal color development before being stopped with 1N NaOH .This color is directly Proportional to the amount of bound sample antigen. The more antigen present in the sample, the stronger the color development in the test wells. Elisa were designed to detect antigen in blood sample by the same immunological reaction between antigen and antibody.

Calculate the Mean Absorbance Value

Find out the absorbance value of each sample in spectrophotometer then calculate the average absorbance values of our samples and then put them in table 3.0 which shows the average absorbance value of our samples .All patients sample are positive as they all develop colour change and their average O.D values are > 0.004 (Table 3.0)which shows the positive results.

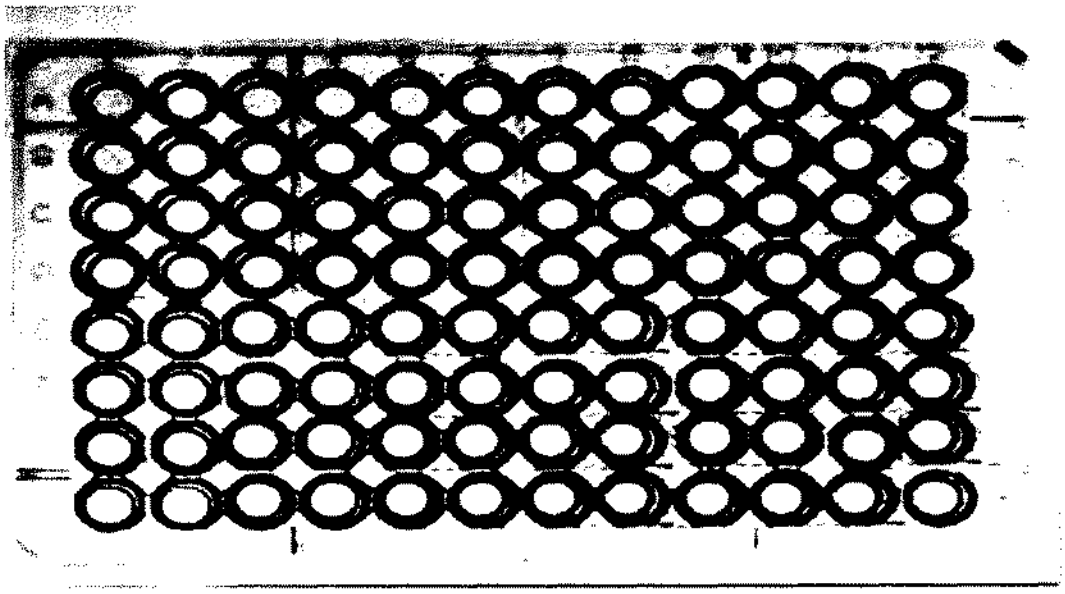

Fig 3. optical change

Fig 3.0:- Optical change occurred and colour development were observed in all samples indicating the presence of specific allergen Gal d 5 in sera of all samples. 96-well plates which permits high throughput results in which samples are added onto a stationary solid phase with special binding properties and is followed by multiple liquid reagents that are sequentially added, incubated and washed followed by some optical change. Colorimetric change confirm the presence of target antigen in sample.

Table 3.0 :- Mean Absorbance Value

Absorbance · Data	1	2	3	4	5	6	7	8	9	10	11	12
A	0.521	0.481	0.712	1.714	0.519	0.872	0.932	0.938	0.986	1.024	0.618	0.763
B	1.768	1.013	0.449	1.148	0.536	0.769	0.882	0.818	1.195	0.843	1.092	0.943
C	0.542	1.789	0.873	0.927	0.753	0.953	1.086	1.131	O.626	0.818	0.942	1.107
D	0.816	0.548	0.925	0.812	0.597	0.629	0.538	0.846	1.413			
E												
F												
G												
H												

Table 3.0 shows the mean absorbance value of our sample. When Enzyme reaction is completed the whole plate is placed into the plate reader and O.D value is determined for each of well. The amount of color produce is directly proportional to the amount of targeted antigen present in the sample.

Statistical Analysis

Data was documented and statistically analyzed using SPSS 20, a statistical software package (SPSS, Inc., USA).The frequency of egg allergic patients from different hospitals was determined. All of the patients got allergic symptoms immediately after eating eggs (Table 3.1)The symptoms varied in patients but all of them developed allergic symptoms immediately after eating egg .The gender frequency of egg allergic patient showed that male were more prone to egg allergy as compared to the females (Table 3.2). The Egg allergic patients were of different age and the average age of patient was 25 years (Fig 3.1) the average height of the patient was 5.3 feet (Fig 3.2) and average weight was 55 Kg (Fig 3.3). Furthermore different occupation of egg allergic patient was described in (Table 3.3).The Most of Egg allergic patients were students and it is correlated with our findings that student develop allergy in younger age as average age of our patients is 25 years (Fig 3.4).A frequency of egg allergic patients in different cities was determined. The highest number of our patients was presented in Wah cantt (Fig 3.5).The patient history showed that most of egg allergic patients were geographically located in Punjab, so most of Punjabies were found to be allergic with egg (Table 3.4). The cross tabulation of cast represents that Awan, Malik and Mughals were more frequently allergic to egg than other casts (Table 3.5).As far as dietary habits of our patients were concerned most of egg allergic patient were omnivore (Fig 3.6).Our results showed that relatively less number of patients consumed junk food items in their daily routine (Table 3.6). Our finding showed that very less no of of patient go to an allergist after having symptoms, mostly people allergic to egg completely avoid the eggs and do not take it at all as they are sensitive to egg proteins and developed allergic reactions after eating it (Table 3.7). Most of the patients do not consult with an allergist when they have allergic symptoms (Fig 3.7).Very less percent of people undergo skin testing (Table 3.8). Our finding also show that above 70% of patients don't take any medicine to get relief (Fig 3.8). Patient taking medicine were also not satisfied with drugs as medicine didn't provide relief to them (Table 3.9). Those patients who use drugs don't have any reaction against the allergy medication (Table 3.10). Anaphylaxes is the severe, life-threatening condition that impairs breathing and can send the body into shock. About 90% of our patient didn't have anaphylaxes (Table 3.11) our finding showed that mostly kids were allergic to Eggs and at the age of 3 to 12 most of the children got allergic

symptoms after eating eggs and were become allergic to egg (Fig 3.9). Different patients experienced different symptoms after eating eggs but our finding showed that most common symptoms were rashes, hives & itching. Some of the patient experienced abdominal pain, vomiting, swelling & pimples and frequently less people experienced breathing problem, cough, constipation, diarrhea and pain in whole body (Table 3.12).Allergic symptoms appeared on different parts of the body but face and arms were most effected region in most of the patients (Fig 3.10). It was revealed in study that in most of the cases symptoms remains in body till the whole day. After eating egg for a whole day most of the people remains suffered from allergic symptoms(Table 3.13).Mostly people experienced severe kind of allergic symptoms after eating egg, that can't be bear (Table 3.14).Allergic symptoms were of very severe type that people have disturbance in their sleep so most of the allergic patient can't sleep due to severity of the allergic symptoms (Fig 3.11).Few of egg allergic Patients were visited to an allergist for proper medication but in most of cases people don't take any kind of medicine (Fig 3.12). Most of egg allergic patients avoided to go to an allergist for treatment and use social mean to cure the symptoms(Fig 3.13).Family history of the patients showed that about 90% of Egg allergic patients does not have any disorder in their family(Fig 3.14).In some cases other family member of the patients may also effected from egg allergy but not in all cases (Table 3.15).The statistical data showed that other types of allergy are also found among family members of egg allergic patients(Table 3.16).The cross tabulation of family members having another allergy represented that some of family member may have skin ,drug and food allergy but most of them have asthma ,so Asthma is found to be more common among family members of Egg allergic patients(Table 3.17).

Table 3-1: Did eating egg initiates the symptoms

Did eating egg initiate the symptoms	No of Patients	Percent Age
Yes	45	100.0
No	0	00.0
Total	45	100.0

Table elaborates that in all of the patients eating egg initiates the allergic symptoms. In all cases eating egg is precursor for allergic symptoms, patient's experiences the symptoms immediately after eating eggs.

Table 3- 2: Gender frequency of egg allergic patients

Gender	No of patients	Percent Age
Male	30	66.7
Female	15	33.3
Total	45	100.0

The above frequency table describes the percentage numbers of Egg allergic patients in male and female. The males are highly affected with Egg allergy in our local Pakistani population.

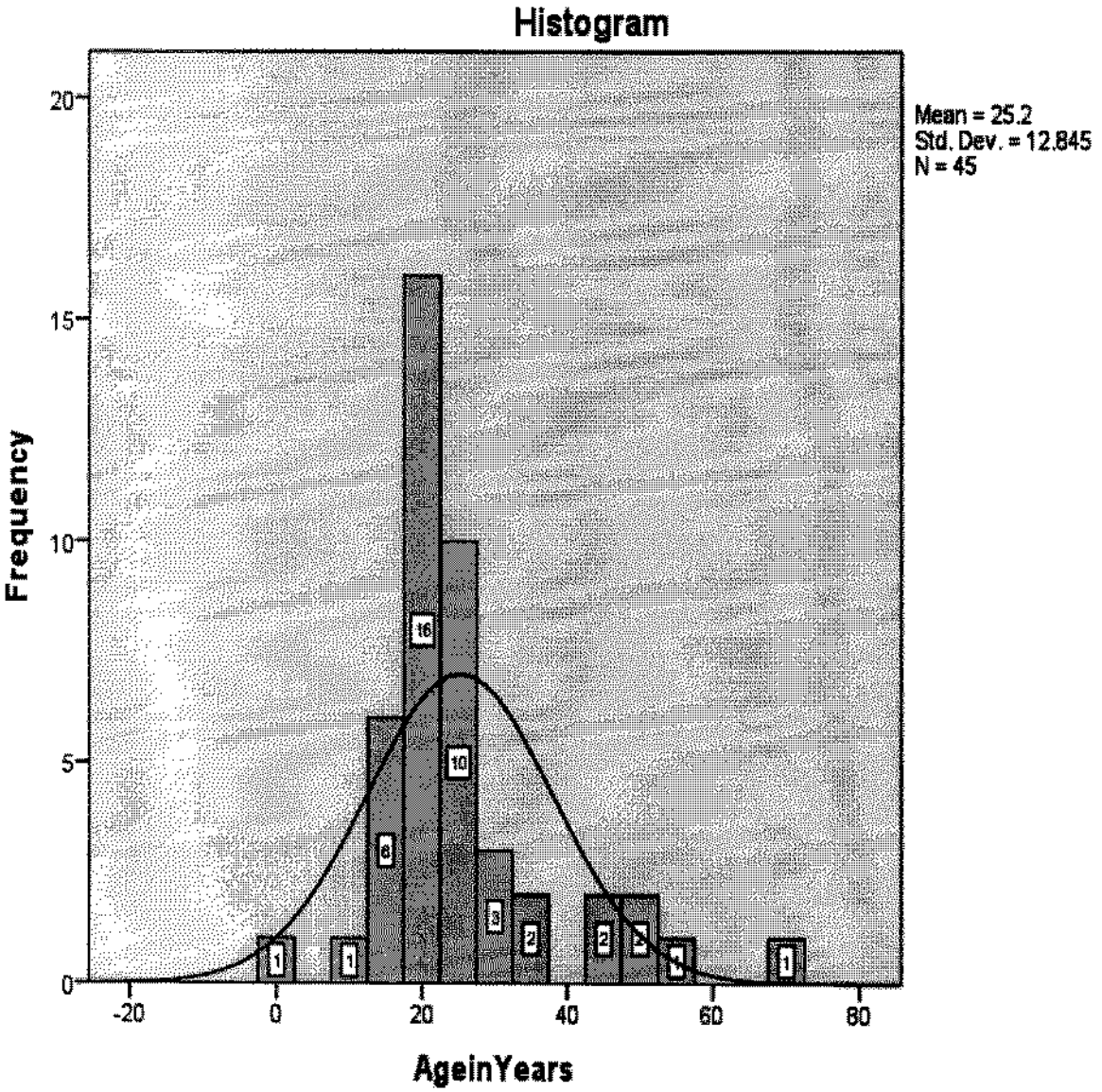

Figure 3-1: Histogram shows that age of Egg allergic patient and it describes that age lies between 1 to 70 years and average age of patient is 25 years.

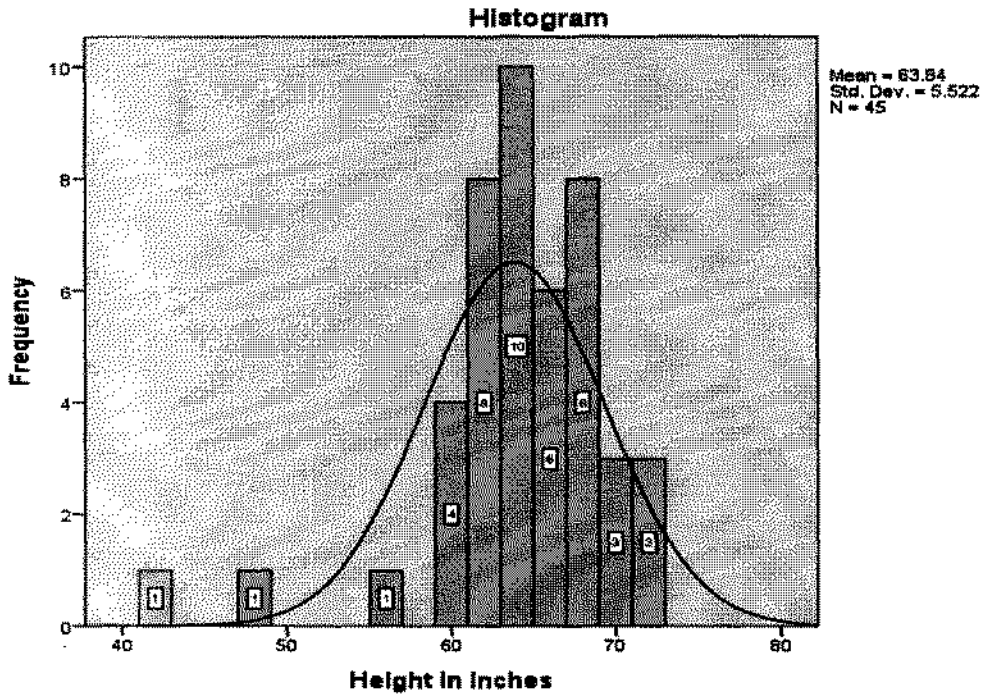

Figure 3-2 : Histogram shows the height of Egg allergic patient .The average height of the patient is 63.84 inches or 5.3 ft. (as 1 ft. =12 inches)

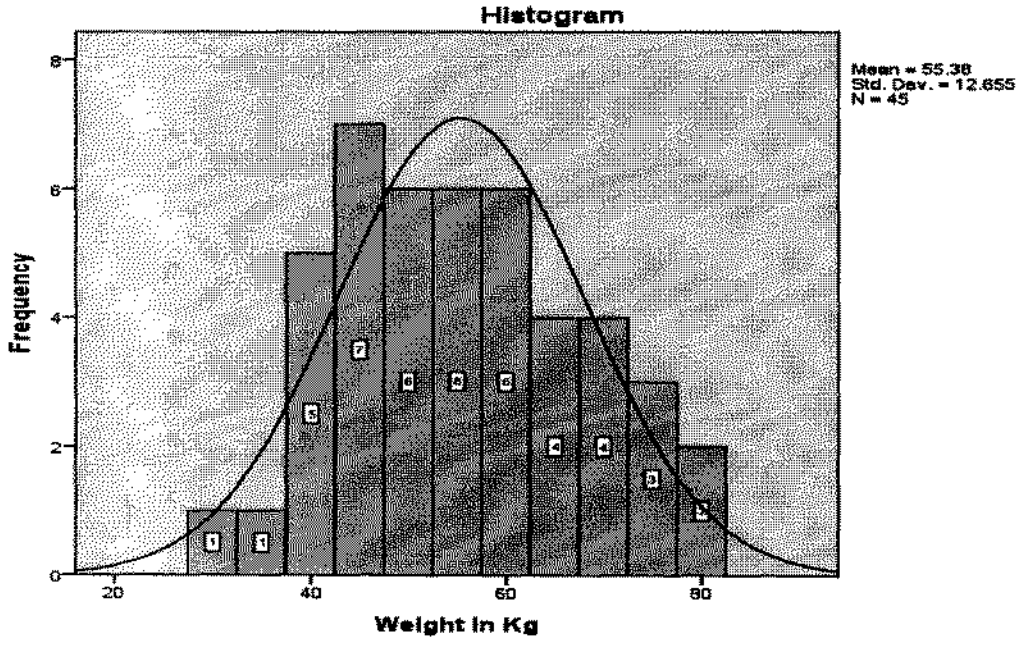

Figure 3-3 : Histogram shows that weight of Egg allergic patient's lies between 25 to 80 kg and Average weight of Egg allergic patient is 55 kg.

Table 3-3: Occupation of Egg allergic patients

Occupation	No of Patients	Percent Age	Cumulative Percent Age
Cook	1	2.2	2.2
Govt Employee	2	4.4	6.7
Hostel owner	1	2.2	8.9
House Wife	5	11.1	20.0
MTO (PTA)	1	2.2	22.2
N.Qasid	1	2.2	24.4
Nursing	2	4.4	28.9
Private job	1	2.2	31.1
Retired Govt Officer	1	2.2	33.3
Sanitary worker	1	2.2	35.6
Student	25	55.6	91.1
Teacher	4	8.9	100.0
Total	45	100.0	

Table 3-3: shows the frequency of occupation of our patients. Table describes that patients are having different occupation but mostly students are prone to egg allergy.

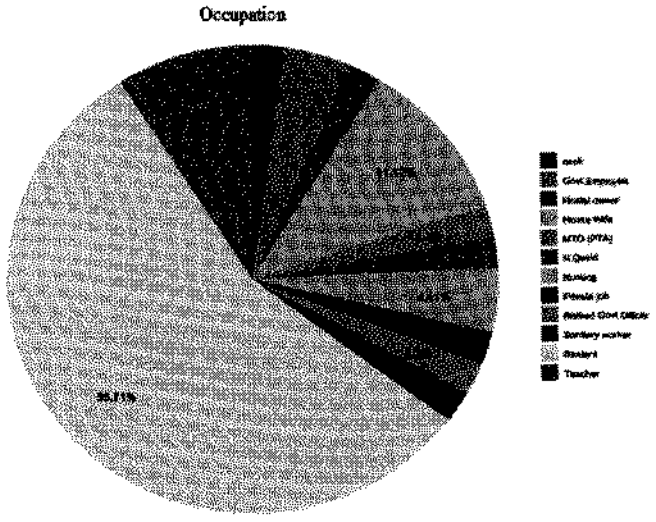

Figure 3-4 : This Histogram represents the occupation of our patient with different colors. The colors shows the different occupation of Egg allergic patients. This shows that students are more allergic to Eggs than others.

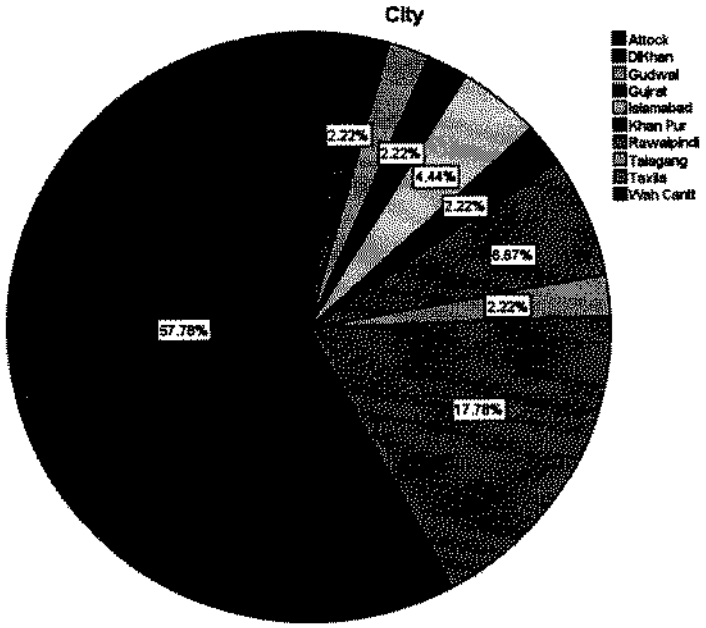

Figure 3-5 : This Histogram elaborates the Egg allergic patients in different cities of Pakistan. In this Histogram, wah cantt has the largest number of patient affected with Egg allergy. The lowest numbers of Egg allergic patients were presented in Talagang.

Table 3-4 : Geographical Cast of Egg allergic patients

Cast	No of Patients	Percent Age	Cumulative Percent Age
Baloch	1	2.2	2.2
Kashmiri	4	8.9	11.1
Pathan	5	11.1	22.2
Punjabi	32	71.1	93.3
Sindhi	3	6.7	100.0
Total	45	100.0	

This table shows the geographical cast of our Egg allergic patients. Table describes that most of egg allergic patients are Punjabi and Baloch people are relatively very less allergic to egg.

Table 3-5 : Sub cast * Cast Cross tabulation

Sub Cast	Cast					
	Baloch	Kashmiri	Pathan	Punjabi	Sindhi	Total
Abassi	0	0	0	3	0	3
Arain	0	0	0	1	1	2
Awan	0	1	0	7	0	8
Chaudhary	0	0	0	1	0	1
Jutt	1	0	0	1	0	2
Khahut	0	0	1	0	0	1
Khan	0	0	3	1	0	4
Kiani	0	1	0	0	0	1
Malik	0	2	0	4	0	6
Mughal	0	0	3	1	1	5
Orakzai	1	0	0	0	0	1
Qureshi	0	1	0	0	0	1
Raja	0	0	0	1	0	1
Rao Rajput	1	0	0	1	0	1
Saraeki	0	0	1	0	1	2
Sardar Rajput	0	0	0	1	0	1
Satti	0	0	0	1	0	1
Sountry	0	0	0	0	1	1
Syed	0	1	0	1	0	2
Total	3	6	8	24	4	45

This cross tabulation represents the frequency distribution of egg allergic patients among different casts and it illustrates that with relevance to our data Awan, Malik and Mughals are more frequently allergic to egg than other casts.

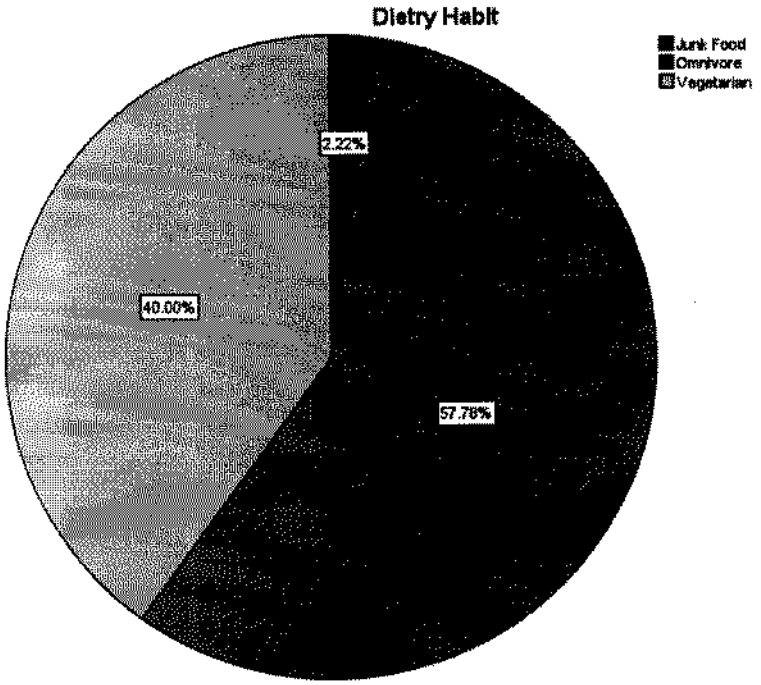

Figure 3-6 : This Histogram describes the dietary habit of Egg allergic patients. This shows that large no of Egg allergic patients are omnivore in nature

Table 3-6 : Frequency Table of Dietary habit of patient

Dietary Habit	No of Patients	Percent Age	Cumulative Percent Age
Junk Food	1	2.2	2.2
Omnivore	26	57.8	60.0
Vegetarian	18	40.0	100.0
Total	45	100.0	

Table shows the dietary habits of egg allergic patients. This shows that large no of Egg allergic patients are omnivore in nature and relatively less patients likes to eat junk food items in their daily life.

Table 3-7 : Frequency Table of Egg intake of Allergic patient

Egg intake	No of Patients	Percent Age	Cumulative Percent Age
1 in a week	2	4.4	4.4
1 in 6 months	1	2.2	6.7
1 in a week	6	13.3	26.7
2 Per week	1	2.2	28.9
3 Per Week	1	2.2	31.1
Avoid	26	57.8	88.9
Some Time	1	2.2	91.1
Very Less	4	8.9	100.0
Total	45	100.0	

Tables shows Egg intake of Allergic patients .The ratio describe that most of egg allergic patients totally avoid egg in their diet so they may protect themselves from harmful effect of allergen present in egg.

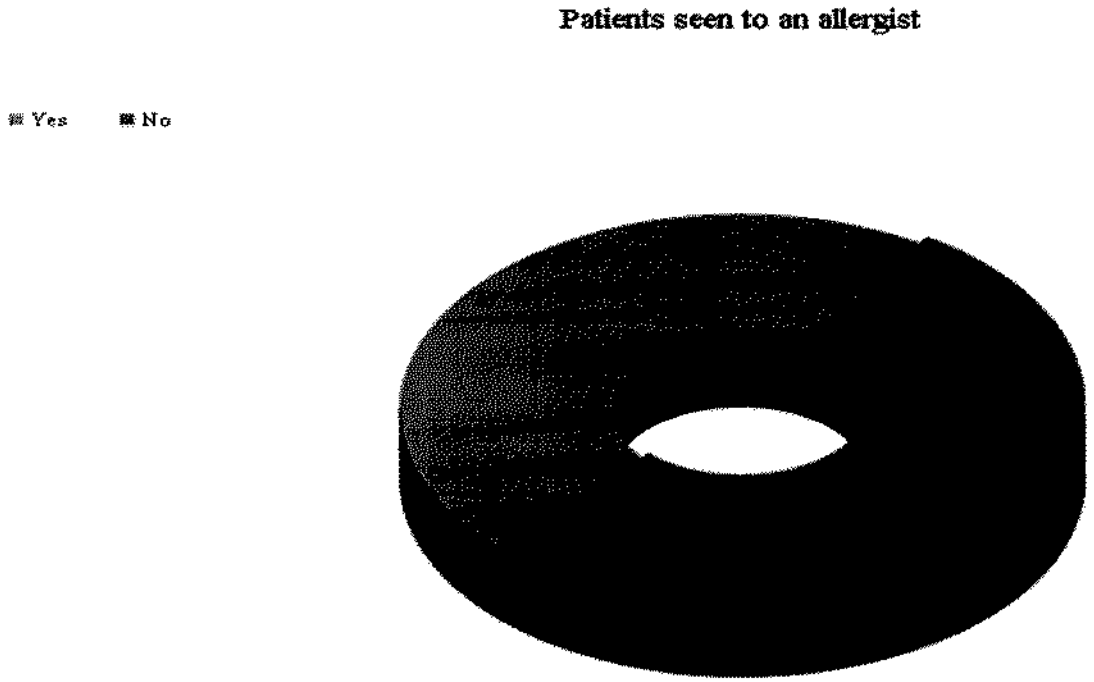

Figure 3-7 : This Pie chart describe the ratio of patient go to an allergist after having symptoms. The finding shows that mostly people does not seen to an allergist when they have allergic symptoms after eating eggs.

Table 3-8 : Frequency Table of skin testing

Skin testing	No of Patients	Percent Age	Cumulative Percent Age
No	34	75.6	75.6
Yes	11	24.4	100.0
Total	45	100.0	

This table shows the frequency that how many people go for skin testing. Ratio indicates that less number of patients undergone skin testing.

On Allergy Medication

Figure 3-8 : The pie chart showing that above 70% of our patients does not take any medicine. Less no of patients take medicine to get relief of allergy symptoms

Table 3-9 : Frequency Table of Drugs improves the symptom

	Drugs improve the symptom	No of Patients	Percent Age	Cumulative Percent Age
	No	25	55.6	55.6
	Yes	20	44.4	100.0
	Total	45	100.0	

Table describes either drug improves the allergic symptoms or not and it indicates that in most of cases medicine can't reduce the symptoms of Egg allergy.

Table 3-10 : Frequency Table of reaction to medication

Reaction to medication		No of Patients	Percent Age
	Yes	0	0
	No	45	100.0
	Total	45	100.0

Table reveals that patient that use drugs none of them have any reaction against the allergy medication. None of our allergic patient ever experience any side effect of allergy medication.

Table 3-11: Frequency Table of Having Anaphylaxis

	Having Anaphylaxis	No of Patients	Percent Age	Cumulative Percent Age
	No	40	88.9	88.9
	Yes	5	11.1	100.0
	Total	45	100.0	

Table describes the anaphylaxis ratio in our patients. Table shows that high frequency of our patient does not have anaphylaxis.

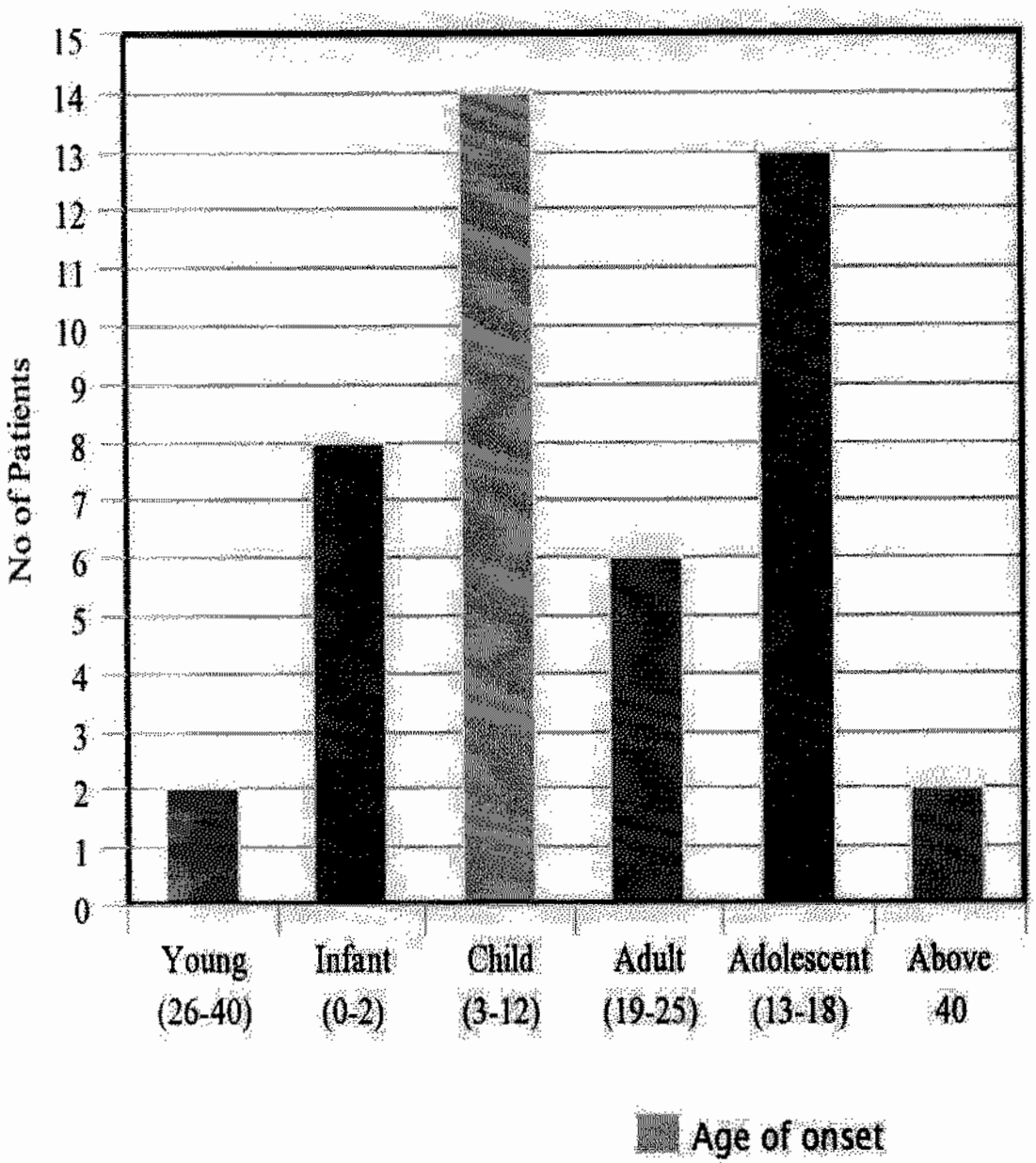

Figure 3-9 : This bar graph shows the age of onset of Egg allergy. This graph elaborates that mostly kids are allergic to Eggs and at the age of (3-12) most of people get allergic symptoms after eating eggs and become allergic to egg.

Table 3-12 : Frequency Table of Symptoms of egg allergy

Symptoms	No of Patients	Percent Age	Cumulative Percent Age
Abdominal Pain	6	13.4	13.4
Breathing Problem	2	4.4	17.8
Cough	1	2.2	20.0
Headache	2	4.4	24.4
Itching	6	13.4	37.8
Pimple	6	13.4	51.2
Swelling	5	11.1	62.3
Rashes & Hives	8	17.8	80.1
Constipation	1	2.2	82.3
Vomiting	4	8.9	91.2
Diarrhea	2	4.4	95.6
Pain in whole body	2	4.4	100.0
Total	45	100.0	

Table elaborates different symptoms of egg allergy.it reveals that different patients experience different symptoms after eating eggs but most common symptoms are rashes and hives. Abdominal pain & itching are also commonly observe.

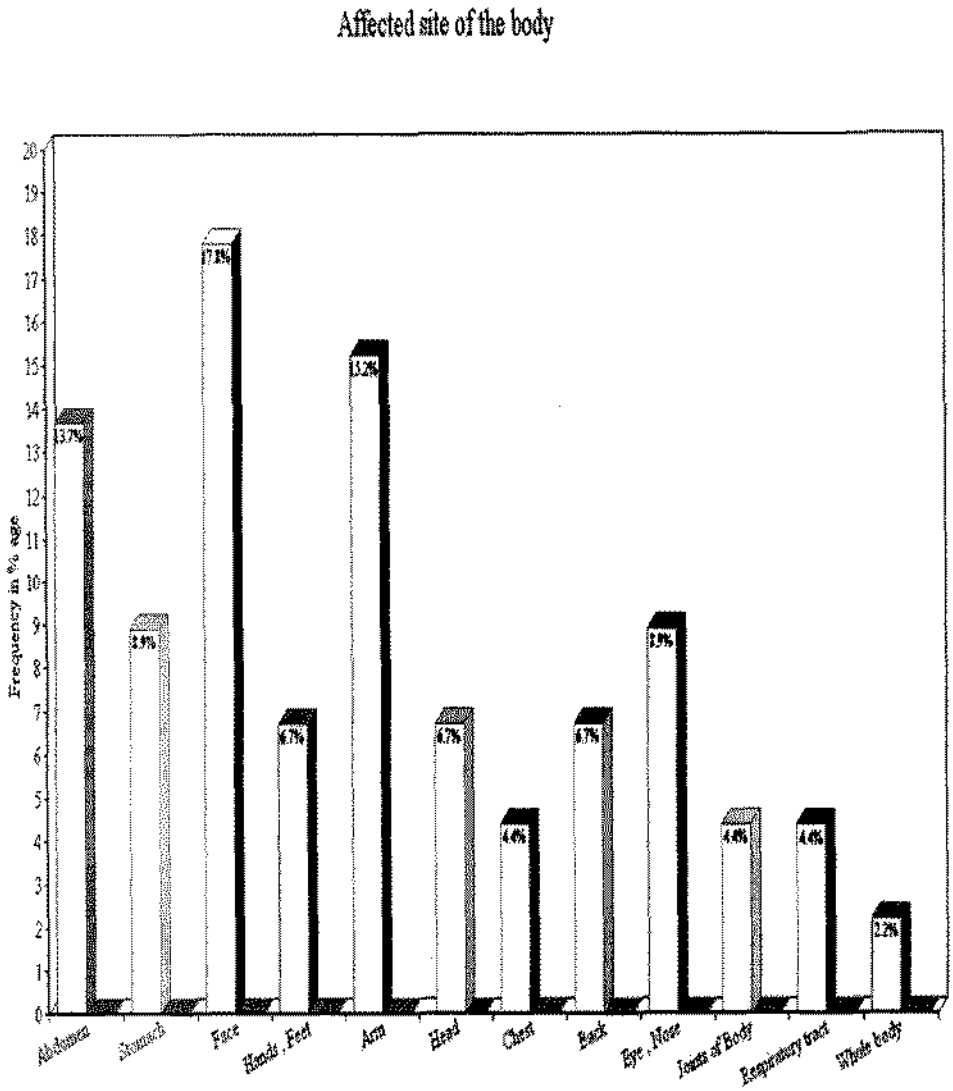

Figure3-10 : This bar chart shows the affected sites of the body. The chart tell us that different people have different areas that are effected by allergic symptoms but in most of the cases symptoms are shown on the face of people.

Table 3-13 : Frequency Table of Duration of symptoms

Duration of the symptoms	Frequency	Percent	Cumulative Percent
1 Day	9	20.0	20.0
1 Hour	2	4.4	24.4
1 Month	4	8.9	33.3
1 Week	4	8.9	42.2
1-2 Hours	1	2.2	44.4
1-2 Weeks	1	2.2	46.7
2 Days	1	2.2	48.9
2 Hours	1	2.2	51.1
2 Weeks	2	4.4	55.6
2-3 Days	2	4.4	60.0
2-3 Hours	4	8.9	68.9
2-3 Months	1	2.2	71.1
20-30 Minutes	1	2.2	73.3
3-4 Days	2	4.4	77.8
3-4 Hours	1	2.2	80.0
4-5 Days	1	2.2	82.2
4-5 Hours	1	2.2	84.4
5-6 Hours	1	2.2	86.7
Half Day	1	2.2	88.9
Till 1 Hour	2	4.4	93.3
Till take medicine	2	4.4	97.8
Whole Body	1	2.2	100.0
Total	45	100.0	

Table shows the duration of symptoms and it reveals that in most of the cases symptoms remains in body till the whole day. After eating egg for a whole day mostly people suffer from allergic symptoms.

Table 3-14 :- Frequency Table of Intensity of symptoms

Intensity of the symptoms	No of Patients	Percent Age	Cumulative Percent Age
Bearable	8	17.8	17.8
Extreme	2	4.4	22.2
Intense	3	6.7	28.9
Mild	3	6.7	35.6
Normal	1	2.2	37.8
Severe	26	57.8	95.6
Un Bearable	1	2.2	97.8
Much Severe	1	2.2	100.0
Total	45	100.0	

Table describe the intensity of symptoms after eating Egg. Our finding shows that most of the patients have severe kind of allergy symptoms after eating Egg.

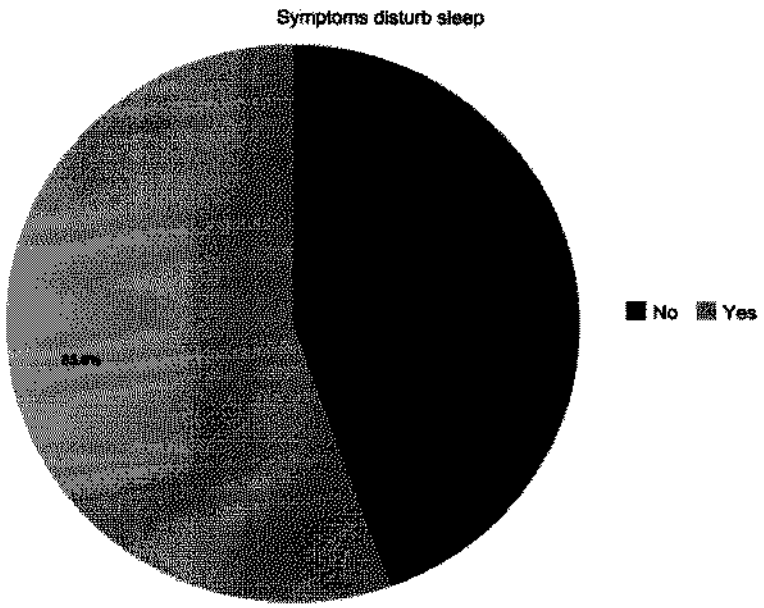

Figure 3-11 : The pie chart describes the disturbance frequency of the patients after having allergic symptoms and it reveals that in most of cases patient can't sleep due to severity of the symptoms.

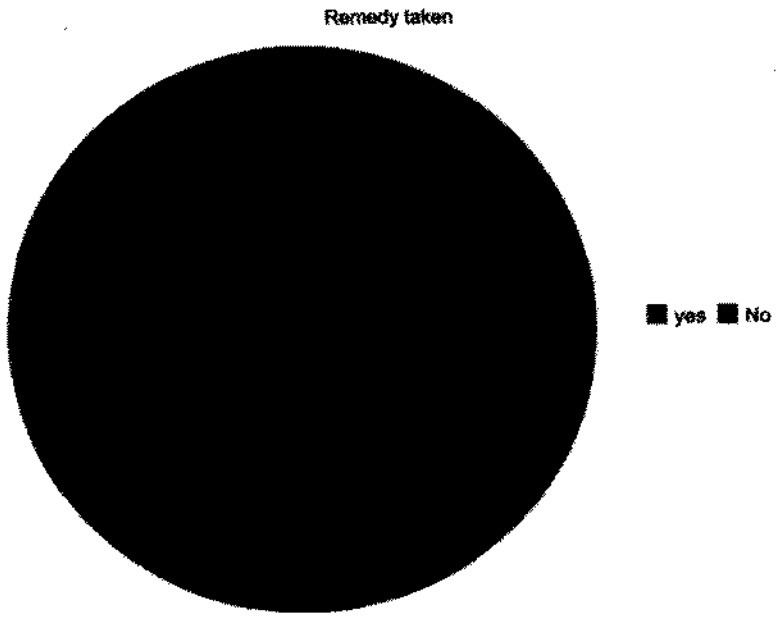

Figure 3-12 : The pie chart shows that either Egg allergic patient take remedy to cure Egg allergy or not and the finding shows that most of the patient does not take remedy to cure the allergy.

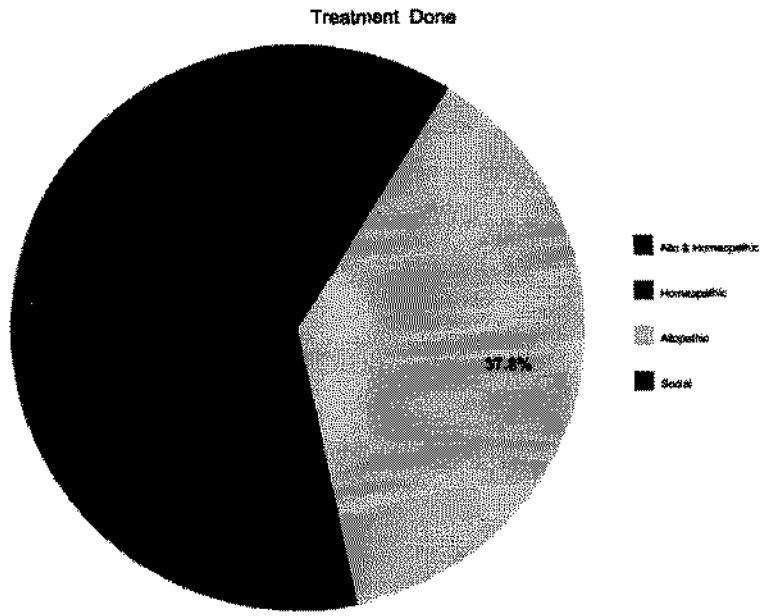

Figure 3-13 : The pie chart illustrates different types of treatment used by patients. It describes that most of Egg allergic patients does not go to doctor for treatment and use social mean to cure the symptoms.

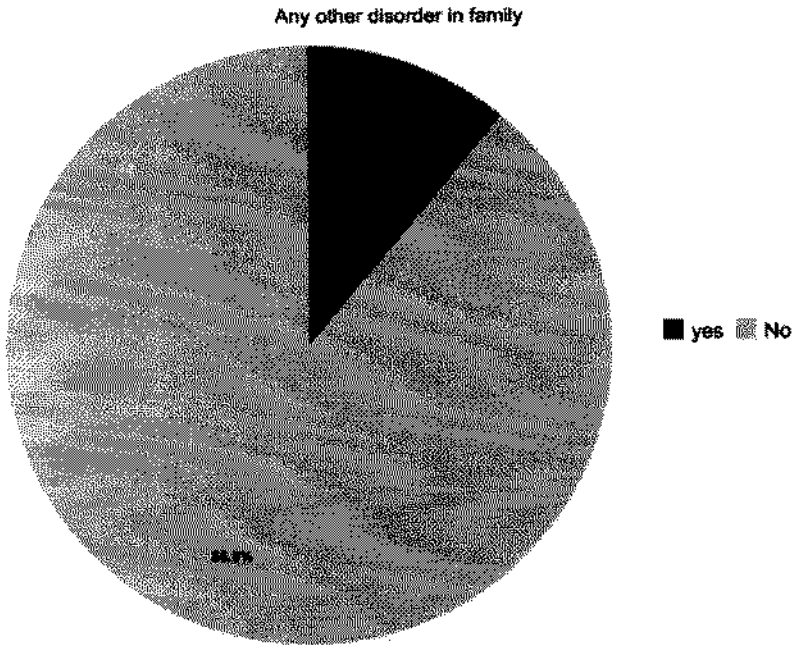

Figure 3-14 : The pie chart shows other disorder present in family and it explains that about 90% of Egg allergic patients does not have any disorder in their family.

Table 3-15 : Frequency Table of Other Family member effected

Other Family member effected	No of Patients	Percent Age	Cumulative Percent Age
Brother	3	6.7	6.7
Sister	5	11.2	17.9
Son	1	2.2	20.1
Daughter	1	2.2.	22.3
Mother	2	4.4	26.7
Father	2	4.4	31.1
Grand Parents	1	2.2	33.3
Maternal Aunt	2	4.4	37.7
None	28	62.3	100.0
Total	45	100.0	

Table shows the affection of other family members and it describes that in most of cases not any person in the family is effected by the egg allergy.

Table 3 -16 : Family member having another allergy

Family member having another allergy	No of Patients	Percent Age	Cumulative Percent Age
Asthma	8	17.8	17.8
Drug Allergy	1	2.2	20.0
Food Allergy	1	2.2	22.2
Skin Allergy	6	13.4	35.6
None	29	64.4	100.0
Total	45	100.0	

Table describes the family members suffers from another allergy and it reveals that family member of the Egg allergic patients are having different allergy too Asthma is more common among family members of Egg allergic patients.

Table 3-17 : Cross tabulation * Family member having another allergy

	Family member having another allergy					
Other Family member effected	Asthma	Drug Allergy	Food Allergy	Skin Allergy	None	Total
Brother	1	1	1	1	0	3
Child	0	0	0	0	1	1
Daughter	0	0	1	1	0	1
Father	1	0	0	0	1	2
Grand Parents	1	0	0	0	0	1
Khala	0	0	0	0	0	1
Mother	0	0	1	1	1	2
None	2	0	3	3	24	29
Sister	3	0	0	0	2	5
Total	8	1	1	6	29	45

The Cross tabulation of Family member having another allergy represents that Asthma is found to be more common among family members of Egg allergic patients while skin, drug and food allergy is also present in family members of the patients.

Discussion

Egg allergy is the most prevalent, IgE-mediated, food allergy in infants and children. Experts calculated that as much as 2 percent of infants are having egg allergy. . Egg allergy is the most famous food allergy in infants. Food allergic reaction is actually trigger simply by allergen seen in the food. The things that trigger allergies within food items happen to be naturally occurring protein. More than one hundred sixty food items have been highlighted while causing food hypersensitivity, nevertheless allergic reaction experts only consider a limited amount of individuals being connected with general public health and fitness worry. (Taylor and Hefle *et al.*, 2001).

Egg allergy s possess a cumulative incidence of approximately a couple of 2.8% simply by 2.5 of years old. (Eggesbo *et al.*, 2001).Egg allergy generally effects the children & considered to have a good prognosis, and parents are typically suggested that their children will outgrow the allergy by the early school-age years. Our results from this analysis of 45 patients with IgE-mediated egg allergy does not support the idea that the majority of children with egg allergy will develop tolerance over time. However, our data suggest that in most of the cases children does not get relief from this allergy. This was does not happen as previously thought. We reviewed in literature that egg tolerance was achieved by kids they found that 4% of patients developed tolerance by age 4 years, 12% by age 6 years, 37% by age 10 years, and 68% by age 16 years.

Our study of the egg allergy, from a cohort of 45 patients, showed that the Egg allergic patients were of different age, the average age of patient was 25 years the average height of the patient was 5.3 feet and average weight was 55 Kg (Fig 3.3). Furthermore different egg allergic patient were having different occupations but most of them are students this means majority of the patients are young and they don't attained tolerance indicating that allergy may remain with them till last until or unless proper treatment or drugs have been developed for egg allergy.

The allergic reaction is easily activated in people with egg allergy when they come in contact with the egg either through touching, smelling or eating. Reactions usually occur soon

Or immediately after food ingestion (Scurlock AM *et al.*, 2005 & Allen JA *et al.*, 2006). In our study all of the patients were highly sensitive to egg too, the symptoms varied in patients but all of them developed allergic symptoms immediately after eating egg. It may include skin reaction, nasal congestion and digestive problem and in very rare cases it may cause anaphylaxis, but most of our patient's experiences reaction on skin either in form of Itching, Hives, or Swelling. Abdominal pain was also frequently observed.

Anaphylaxes is the severe, life-threatening condition that impairs breathing and can send the body into shock. About 90% of our patient didn't have anaphylaxes .Most of the reactions affect the skin while it was illustrated in literature that Egg accounted regarding 7% of serious anaphylactic side effects with newborns and children in a German study (Mehl A *et al.*, 2005) in our study it was also observed in frequently less patients.

The patient history showed that most of egg allergic patients were geographically located in Punjab, so most of Punjabies were found to be allergic with egg. The cross tabulation of cast represents that Awan, Malik and Mughals were more frequently allergic to egg than other casts but not such data were observed in literature that showed that egg allergy is cast specific. As far as dietary habits of our patients were concerned most of egg allergic patient were omnivore. Our results showed that relatively less number of patients consumed junk food items in their daily routine. Dietary habit may link with sensitisation so it may be happen that eating certain type of food item may develop immune system to become sensitive and cause allergic reaction.

Most of the patients (about 76%) do not consult with an allergist when they have allergic symptoms Very less percent of people undergo skin testing. Our finding also showed that above 70% of patients don't take any medicine to get relief when they have symptoms while Patient taking medicine were also not satisfied with drugs as medicine didn't provide relief to them .Age of onset of this allergy frequently found in children mostly kids are allergic to Eggs and at the average age of (3-12) most of people get allergic symptoms after eating eggs and become allergic to egg. This finding is supported by previous research. Egg allergy has a cumulative prevalence of approximately 2.6% by 2.5 years of age (Wüthrich *et al.*, 1993).

Several clinical and laboratory parameters were related to egg allergy outcome. The presence of other atopic disease was significantly related to the persistence of egg allergy. In addition, allergy to other foods was significantly related to the persistence of egg allergy. We found that patients effected with egg allergy either themselves and in most of the cases their family members are suffered from asthma. This indicate the possibility that there might be a linkage between these two harmful conditions. Oral immunotherapy has been successful in desensitizing patients to several food allergens in small clinical trials but No ultimate cure is available till today.

Egg allergen are responsible for allergic reaction in egg allergic patients. An Allergen is always a protein. To find out the antigenic protein we developed an indirect Elisa in which specific antibody was used to check out the presence of Gal d 5.Antigen antibody reaction was occurred and color development was observed in all of our patients samples confirming the presence of targeted Gal d 5 in all samples while control remained un effected.

Our study had some limitations. First, it was a short time period study, and hence, an exact assessment of the prevalence and incidence of Egg allergy and its complications was not possible. Second, it was based on characterisation of egg yolk allergen for that purpose we specifically design Elisa for Gal d 5 which is the key allergen present in egg yolk therefore, the study population did not reflect the characteristics of the entire allergic community as egg is having too many allergens that may responsible for harmful reaction.

In conclusion, we can said that less no of patients with egg allergy eventually develop egg tolerance. The rate of development of tolerance is slower than described previously, Asthma is found to be more common in family of egg allergic patient's. So it reflects the high degree of atopy in our study population. Till yet there is no proper treatment of egg allergy still avoidance is the key option for allergic people.

Conclusion

As the prevalence of Egg allergy rises worldwide, it is important that physicians are able to accurately diagnose and manage this problem at the primary level. Patient's history is the most significant element of the evaluation; this is followed by physical examination, which often reveals signs of allergic reaction associated with hypersensitivity. We should also continue to inform those mixed up in care on the child, moreover prepare the crooks to handle an emergency -such as anaphylactic shock. As novel and effective approaches emerge for modulating the immune system, we can say that the future is very promising for those suffering from Egg allergy but till yet avoidance is the only key remain associated with therapy, so labelling should be performed. Indirect Elisa method used to check out the presence of Gal d 5 antigen present in patient's sera. Antigen/Antibody reaction, optical change and O.D values which are > 0.04 in all patient samples confirm the presence of Gal d 5 which is the major egg yolk protein and is responsible for antigenic and allergenic activity in our patients. Like the successful development of Elisa for detection of Gal d 5 in patient sera using specially designed antibody IgE & anti-human IgE conjugated alkaline phosphatase some other sort of certain antibodies may possibly be capable to the use within the particular ELISA development method associated with drug and cure.

Future Prospective

Egg allergy that is believed to be responsible for most immediate-type food-induced hypersensitivity reactions but till yet not an accurate method developed to treat the reactions significantly. Oral immuno therapy should need certain modification to treat the un-bear able condition, use of ovomucoid specific IgE concentration might be used to increase the accuracy of treatment. Still avoidance of Egg is the most effective and the leading way of treatment .The developed ELISA is highly sensitive and can be utilized by the food industry to detect and quantify allergens. Elisa format might be used to diagnose the better treatment of egg allergy by using IgE antibody which is the leading cause of allergic reactions.

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ABSTRACT:

Adverse reactions to egg i.e. egg allergy and intolerance have gained sizeable consideration. This study focuses on the particular characterisation and management associated with IgE mediated Egg allergy that is believed to be responsible for most immediate-type food-induced hypersensitivity reactions. Clinically, these reactions are characterised by a variety of signs and symptoms that occur within minutes as well as hours after consumption of egg. Reactions may be constrained and up generalised together with involvement of the skin, nose, eyes, and/or lungs. In more severe and worse cases, anaphylactic shock can occur.