

CHARACTERISATION OF GRASS POLLEN ALLERGENS IN ZIMBABWE

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Declaration

I, Caroline Chasara declare that this Honors thesis entitled “Characterization of grass pollen allergens in Zimbabwe”, is my document. This thesis contains no material that has been submitted previously, in whole or in part, for the reward of any other academic degree or diploma. Except where otherwise indicated, this thesis is my own work.

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List of abbreviations

UV	Ultraviolet
SDS	Sodium dodecyl sulphate
Df	Dermatophagoides farina
Dp	Dermatophagoides pteronyssinus
AAIC	Asthma Allergy and Immune Dysfunction clinic
MW	Molecular weight
AP	Alkaline phosphatase
TEMED	Tetramethylethylenediamine
OAS	Oral Allergy Syndrome

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ABSTRACT

IgE mediated hypersensitivity affects more than 25% of the of the industrialized countries. The first wave was that of inhalant allergies and this has been recorded as a series of ISAAC studies. The second wave is emerging and it is affecting continents such as Europe, Asia and Africa. The environmental proteins that trigger allergic responses differ with the climate, environment and seasons during which they occur. Pollen allergy causes type 1 hypersensitivity. The common clinical manifestations include asthma, conjunctivitis, allergic skin inflammation, ocular allergy anaphylaxis and allergic rhinitis. Pollen grains only represent a small fraction of the total amount of the viable biological particles present in the air, but pollen are the most important aeroallergens in the outdoor environment. The prevalence of pollen allergy has been documented globally with limited evidence from Africa and Zimbabwe. Some of the reasons for non-documentation of allergy in Africa are the limited number of allergy specialists and the limited number of allergy centers. This study was conducted to access the burden of pollen allergy in Zimbabwe and to characterize common grass pollen allergen extracts.

A retrospective study was carried out on 2735 patients. Data analysis was carried out using a software known as SPSS. General sensitization trends were deduced. Sensitization was seen to increase between the ages 9-39. Grass pollen allergens were seen to be the third commonest inhalant allergen source after house dust mites and the frequently manifested clinical condition was asthma. The laboratory detection of IgE was done on 55 serum samples using the Line blot assay. Ten pollen proteins were extracted using the alkaline lysis extraction method. Bermuda grass proteins were separated by SDS PAGE. Low molecular weight (5-30kDa) proteins were separated.

Key words

Pollen allergy, environment

CHAPTER 1

1.0 INTRODUCTION

1.1 Pollen allergy burden

Pollen allergy continues to be a global burden. A quarter of most industrialized countries are known to be affected by diseases such as allergic rhinitis, conjunctivitis, asthma and dermatitis. Inhalant allergy is encountered globally and these cases are increasing in the Central African region. Allergic rhinitis (AR) is prevalent in African countries such as Nigeria. However, little information exists on the allergens causing this disease. Most AR patients presenting for treatment in Abuja, Nigeria, have moderate-severe persistent AR. (Potter and Cadman, 2001). The patients also show similar SPT sensitization patterns with other countries having similar climatic conditions. While the prevalence of these diseases has been documented in various other countries, there is limited information reported in Africa. Under reporting of allergies in Africa are due to the shortage of allergy centers and limited allergy specialists in Africa. Concurrent short studies have been carried out in Zimbabwe. There is one specialist allergy clinic in Zimbabwe (Asthma, Allergy and Immune Dysfunction Clinic), and therefore patients attending this clinic may be representative of the pattern of sensitization across the country. Patients have been investigated for suspected pollen allergies. Physical and clinical investigations were carried out. The conditions diagnosed included asthma, allergic rhinitis, conjunctivitis and dermatitis.

The human immune system is a system that triggers responses against dangerous foreign substances. The word allergy came into use in the early 1900s to describe shortness of breath (Cohen and King, 2001). However, with time its meaning has changed to a condition in which antibody-mediated immunologic responses are elicited.

1.2 Allergenic molecules

Allergens are usually protein molecules. Macromolecules such as polysaccharides can also act as allergens although these macromolecules usually generate poor antibody response.

Polysaccharide are not usually involved in allergies that are IgE mediated. Extracts of pollen grains are mixtures of substances such as proteins, glycoproteins and fatty acids. However, the well-characterized pollen antigens responsible for immediate hypersensitivity reaction are mostly proteins (Jenkins and Vickers, 2000).

Sensitization to many pollen allergens occurs via the gastro-intestinal tract route. The onset of food allergy is related to inhalant allergies in adults. Inhalants such as tree, grass and weed pollen, house dust mites, animal fur and molds contribute to the increasing trend of inhalant allergies. Recent evidence suggests that the dermal route of exposure may also be relevant (WHO, 2009). Pollen, latex and other various fruits and vegetables may develop cross-reactive allergens. The cross reactivity between fruits and vegetables is known as pollen-fruit and latex-fruit syndromes.

Aeroallergens play a pivotal role in respiratory allergic disease pathogenesis. Diseases such as asthma and rhinitis are caused by a large percentage of aero allergens. Pollen grains, fungi, animal dander, house dust mites, domestic pets and insects are of particular importance as triggering factors. Pollen grains are well studied as allergens among all other aeroallergens and are important source of pollinosis (Chua *et al.*, 2001).

Specific immunotherapy is amongst treatment which change the immunological profile of an individual from Th2 to Th1. Various allergenic proteins have been purified and characterized. Diagnosis and immunotherapy is practiced with commercially accessible extracts from different natural sources, these sources may contain varying amounts of an individual allergens in batches. Each extract usually contains multiple allergenic proteins in an extract. The quality and the quantity of allergens differs from extract to extract. The differences are due to a change in source material, the extraction protocol and storage conditions used (Valenta, 2002).

It is commonly thought that for one to develop the atopic asthma or allergic disease, both the genetic predisposition and allergen exposure are required. It is important to understand how future climate changes affect aeroallergens that elicit the onset of disease and precipitate symptoms. Climate change partly gives rise to variations in the temperature pattern characterizing the different seasons throughout the year. Plants may vary their pollination schedule advancing and also prolonging their pollination period (Maikowska *et al.*, 2007).

Environmental conditions such as the presence of pollutants are known to up-regulate plant proteins and some pollen proteins (Horiuti *et al.*, 2000). Grasses are the commonest cause of pollen allergy. Approximately twenty grass species from five subfamilies are considered as the most common cause of grass pollen allergy. Allergic relationships among these grass families closely follow their Phylogenetic relationships.

Amongst the factors that increase susceptibility to allergies are immune compromise, pregnancy and adolescence. The weakening of the body's immune defense usually after viral infection increases allergic potency to environmental allergens. (Amalia and Knifemen, 2007).

Allergy is diagnosed by a combination of history, physical examination, and confirmatory testing. Modalities for testing include skin testing, in vitro assessment, and challenge testing of the conjunctiva or nasal mucosa. Challenge testing is primarily reserved for research.

1.3 Pollen distribution

Pollen are shed by the male part of flowers and their release is often triggered by changes in atmospheric humidity levels. Pollen may be released in a single burst or be released gradually. Usually, pollen release occurs early in the morning so your symptoms may be worst early in the day.

The distance pollen grains travel from the plant depends on the nature of the carrying agent for example, insects, environmental conditions and pollen grain size. For example, most grass pollen is deposited within 3 meters from the source; only 1% of grass pollen manages to travel a distance of 1 km from the source. However, depending on the characteristics of the wind, pollen grains from some plants can be found up to an astounding 20 km away from the plant that released them (Amato *et al.*, 2007).

1.4 Interaction between thunderstorms and asthmatic attacks in pollen sensitive individuals

Thunderstorms have been recently linked to asthma epidemics, particularly during the pollen seasons. These epidemics have been reported in different cities found in western Europe (Birmingham and London in UK and Naples in Italy) and Australia (Melbourne and Wagga Wagga). Since pollen grains are microscopic, they can be easily carried at ground level by strong air currents associated with thunderstorms. During these events, the possibility of pollen grain bursting and releasing allergens is high. These allergens can be released as fine particles in air and water mixtures known as aerosols. The aerosols dispersed can be readily inhaled by an individual. They can also penetrate deeply into the lower airways hence inducing asthma reactions in pollen patients (Amato *et al.*, 2007).

1.4 Electrophoresis

Polyacrylamide gel is a synthetic, thermo-stable, transparent, strong, chemically relatively inert gel, and can be prepared with a wide range of average pore sizes. The pore size of a gel is determined by two factors, the total amount of acrylamide present and the amount of cross-linker. Acrylamide is extremely toxic, causing central nervous system paralysis. Therefore, being careful is essential. Where agarose gels are best for running larger molecules, like DNA, SDS-PAGE is better suited for running smaller ones, like proteins. SDS-PAGE has a number of uses, which include establishing protein size, protein identification, determination of sample purity (Suvra and Vikash, 2012).

SDS-polyacrylamide gel electrophoresis (SDS-PAGE), is a commonly used technique that can help in identifying protein sizes, and protein quantity. The purpose of SDS-PAGE is to separate proteins according to their size, and no other physical feature.

SDS is an anionic detergent, therefore when dissolved its molecules have a net negative charge within a wide pH range. A polypeptide chain binds amounts of SDS in proportion to its relative molecular mass. The negative charges on SDS destroy most of the complex structure of proteins and are strongly attracted toward an anode (positively-charged electrode) in an electric field.

Polyacrylamide gels restrain larger molecules from migrating as fast as smaller molecules. Because the charge-to-mass ratio is nearly the same among SDS-denatured polypeptides, the final separation of proteins is dependent almost entirely on the differences in relative molecular mass of polypeptides.

In a gel of uniform density, the relative migration distance of a protein is negatively proportional to the log of its mass. If proteins of known mass are run simultaneously with the unknowns, the relationship between relative mobility and mass can be plotted, and the masses of unknown proteins estimated. The key to accurate molecular weight determination is selecting separation conditions that produce a linear relationship between log MW and migration within the likely MW range of the unknown protein.

After separation, the relative migration distance (R_f) of the protein standards and of the unknown protein has to be determined. R_f is defined as the mobility of a protein divided by the mobility of

the ion front. Because the ion front can be difficult to locate, mobilities are normalized to the tracking dye that migrates only slightly behind the ion front:

$$R_f = (\text{distance to band}) / (\text{distance to dye front})$$

Using the values obtained for the protein standards, when a graph of log MW vs. R_f is plotted, the plot should be linear for most proteins, provided they are fully denatured and that the gel percentage is appropriate for the MW range of the sample. The standard curve is sigmoid at extreme MW values because at high MW, the sieving effect of the matrix is so large that molecules are unable to penetrate the gel; at low MW, the sieving effect is negligible and proteins migrate almost freely. The accuracy of MW estimation by SDS-PAGE is in the range of 5–10%.

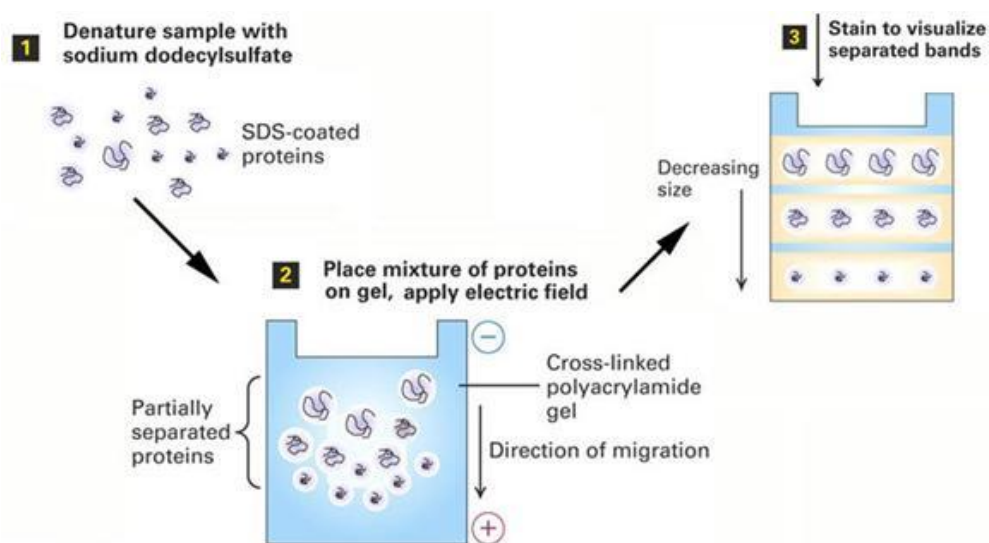


Figure 1: Steps involved in SDS PAGE analysis

After visualization by a protein specific staining technique, the size of protein can be estimated by comparison of its migration distance, with that of a standard of known molecular weight. It is possible to blot the separated protein onto a positively charged membrane and to probe with protein specific antibodies in a procedure termed western blotting (Mothes *et al.*, 2004).

CHAPTER 2

2.0 LITERATURE REVIEW

2.1 Pollen allergy epidemiology

a) Global overview of pollen allergy

IgE mediated hypersensitivity affects more than 25% of industrialized countries. The worldwide trend of allergic diseases is increasing and remains a huge medical concern. In the developed world, Australia and New Zealand have the highest recorded prevalence of allergic diseases in the developed world. An ASCIA-Access Economics Report(ASCIA)estimated that in 2007 4.1 million Australians (19.6% of the population) had at least one allergic disease. The highest prevalence of allergic disorders was observed in the older people particularly the working population. The proportion of people with allergies was 45% and the age range was between 15 and 64 years.

Allergic disease prevalence and symptoms vary between various studied populations. ECRHS and ISAAC studies have suggested that regional differences give rise to many new hypotheses about this fact. In a report by the World Health Organization (WHO), Ukraine and United Kingdom reported the highest allergic disease prevalence. The percentages recorded were of (40%) and (32%) respectively. Azerbaijan (3%) had the lowest prevalence allergic symptoms. The highest rates recorded for rhinitis were from Ukraine (40%) and Portugal (37%) with the lowest rate reported in Bangladesh (5%).Venezuela and United Kingdom (32%) reported the highest asthma prevalences, while the lowest prevalence was in Iceland and Mongolia (2%). For eczema the highest were reported from Ukraine (35%) and United Kingdom (27%) and the lowest Russia and Poland (<2%). This survey shows that in some nations allergy constitutes to be a relevant public health problem (WHO, 2009).

In a study by Vanessa Wong *et al.*,1953 it was concluded that over the past decades, in developing countries, the prevalence of allergic diseases has increased considerably. Weeds have been seen to play a pivotal role in allergies in children living in the Great Basin with grasses playing an almost an equal role. The prevalence of sensitization to at least one grass was lower

than that of the weeds, but still was significantly high at 57% in children age 2-3 years old. The early grass sensitizers, Timothy and Alfalfa, are both grasses that are common to the Great Basin. Timothy grass is native to the region. Alfalfa is a perennial grass and is planted for hay by cattle ranchers (Do, Pollen, & Individuals, n.d.)

Pollen allergy has a huge clinical impact in Europe and recent evidence suggests that the prevalence of respiratory allergic reactions induced by pollen allergens in Europe has been continuously increasing during the past decades. (Amato *et al.*, 2007) Recent findings of the phase three of the International Study of Asthma and Allergies in Children (ISAAC) study however showed that there were no changes in prevalence of asthma symptoms, rhino conjunctivitis and eczema in the European population. High prevalence was observed among the older children (Cantani and Micera, 2000).

The prevalence of pollen allergy in Europe is currently estimated at 40%. Factors such as air pollution may contribute to asthma. However, exposure to allergens represents a key factor among the environmental determinants of asthma. (Aedrychowski *et al.*, 2001). Airborne-induced respiratory allergy does not recognize national frontiers, the study of pollinosis cannot be limited to national boundaries, as obviously happens with most diseases that can be prevented by avoiding exposure to the causative agent. The main pollination period in Europe covers about half the year, from spring to autumn. The distribution of airborne pollen taxa of allergology interest is related to five vegetational areas.

The better understanding of molecular mechanisms of allergy in Europe has led to the progression of knowledge of molecular mechanisms of allergy. The structure and functions of some allergens have been identified. The knowledge of both qualitative and the quantitative allergy prevalence is important. Their seasonal and annual differences is important in the diagnosis and management of pollen related allergens. Several studies have provided explanations about the relationship between allergic sensitization, allergen exposure and clinical observations such as allergic cross-reactions. Allergens have been recently cloned and their expression has been achieved by as recombinant molecule. (Valenta, 2002). Scientists involved in allergy research and Type I hypersensitivity studies are still searching for the components that

are responsible for making an antigen an allergen. A lot of research has been focused on determining allergenic structures and epitope mapping of IgE-binding epitopes.

In countries such as India, cases of respiratory allergies show an increase from 10% in 1964 to approximately 30% in the year 2000. Patients diagnosed of grass pollen allergies, often show reactivity to pollen allergens from various grass species. The grass family comprises of approximately 8000 wide spread species. The grass family covers about 20% of the total world's surface. Various conditions include the production of pollen by plants. These conditions include the ability of a plant to carry out wind pollination, its ability to produce allergenic pollen in sufficient amounts, and the proximity of the pollen to humans (Vieira,2002).

About 40% of allergic patients are sensitized to grass pollen allergens. *Lolium perenne* is a significant allergen source in regions that have temperate climate for example regions in North America, Europe and in other parts of Australia (Vieira, 2002; Smart *et al.*, 1979; Wüthrich *et al.*, 1995).

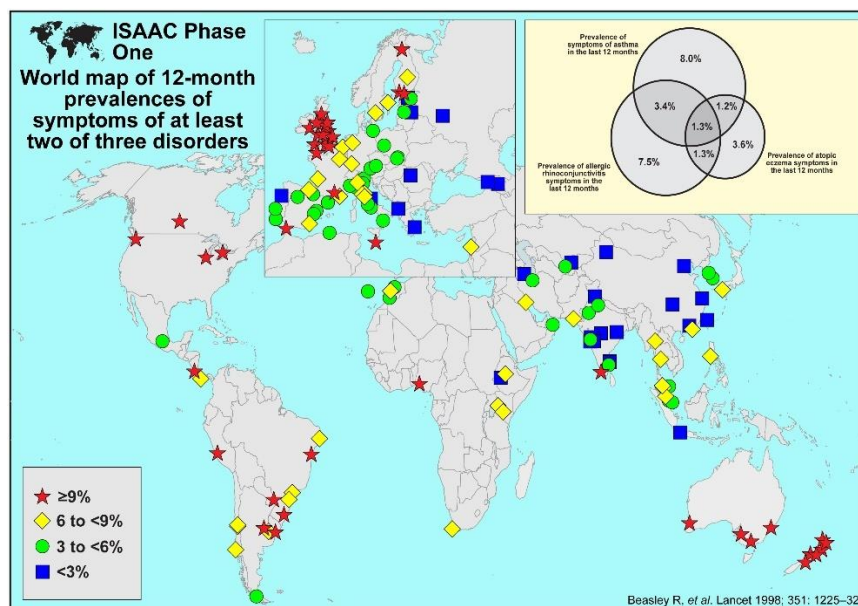


Figure 2: ISAAC Phase 1 world map

b) Pollen allergy in Africa

Pollen grains represent small fractions of the total amount of the viable biological particles present in the air. In Africa, pollen are the key aeroallergens in the outdoor environment. Diseases such as dermatitis, conjunctivitis, asthma and rhinitis affect a number of countries.

The prevalence of these diseases has been documented in various other countries. However, there is limited information reported on Africa. Not much has been documented on allergy in Africa and yet the prevalence of pollen allergy is increasing. One of the reasons for minimal reports on pollen allergy is that there are limited allergy centers in Africa. The shortage of allergy centers in the African countries leads to a reduction in the studies carried out on pollen allergy. A huge limitation in diagnosis has been due to the fact that there is limited relevant personnel in this field.

Allergic rhinitis (AR) is persistent in Nigeria. However, little information exists on the allergen sources. Most AR patients presenting for treatment in Abuja, Nigeria, have moderate-severe persistent AR. These patients show similar SPT sensitization pattern with other countries having similar climatic conditions. The study carried out in Nigeria led to the conclusion that sensitization patterns are not always related to ARIA classification or any predominant AR symptoms but rather may rely on the environmental condition of study area and genetic makeup of the study population. The study also revealed the concept of multiple sensitization for allergens among the patients. A patient with positive sensitization to pollen could also be sensitized against an insect, food or fungi allergens. (Berger, 2002).

Concurrent studies carried out in South Africa show that pollens that cause seasonal symptoms come from trees, grasses and weeds. Different areas were sampled in South Africa. From the sampled regions, the most common allergen was found to be grass. (Berman, *et al.*, 2007) It was observed that the highest tree pollen concentrations are commonly present from early spring to early summer. This season is followed by the rainy season which usually occurs during mid-summer. In South Africa, the rainy season varies according to the climate. The weed season is less important. Major weed allergens like ragweed are not predominant sources of allergy in South Africa. Weeds pollinate in late summer and autumn. Fungal spores, or molds, are usually sampled concurrently with pollen grains, so that the pollen catch and fungal spore loads as well as their seasonal peaks, are noted for specific areas. (Potter and Cadman, 1996).

c) Pollen allergy in Zimbabwe

One of the most common forms of seasonal respiratory diseases in Zimbabwe is pollen allergy. (Sibanda, 2013) In Zimbabwe, pollen is released from grasses, trees and weeds during the windy season. The month of August is usually more windy than other months during the year leading to an increase in the cases of pollen allergies during this season. Windy conditions stir up dust and pollen leading to the activation of various inhalant allergies and pollen allergies since some plants start pollinating during the month of August (allergies can be controlled)

2.2 Mechanisms and diseases manifested

The environmental proteins that trigger allergic responses differ with the climate, environment and seasons during which they occur. Common clinical manifestations include allergic asthma, eczema, allergic skin inflammation, allergic rhinitis and allergic anaphylaxis. (Cromwell *et al.*, 2000). Type I hypersensitivity causes anaphylaxis to drugs and insect venoms, and the skin test responses. It also causes asthmatic reactions to allergens such as pollens and house dust mites.

The hallmark of type I hypersensitivity is induction of IgE antibodies. IgE exists in small quantities in serum but it attaches by its Fc region to high-affinity receptors (FcεRI) on tissue mast cells and the basophilic polymorphonuclear cells (basophils) in the blood. Only certain immunization regimens induce IgE antibody. However, not all people exposed to allergens become allergic. The reasons for this are not completely known. There are strong genetic factors and agents such as aluminium hydroxide act as adjuvants for IgE immune responses. Mast cells and basophils contain several highly active pharmacological mediators in large cytoplasmic granules. When antigens bind to the antigen binding sites of the divalent IgE molecules they crosslink the Fc receptors and induce a signal for the granules to be extruded from the cells and release their contents. (Mark *et al.*, 2006).

Localization of allergic reactions to target regions may occur. The etiology of immune responses to allergy have been shown to be complex, Various factors have been shown to influence this etiology. These include genetic susceptibility, allergen dose, the route of exposure and structural properties of the allergen. In a landmark experiment by Prausnitz and Kustner, 1921 it was shown

that Type I allergy is a classical IgE mediated disease. Type I allergy diseases require at least two components. These components include a disease-eliciting antigen (known as an allergen) and an exchangeable serum factor that differentiates the allergic patients from the healthy individuals. (Mark *et al.*, 2006)

2.3 Pollen allergy diagnosis

Serology assays and techniques such as skin prick tests are currently being used for the diagnosis of pollen allergy. However, improvements are being seen in micro array technology (Berger, 2002) Progress in microarray technologies may soon lead to their application in clinical practice. These high-throughput technologies are expected to have a major impact on molecular pathology and improve disease diagnosis.(Amalia and Klinieken, 2007).

2.4 Pollen allergy diagnosis methods

a) Physical examination

Physical examination of the patient signs can be analyzed. Physical investigations are done by a clinician. However physical examination on its own cannot lead to a conclusion on a certain type of allergy.

b) Skin Prick testing

Skin allergy testing or Skin prick test is a method for medical diagnosis of allergies that attempts to provoke a small, controlled, allergic response. In the prick, scratch and scrape tests, a few drops of the purified allergen are gently pricked on to the skin surface, usually the forearm. This test is usually done in order to identify allergies to pet dander, dust, pollen, foods or dust mites. Intradermal injections are done by injecting a small amount of allergen just beneath the skin surface. The test is done to assess allergies to drugs like penicillin or bee venom(Berger,2002).

To ensure that the skin is reacting in the way it is supposed to, all skin allergy tests are also performed with proven allergens like histamine or glycerin. The majority of people do react to histamine and do not react to glycerin. If the skin does not react appropriately to these allergens

then it most likely will not react to the other allergens. These results are interpreted as false negatives.

d) Laboratory diagnosis

i)Western Blot

The Western blot is commonly used to identify, quantify, and determine the size of specific proteins. Western blotting evolved from Southern blotting, which is used to detect specific DNA sequences among DNA fragments separated by gel electrophoresis, and northern blotting, which is used to detect and quantify RNA and to determine its size, and also involves gel electrophoresis to separate RNA. In the late 1970s, (Towbin *et al.*,1979) enabled proteins to be electrophoretically separated using polyacrylamide–urea gels and transferred onto a nitrocellulose membrane. Burnette (1981) later employed the more widely used sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE), which eventually led to this method being termed western blotting. It is also called protein blotting or immunoblotting and has rapidly become a powerful tool for studying proteins. Basically, gel electrophoresis is used to separate native or denatured proteins. The proteins are then transferred to a membrane for detection using antibodies specific to the target protein. The technique has continued to evolve, and there are many reports on troubleshooting and improving the technique (Kurien and Scofield, 2009).

ii)ELISA

Over the past decades, the use of IgE testing to determine pollen allergies has been practiced. There is good reason for this. Today, a number of clinical practices regularly diagnose pollen allergies using IgE testing offered by diagnostic labs under a variety of trademarks. Recently drugstore pharmacies have joined the fray, offering in-store blood draws with follow-up test results.

A few studies have shown a possible correlation between self-reported pollen allergy and IgE testing with ELISA. The western blot assay is the most frequent used technique to detect IgE against inhalant allergens.

iii) Micro arrays

Microarrays have been a significant advance because of their small size. They are therefore useful to survey a large number of genes quickly or when the study sample is small. Microarrays may also be used to assay gene expression within a single sample and to compare gene expression in various cell types and tissue samples, for example in healthy versus diseased tissue. A microarray can be used to examine the expression of hundreds or thousands of genes at once and because of this it promises to revolutionize the way gene expression is routinely examined (Akhtar, 2009).

2.5 Pollen and food cross reactivity

Cross-reactivity occurs when an adaptive immune response to a particular antigen causes reactivity to other antigens that are structurally related to the inducer. CR represents an advantage in defense against infections, but has negative effects in some immune disorders, the most notable of which are autoimmune diseases and allergic disorders, in which both diagnosis and treatment can be affected. The antigen–antibody reaction is based on the spatial complementarity of the epitope with the idio type. Epitopes, which are made up of fragments of 5-7 amino acids, may be linear or conformational, although the latter are more frequent and variable. (Agarwal *et al.*, 2004). The concept of homology is based on the fact that the similarity in the sequences observed between molecules arises from their common origin. They therefore share the same function, and so must conserve the same overall folding. Consequently, aspects that are essential for the stability of the core (double-stranded β helices, α helices, β -pleated sheets, and disulphide bridges) must be conserved, and this is possible with 35% sequence similarity. In contrast, the external loops that are more exposed and house most immunoglobulin (Ig) E epitopes are more open to evolutionary changes (Cromwell *et al.*, 2011).

Up to 70% of patients allergic to pollen from birch and other females show symptoms of allergy to plant foods. Although the list of foods described is considerably long, those most frequently involved are the Rosaceae (especially apple), nuts (mainly hazel nut), and vegetables from the Apiaceae family (mainly celery and carrot). Pollinosis precedes the symptoms induced by the foods. These tend to be slight, characteristically OAS, and occur following ingestion of the raw food. The main culprit allergen, which is involved in more than 90% of patients with allergy to plant foods associated with allergy to birch pollen, is Bet v 1, a PR-10, which gives rise to CR

with its homologs in these foods. Less than 25% of patients with this syndrome are sensitized to Bet v 2 (birch profilin), although its contribution to symptoms remains unclear (Ausse *et al.*, 1992).

Characterization of various allergens marked a significant milestone in the field of pollen allergy. The quality of the isolated allergen extracts has since improved over the past two decades due to improved characterization of the allergens. However, products derived from natural sources are usually diverse. Some products contain nonallergenic molecules, which may vary in their arrangement and quantity (Bonds *et al.*, 2008).

Pollen-Food Allergy (also known as Oral Allergy Syndrome or OAS) results from a cross-reactivity reaction between allergy antibodies that your body raises against pollen proteins, with similar proteins found in other (edible) parts of plants. Common OAS symptoms include itchiness of the mouth and throat associated with mild swelling immediately after eating the fresh fruits or vegetables to which you have become sensitive. If you are allergic to ragweed pollen, you might experience these symptoms when consuming banana, cucumber, melon, zucchini/courgette, sunflower seeds, chamomile tea or Echinacea due to cross-reactivity.

OAS is common in people with birch tree pollen allergies. Foods that can trigger a reaction in people with this allergy are: peach, apple, pear, cherry, carrot, hazelnut, kiwi or almonds.

2.6 Rationale

A significant number of patients in Zimbabwe show allergic symptoms caused by grass and tree pollen. The prevalence of pollen allergy is increasing. This could be due to the use of European diagnosis kits (EUROIMMUN) to diagnose locally relevant allergies. There is need to characterize grass and pollen from Zimbabwe to confirm reactivity and in turn produce diagnostic kits that are relevant to the local population. Confirmation of the existence of cross-reactivity between different antigenic sources facilitates the use of monovalent vaccines, which are easier to standardize and at the same time prevent further sensitization of patients and reduce adverse reactions. Cross reactivities are occurring between pollen and food allergen sources. There is need to study cross reactivity patterns so as to explain some sensitization patterns and improve immunotherapy hence reducing the burden of pollen allergy in Zimbabwe.

Cloning of recombinant allergens has helped in the identification and confirmation of homologous proteins and has clarified their functions. It has been suggested that protein content, or molecular classification, is a superior way to address cross-reactivity issues rather than botanical taxonomy (Mothes *et al.*, 2004)

The characterization of many important allergens marked an important milestone in the field of allergy. The quality of natural allergen extracts has improved over the past two decades with increased standardization and characterization. However, products from natural sources are often heterogeneous and may contain many nonallergenic molecules, which can vary in composition and quantity.

2.7 Research question

What is the burden of pollen allergy in Zimbabwe and the specific proteins associated with the common conditions presented by patients?

2.8 Main objective

To determine the burden of pollen allergy in Zimbabwe and characterize grass pollen allergens

2.9 Specific objectives

- a) To determine the prevalence of pollen allergy in Zimbabwe
- b) To determine the demographic characteristics of affected patients
- c) To establish the common conditions presented

d) To identify the specific reactive proteins from grass pollen

CHAPTER 3

3.0 MATERIALS AND METHODS

3.1 Ethics

Ethical approval to conduct this study was sought from the Medical Research Council of Zimbabwe (MRCZ/B/1495). The patients involved in this study had given consent. In situations where children could not give consent, the parents and guardians gave consent on their behalf. Patients with suspected pollen allergies consented to the use of their serum samples. Patients were assured of confidentiality, unique identification of samples and sample disposal after use consent on behalf of the children. The patients' names were not used. Instead, laboratory numbers were allocated for identification.

3.2 Study area

The study was carried out using serum samples of people suspected to have pollen allergies. The people involved in this study attended the Asthma, Allergy and Immune Dysfunction clinic (AAIC), Harare, Zimbabwe. Convenient selection of the study was done because of the continuous increase in pollen allergy cases in Zimbabwe.

3.3 Sampling

Sample size calculation

The sample size for this study was calculated using the following formula

The total number of participants required n to determine the prevalence of pollen allergy at 95% confidence level is given by:

$$n = \frac{z^2 p(1-p)}{\Delta^2} = 385$$

Where n -sample size

Z -1.96 standard score value from the normal distribution at 95% confidence interval

p -0.5 standard value used for an unknown prevalence of pollen allergy

Δ -0.05 precision of the estimated prevalence

$$n = (1.96/0.05)^2 \cdot 0.5(1-0.5) = 38$$

Sampling frame

Random sampling was done on 2576 serum samples of patients referred to the Asthma Allergy and Immune Dysfunction clinic from 2010 to 2017

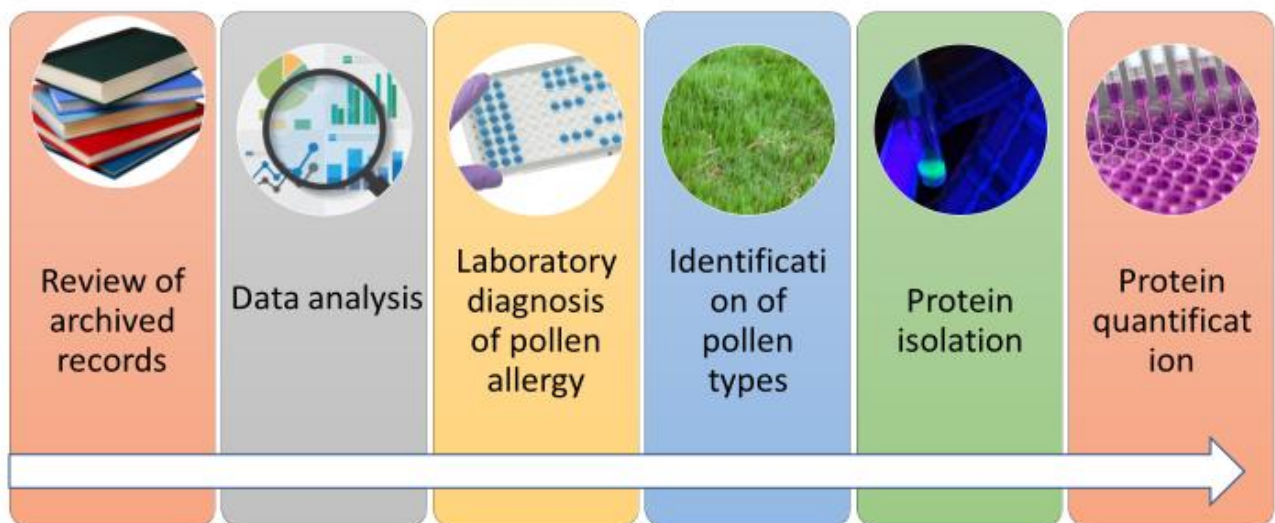
Study population

The study population were patients with suspected pollen allergies

Sampling unit

The sampling unit comprised of suspected pollen allergies serum samples

The procedures that were followed throughout the study



are summarized below:

Figure 3: Summary of all the methods used throughout the study. The steps included the review of archived records, data analysis, laboratory detection of IgE, pollen processing, pollen isolation and protein quantification and separation.

3.4 Review of archived records

The clinical records of 2735 patients referred to the Asthma, Allergy and Immune Dysfunction clinic were reviewed. The data of these patients was entered on a worksheet. The variables that were recorded were:

- a) Patient age
- b) Patient laboratory number
- c) Patient gender
- d) Symptoms presented by a patient upon diagnosis
- e) Date of sampling

3.5 Data analysis

After completing data compilation on a worksheet, data analysis was done using the Statistical Package for Social Sciences (SPSS). SPSS is a software package that is used for interactive, batched and statistical data. The overall inhalant sensitization pattern, pollen sensitization pattern, sensitization patterns by age group and the cross-reactivity patterns between foods and pollen allergens were analyzed.

3.6 Laboratory detection of IgE against pollen allergens

Line Blot assay

A Euro line Mediterranean inhalation assay was used to detect allergen specific IgE in serum against pollen allergens. Serum samples (55) obtained from walk in patients were analyzed by the Line Blot assay. The test is a multiparameter assay containing optimized combinations of relevant allergens enabling the simultaneous analysis of IgE against different allergens.

Reagent preparation and treatment

All reagents were brought to room temperature before use. The universal buffer was prepared by a dilution of 1:10 with distilled water. Due to the special membrane used for the present EUROLINE, the universal buffer was used to dilute patient samples and to wash the test strips. The reagents were stored at 4°C.

Pre-treatment

Test strips were placed in an incubation tray and each channel was filled with 1.0ml working strength universal buffer. The test strips were incubated for 5 minutes at room temperature. The fluid was aspirated after incubation.

Sample incubation

Each channel was filled with 400µl of undiluted sample followed by 60-minute incubation at room temperature on a shaking incubator. The liquid was then aspirated off and washing was

done 3x5 minutes with 1.0 ml working strength universal buffer on a shaker. After washing, 1.0 ml of the enzyme conjugate (alkaline phosphatase -conjugated anti human IgE) was pipetted into each channel followed by a 60-minute incubation at room temperature on a rocking shaker. After incubation, the liquid was aspirated and washing was done 3x5 minutes. After washing, a substrate was added to each channel followed by a 10-minute incubation. This step was followed by 3x1 minute washing. The test strip was air dried.

Interpretation of results

After stopping the reaction using distilled water, the incubated test strips were placed onto the adhesive foil of the green work protocol created in the EURO Line Scan program.

Euro line Scan

The program Euro line was used to quantify the final incubated strips. The final strips were scanned by an automated scanner. After scanning, scanned data was uploaded into a Euro line scan software. This software follows a number of commands and produces a soft copy result.

The position of the test strips was corrected while they were wet and after drying, the strips were struck to the adhesive foil.

The diagnosis concentration grades were divided into the following concentrations:

Table 1: Concentration grades used to diagnose allergen specific IgE levels in serum

Class	Concentration(kU/l)	Result
0	<0.35	No specific antibodies detected
1	$0.35 \leq \text{sIgE} < 0.7$	Very low antibody titer, frequently no clinical symptoms where sensitization is present
2	$0.7 \leq \text{sIgE} < 3.5$	Low antibody titer, clinical symptoms usually present
3	$3.5 \leq \text{sIgE} < 17.5$	Significant antibody titer, clinical symptoms usually present
4	$17.5 \leq \text{sIgE} < 50$	High antibody titer, almost always with clinical symptoms
5	$50.0 \leq \text{sIgE} < 100.0$	Very high antibody titer
6	≥ 100	Very high antibody titer

Twenty allergens were detected by the Line Blot. The following table shows the allergens detected and the respective name of allergen:

Table 2: Position of allergen and allergen name detected by the Line Blot assay

Position	Allergen code	Allergen name
1	g2	Bermuda grass
2	g6	Timothy grass
3	t3	Birch
4	t4	Hazel
5	t9	Olive
6	t11	Plane tree
7	t23	Cypress
8	t210	Privet
9	w1	Common ragweed
10	w6	Mugwort
11	w9	English plantain
12	w19	Wall pellitory
13	d1	Der.pteronyssinus
14	d2	Der.farinae
15	d70	Acarus siro
16	e1	Cat
17	e2	Dog
18	e3	Horse
19	m2	Cladosporium
20	m6	Alternaria alternate
21	CCD	CCD
a	Ind	Indicator band

Waste disposal

Patient samples and incubated test strips were handled as infectious waste and they were disposed in a biohazardous bag for incineration.

3.7 Pollen processing

3.7.1 Collection and storage

Pollen was collected from ten different grasses during the grass blooming season. The grasses were blooming. Pollen was collected from Insiza, Chimanimamni, Arundel and Karoi. Tweezers and scissors were used to cut the grass flowers. The pollen was dried and stored in micro centrifuge tubes set up in a tray.



Figure 4: Protein extracts stored in centrifuge tubes set up in a tray

3.7.2 Protein extraction

Alkaline lysis

Protein extraction was done using the alkaline lysis method. Firstly, 5 grams of pollen were ground. HEPES buffer(5mls) was added to the ground pollen. The mixture was vortexed at the

maximum speed for 5 minutes. After vortexing, the sample was centrifuged at 4°C for 20 minutes. A speed of 10000 rpm was used. After centrifugation, the supernatant was discarded. The debris was reground and the same volume of HEPES buffer was added. Centrifugation was done at the same speed for one hour at 4°C. The sample was then placed on ice for one hour followed by the final centrifugation at 4°C at 18000 rpm. After final centrifugation, the supernatant was collected and stored in a 4°C freezer.

3.7.3 Protein quantification

3.8 BCA assay

A working standard of 1mg/ml bovine serum albumin solution was used to prepare ten different concentrations of BSA ranging from 0 to 5mg/ml. The BCA reagent and protein were added to each tube. The plates were incubated at room temperature for 30min on a float in a water bath. After incubation, the tubes were placed on ice to cool them and to stop the reactions that were taking place. Absorbance values were read off at 562nm.

Table 3: BCA standards prepared using bovine serum albumin (BSA).

The BCA assay was used to determine the concentration of protein in the crude sample. Bovine serum albumin was used as the standard. Absorbance was measured at 562nm. The values recorded showed direct proportionality between the concentration of BSA and absorbance. As the BSA concentration increased, absorbance also increased.

1mg/ml standard(μl)	H ₂ O(μl)	Micro-BCA solution(μl)	BSA total(μg)	Average A ₅₆₂
0	500	500	0	0.150
0.5	499.5	500	0.5	0.168
2.5	497.5	500	2.5	0.423
5.0	495	500	5	0.772
10	490	500	10	1.219
15	485	500	15	1.680
20	480	500	20	1.963

$$\text{Equation } [\mu\text{g protein}] = [\text{extract } A_{562}/\text{BSA standard } A_{562}] \times \text{protein } A_{562}$$

Table 4: Protein concentrations derived from the standard curve

The BCA assay was used to determine the concentration of protein in the crude sample. Absorbance was measured at 562nm. The values recorded showed direct proportionality between the concentration of the protein and absorbance. As the protein concentration increased, absorbance also increased.

Unknown(μl)	Average A ₅₆₂	Protein(μg)
1	0.101	-0.979
2	0.102	-0.969
3	0.105	-0.938
4	0.258	0.667
5	0.389	2.042
6	0.458	2.766
7	0.467	2.861
8	0.568	3.920
9	0.684	5.137
10	0.798	6.333

3.6 SDS

A solution of Acrylamide was prepared by mixing 30 g of Acrylamide with 8g of bis acrylamide in 100ml of distilled water. The mixture was stored at 4°C. A separating gel was prepared by adding, acrylamide (2.00 ml), Tris pH 8.8 (2.24 ml prepared by mixing Tris 6.05 g, HCl 0.94 ml, distilled water 49.96 ml) Distilled Water (1.74 ml), 10% SDS (30.0 μl, prepared by mixing Sodium dodecyl sulphate (SDS) 2.00 g and distilled water 20.0 ml and can be stored at room temperature), Tetramethylethylenediamine (TEMED) (4.00 μl) and 10% ammonium persulphate (20.0 μl which was made by adding ammonium persulphate 0.10 g and Distilled Water 1.0 ml). TEMED was added last. After casting the gel butanol was poured on top of the gel surface to

avoid gel evaporation. The gel was left to solidify for two hours at room temperature. After the gel had set, the butanol was poured off and the gel was rinsed with distilled water.

A stacking gel was made by adding 0.835ml of Acrylamide with 0.625 ml of Tris pH 8.8, 3.515 ml of Distilled Water, 50.0 μ l of 10% SDS and 5.00 μ l of Tetramethylethylenediamine (TEMED) with 25.0 μ l of 10% ammonium persulphate. TEMED was added last. The stacking gel was cast on top of the resolution gel. A comb was inserted on the stacking gel. The stacking gel was left for an hour to solidify at room temperature. The gel cassette was then removed from the casting frame and was placed into the electrode assembly with the short plate facing the same direction. The comb was then gently removed.

The protein samples containing the sample buffer were loaded in the wells. The gel was run at 120 volts for 45 minutes. The gel was then stained using (Coomassie brilliant blue 0.25 g, Methanol 112.5 ml, DDW 112.5 ml, Glacial acetic acid 25.0 ml) for two hours. Destaining was done overnight. After destaining the gel, the bands were viewed under UV.

CHAPTER 4

4.0 RESULTS

4.1 Preliminary study carried out in Zimbabwe at the Asthma Allergy and Immune Dysfunction clinic

Clinical and laboratory data was collected from patients who had attended the Asthma, Allergy and Immune Dysfunction clinic from 2010 to 2017. There is one allergy specialist clinic in Zimbabwe, Harare and hence data reported is representative of Zimbabwean statistics. The patients attending this clinic had been offered clinical consultation by an allergy specialist, skin prick testing and had blood drawn for the determination of allergen specific IgE antibodies to inhaled allergen sources. For the purpose of this study, only those who were exclusively sensitized to pollen were included. Immunoblotting was carried out for specific allergen detection.

4.1.1 Demographics

The frequency of sensitization to common environmental allergen sources was determined by skin prick testing in 2735 patients. Amongst these 1095 (40%) patients were males and 1640 (60%) were females. The proportion of patients reporting to the clinic for allergy profiling decreased with a decrease in age.

The patients (2735), included in this study were born between and 1931 and 2017. The trend observed showed an increase in the number of young patients affected by allergy. The inhalant-allergen sources causing sensitization in patients born between 1931 and 2017 for the respective groups are summarized.

Table 5: Demographic characteristics of study population analyzed between 2010 and 2017

Variable		Frequency	Proportion
Gender	Male	1095	0.4
	Female	1640	0.6
Age groups			
	0-9	506	18.04
	10-19	390	13.9
	20-29	362	12.91
	30-39	551	19.64
	40-49	443	15.79
	50-59	206	7.34
	60 ⁺	122	4.73

4.1.2 Sensitization patterns

The general sensitization pattern of patients to inhalants was analyzed before analyzing the pollen sensitization pattern. Among the analyzed inhalant allergens were house dust mites *Dermatophagoides pteronyssinus* (Dp) and *D. farina* (Df), Grass mix, cat, dog and horse hair and molds *Cladosporium herbarum*, *Alternaria alternata* and *Aspergillus fumigatus*.

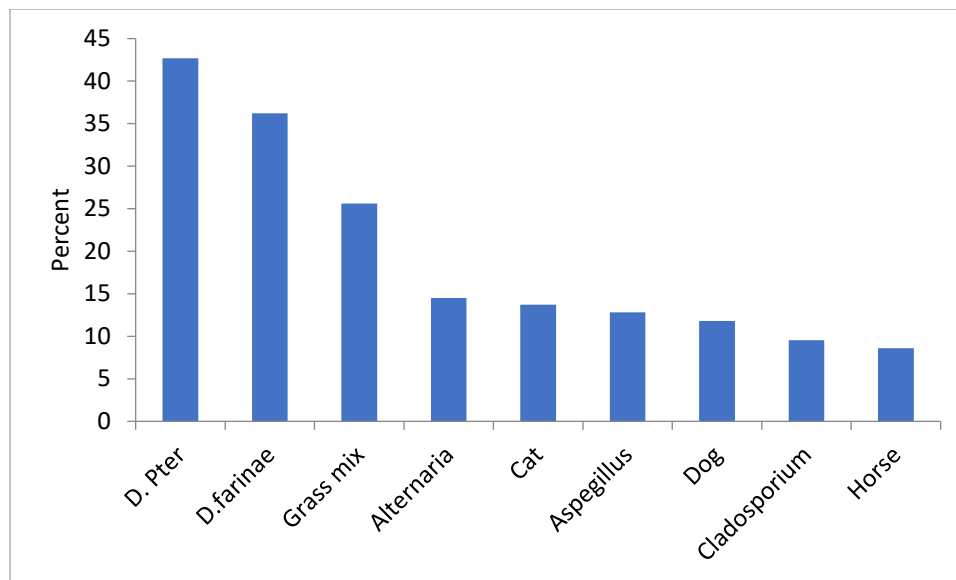


Figure 5: Overall sensitization pattern to various inhalant allergen sources. The most frequent observed source of sensitization to inhalant allergen sources was house dust mites. Sensitization to grass mix was seen to be the third commonest after the house dust mites Df and Dp.

After the analysis of the overall sensitization patterns to various inhalant allergens, the sensitization pattern of pollen allergens was done. The pollen allergens analyzed were Bermuda grass, Birch and Mugwort.

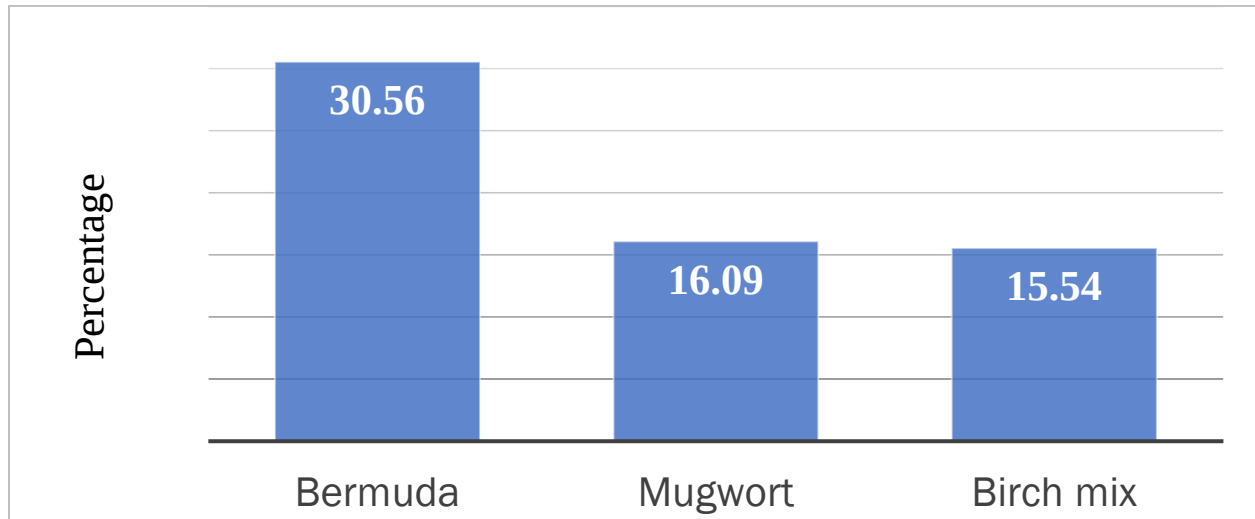


Figure 6: Sensitization pattern to pollen allergens. Most people (30.56%) were sensitized to Bermuda grass. A moderate proportion (16.09%) was sensitized mugwort and the least percentage (15.54%) was sensitized to birch mix.

Sensitization patterns were analyzed for the ages 0-49

Table 6: Sensitization of three examples of pollen allergy in different groups.

Age group		Grass mix	Birch	Mugwort
0-9 years	n= (500)	26.1	15.7	17.3
10-19years	n= (384)	37.6	23.7	24.5
20-29years	n= (362)	28.3	16.4	17.2
30-39years	n= (557)	24.7	16	19.5
40-49years	n= (441)	13.9	10.3	11.6

Percentages of all age groups with serological evidence of allergy

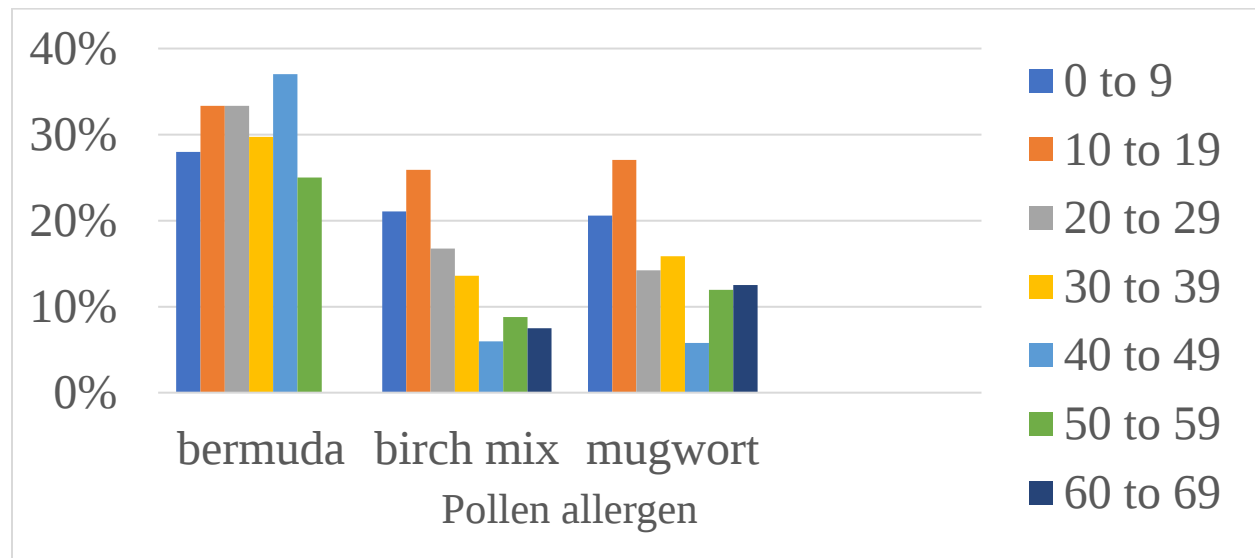


Figure 7: Proportions of various age groups sensitized to a specific pollen allergen. The rates of sensitization generally showed a decrease with an increase in age.

Further analysis of the age group distribution and comparison with the second and third commonest inhalant allergens (Df and Dp)

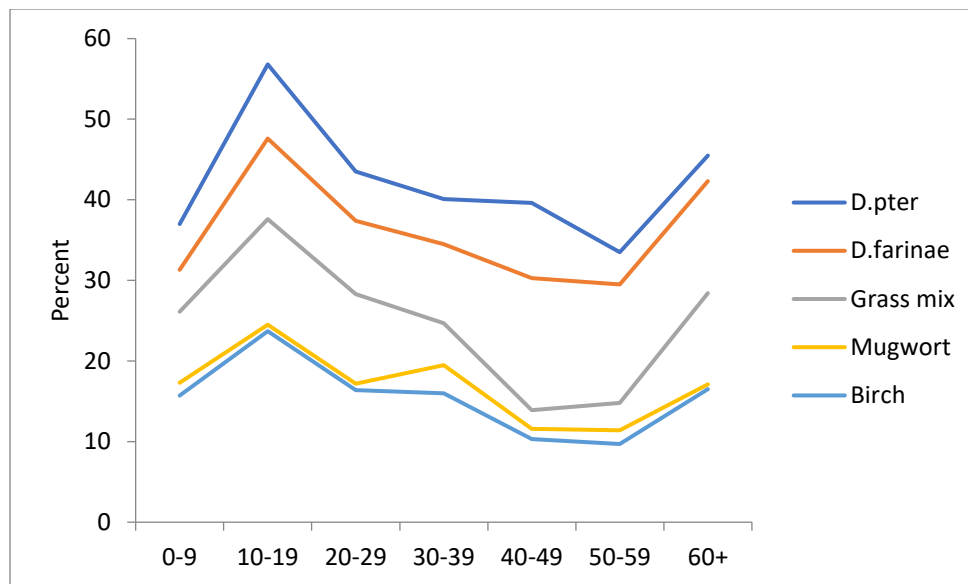


Figure 8: Number of patients (%) with any inhaled allergy plotted against the year of birth (n=2735). An exponential increase in allergen sensitization was observed in patients with age 39 and below.

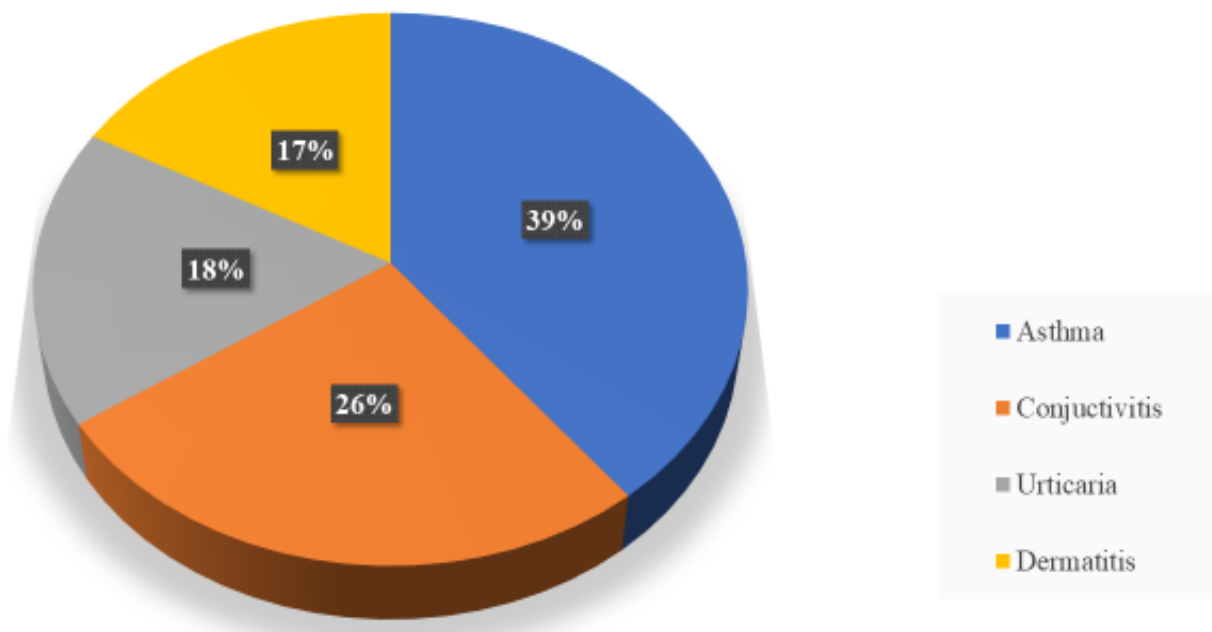


Figure 9: Various symptoms were presented by pollen positive patients. The symptoms manifested by patients presenting with pollen allergy were asthma (39%), conjunctivitis (26%), urticarial (18%) and dermatitis (17%).

4.1.3 Pollen and food cross reactivity

Simultaneous allergy to foods and pollen types is shown in the table below: In Zimbabwe cross reactivity have been observed for various pollen types and foods. However, not much is known about the symptoms manifested by people experiencing the cross reactivities shown in Table 7.

Table 7: Cross reactivity of common foods and pollen allergens in Zimbabwe

TYPE OF POLLEN ALLERGY						
FOOD ALLERGY	Bermuda	Timothy	Olive	Cypress	Plane	Privet
Egg yolk		X			X	X
Beta lactoglobulin		X				X
Peanut	X	X				
Crab	X					
Apple			X		X	
Apricot			X			
Tomato			X		X	
Carrot			X			
Cod fish				X		

Pollen from 10 different grasses was collected. Five of the grasses were identified. These were Bermuda grass (*Cyanidin dactylon*), Beard grass (*Bothriochloa insculpta*), Bahia grass (*Paspalum notatum*), Rhodes grass (*Chloris virgata*) and Rye grass(*Lolium*). Below is a photographic presentation of the grasses from which pollen was obtained.

4.2 Line Blot assay

A line Blot assay was carried out on 55 serum samples. The final incubation results were scanned and uploaded in a Euro line Scan program for evaluation. The results obtained for 10 of the 55 samples are shown below:

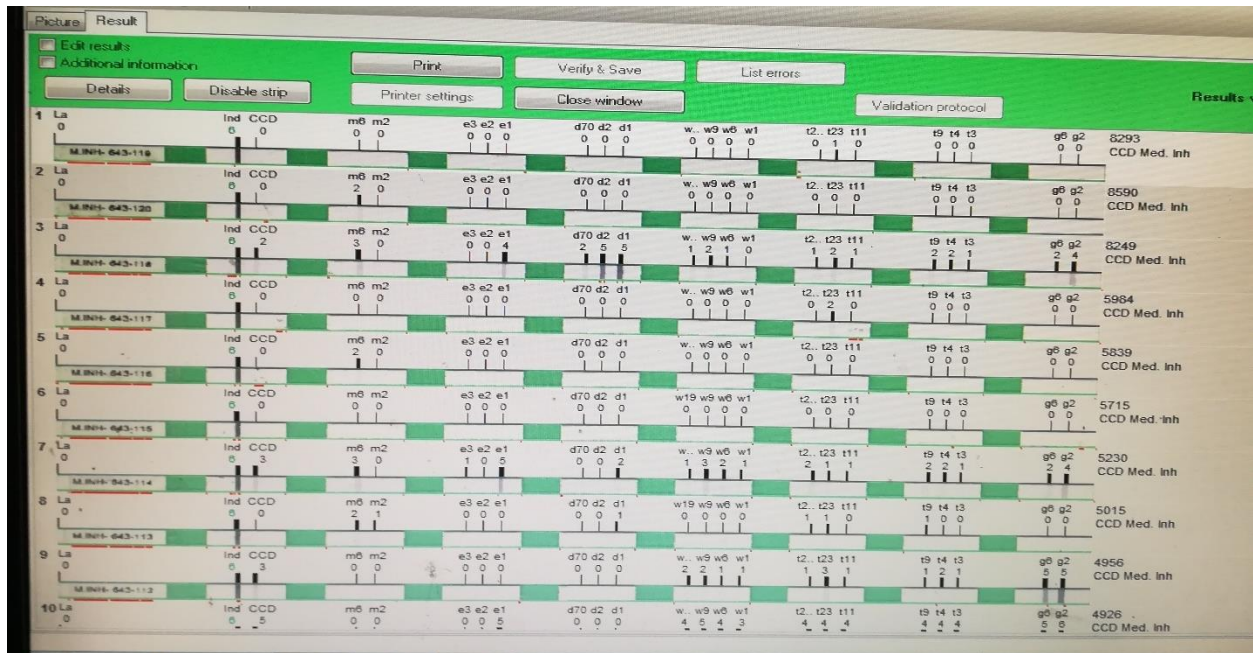


Figure 10: Results obtained from the laboratory detection of IgE against pollen allergens. The bands are representative of antibody and allergen interaction between IgE and various allergens. The list of inhalants is listed in Table 2.

4.3 Pollen collection



Beard



Bermuda



Bahia



Rhodes



Rye

Figure 11: Local grasses from which pollen was obtained.

4.4 BCA assay

A standard curve was plotted using various BSA standards prepared. The standard curve was used to extrapolate the concentrations of the unknown extracted proteins

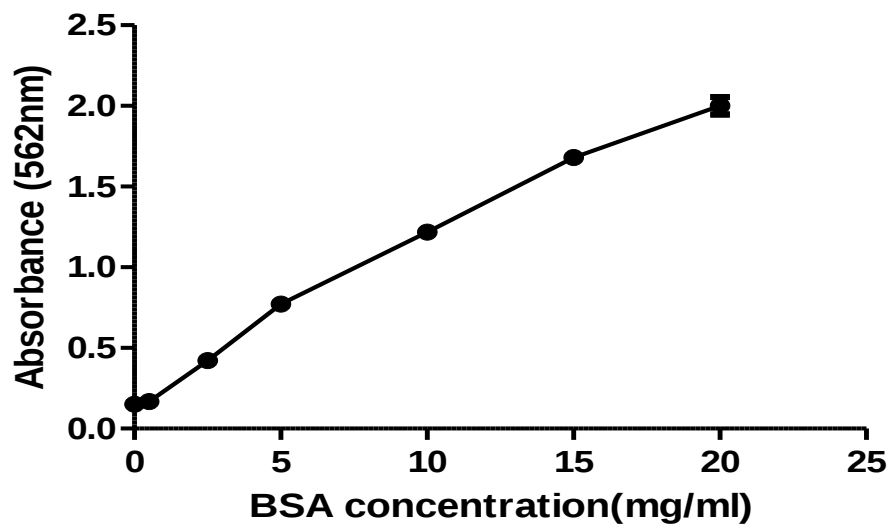


Figure 12: A micro-BCA assay standard curve with triplicate BSA standard points of 0, 0.5, 2.5, 5, 10, 15 and 20 μ l. The data table used to generate the figure and depiction of a typical micro-BCA assay is shown in Table 3.

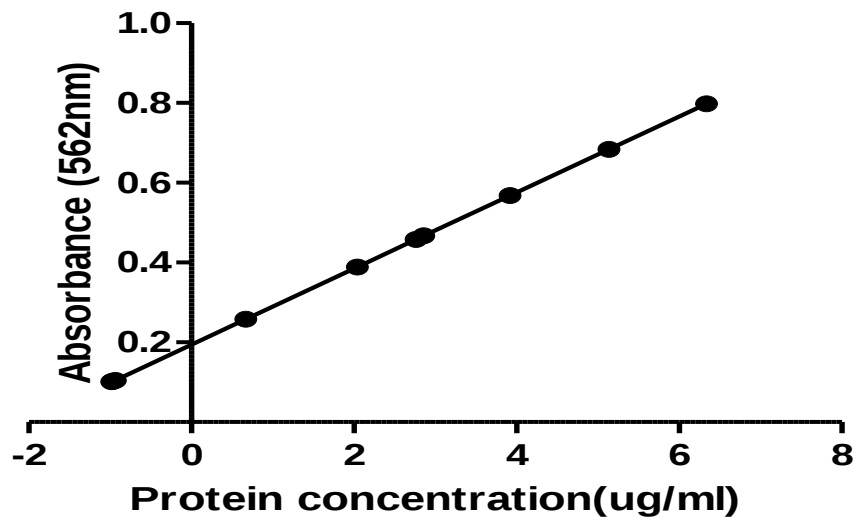


Figure 13: Plot of absorbance against protein concentration of the pollen proteins mentioned above. The protein concentrations increased with an increase in absorbance. Negative protein concentrations were observed. The concentrations ranged from -0.12 to 7ug/ml.

4.5 SDS PAGE

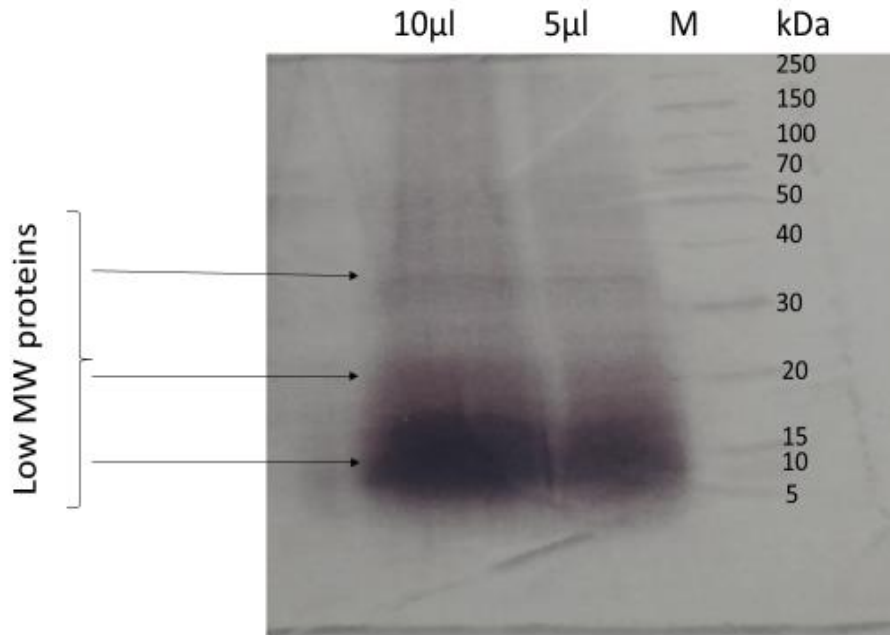


Figure 14: Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE). The gel was stained with Coomassie Blue molecular weight standards. Representative Lane I:10µl Bermuda grass proteins, Lane II:5µl Bermuda grass proteins, Lane III: unstained 250kDa protein marker. MW: molecular weight of extracted proteins ranged from 5 -30 kDa

CHAPTER 5

5.0 DISCUSSION

5.1 Review of archived records

The review of archived records was done so as to give an insight of the prevalence of pollen allergy between 2010 and 2017. The review also gave a direction and highlighted the main focal points for the prospective study. The retrospective study section summarizes the results of an audit review of the profile of allergic sensitization in 2735 patients who attended the AAIC, a specialist referral center in Harare, Zimbabwe. There is one allergy specialist clinic in Zimbabwe therefore an assumption was made that the patients attending the clinic were representative of the whole population. The allergen sensitization patterns are therefore a reasonable representation of allergen sources and should provide a credible panorama of sensitization patterns in Zimbabwe. These patients were clinically evaluated and offered skin-prick tests. Laboratory testing was also carried out if required. Medical practitioners in private practice primarily referred all the patients to the AAIC. The need for referral suggests that the symptoms could have been more severe than can be managed at the level of general practitioners and other physicians.

The demographic distribution shows that the allergy burden affects most females. The distribution also shows that young adults and the middle-aged group are more affected by inhalant allergy. The elderly was less affected by inhalant allergens. An exponential increase in allergen sensitization was observed in patients aged 30-39. This age group consists of the majority of the working class hence this group could be more exposed to outdoor allergens. The number of people with allergic diseases doubled each decade from approximately 2% in 1980, 4% in 1990, 6% in 2000 to approximately 8% in 2010 (Sibanda, 2013). According to results shown in Fig 5, the burden of pollen allergy continues to rise.

Allergy to pollen allergens manifested as respiratory allergy. The conditions manifested were asthma, conjunctivitis, urticaria and dermatitis. Asthma symptoms are usually confused with rhinitis symptoms. Some studies have shown that these conditions usually have the same symptoms hence leading to misdiagnosis. However, some clinicians refer to these symptoms as the obstructive airway disease.

The predominant inhalant allergens were house-dust mites. Although sensitivity to both *D. pteronyssinus* and *D. farinae* were detected, the former predominated. The third commonest

allergen source after the house dust mites was pollen. Sensitization to grass pollen was more prevalent than that to tree or weed pollen. This pattern of sensitization and the corresponding disease severity is similar to what was observed and reported by Ordman¹ in 1972. Presentation with grass pollen sensitization peaked during the last half of the rainy season (February to April). However cross reactivity between pollen and molds (*Cladosporium*, *Aspergillus* and *Alternaria*) could also cause asthma related symptoms hence associated conditions may be reported.

The largest proportion of patients was sensitized to Bermuda grass. The high sensitization levels could be linked to the fact that Bermuda grass is very common and is used as a lawn in most households. Its ability to grow in extreme climate conditions because of its extensive root system makes it easy to maintain. Bermuda grass also has a good tolerance capacity against insects and diseases.

Food and pollen cross reactivity patterns showed that olives are more cross reactive with dicotyledons such as tomatoes. The cross reactivity could have come up due to molecular mimicry. Molecular mimicry is the possibility that sequence similarities between foreign peptides are in sufficient levels to result in the cross-activation of autoreactive T or B cells by pathogen-derived peptides. Cypress and Bermuda grass had the least cross reactivity with food allergens.

Patients sensitized to mugwort and peach usually develop clinical symptoms such as pruritus, erythema, and angioedema following ingestion of mustard. Additionally, the mugwort-peanut sensitization syndrome is known to occasionally cause severe systemic reactions, including anaphylaxis in Europe. Kaila and Isolauri demonstrated that 97% of 38 patients with mustard allergy have at some stage had been sensitized to mugwort pollen and that all of these patients were allergic to other foods belonging to the Brassicaceae family, such as cauliflower and cabbage (Kaila and Isolauri, 1997).

In a study by Yuri *et al*, it was observed that mugwort allergens are thermostable and are also resistant to peptic digestion. These allergens are believed to cause oral allergy syndrome and severe systemic reactions.

However, some allergens could not be readily detected. The test panel which was used is specific for certain standard allergens. It does not permit selective testing of some clinically suspected

allergen sources. Considering that most patients included in the study were referral patients, there is a possibility that some patients could not afford private health care even if they may have suffered more severe conditions than those of people attending the clinic.

5.2 Line Blot assay

The Line blot uses the principle of antigen and antibody interaction. In this case, the interaction of IgE and the pollen allergens led to the production of bands on various positions depending on the position of the standard allergen. The enzyme conjugate used was alkaline phosphatase-labelled anti-human IgE(mouse). Nitro blue tetrazolium chloride was used as the substrate solution. Functioned by Alkaline phosphatase (AP) catalyzation, a colorless substrate BCIP is converted to a blue product. In the presence of H₂O₂, 3-amino-9- ethyl carbazole and 4-chlorine naphthol will be oxidized into brown substance and blue product respectively under the catalyzation of HRP. Methods such as enhanced chemiluminescence employs HRP detection and can be used as an alternative method. Using HRP as the enzyme label, luminescent substance luminol will be oxidized by H₂O₂ and will luminesce. Moreover, enhancers in this substrate will enable a 1000-fold increase in light intensity. HRP will be detected when the blot is sensitized on photographic film (Kurian and Scofield, 2009).

Out of the 55 samples analyzed, bands were observed on position g2 followed by position 10 and position 3. The bands were representative of a Bermuda grass allergen, Mugwort allergen and Birch allergen respectively. Unexpected bands were observed in Fig 10 row 6 and 7. The Line Blot assay can be affected by various problems leading to a distortion or unexpected results. One of the problems is observing unusual or unexpected bands. These bands could have arisen due to degradation of proteases leading to a production of bands at unexpected positions. In order to overcome this challenge, fresh enzyme solutions could be used. Alternatively, altering the antibody could help. Reheating the serum samples can aid in breaking the protein quaternary structure.

Unclear bands were dominant. This could be due to air bubbles encountered during incubation. A universal buffer of concentration (1:10) was used. To improve band intensity increasing the concentration of the buffer can improve the incubation efficiency hence improving band intensity. White bands could have been due to too much antibody used.

No bands were observed in row 2. Not observing any bands could be due to no allergen-antibody interaction or due to problems related to either the serum or the buffer used. Considering that in the case of a Line blot, antigens are embedded in the test strip, they may not have much influence in this result. If the concentration of the antibody in the serum is low, bands may not be observed. Prolonged washing may also lead to a decrease in the signal hence leading to invisible bands. Contamination of buffers by substances such as sodium azide may lead to distortion of results. Sodium azide is an inorganic compound. When mixed with water or an acid changes into a toxic gas.

High background could have been caused by the buffers, which may have been too old. In this case, increasing washing time can decrease background noise. To achieve an optimum washing rate, various exposure times can be analyzed. Due to similar allergen properties, cross reactivity can occur between allergens. This will cause identical epitopes of homologous protein allergens to attach to a patient's specific IgE antibodies developed.

Allergy diagnosis using the Line Blot assay is effective. However, final diagnosis should not only be based on this diagnosis. Other diagnostic tests such as skin prick testing and the diagnosis of clinical symptoms presented can help in diagnosis. Provocation tests are important.

Negative invitro results may occur when symptoms are not IgE mediated and when a serum sample was collected when before a certain organism was able to produce antibodies against the antigen. One of the reasons for negative invitro results could be the fact that the IgE concentrations reached a minimum a long time after sensitization.

Besides the Line blot assay, assays such as the ELISA can be used. ELISA (enzyme-linked immunosorbent assay) is a plate-based assay technique designed for detecting and quantifying peptides, proteins, antibodies and hormones. In an ELISA, an antigen must be immobilized to a solid surface and then complexed with an antibody that is linked to an enzyme. ELISA tests are more accurate. They are considered highly sensitive, specific and compare favorably with other methods used to detect substances in the body, such as radioimmune assay (RIA) tests.

Specificity of ELISA is because of the selectivity of the antibody or antigen (Bugajska *et al.*, 1998).

5.3 BCA assay

Accurate measurement of protein concentration is critical since the results are used in other calculations, such as determination of enzyme activity and SDS PAGE. Errors in protein concentration determination tend to amplify overall errors in these calculations.

The absorbance for several concentrations of BSA were measured in triplicate and averaged. Fig 12 shows that there is little variation between measurements at low standard concentrations with respect to the change in average absorbance observed from standard to standard. The data graphed in Figure 12 is not very linear. This could be due to the similarities in absorbance for the highest concentrations of BSA. These observations indicate that lowering the concentration range of the BSA used could lead to better linearity with the absorbance change.

Fig 13 shows a linear relationship between the protein concentrations and the absorbances recorded. Although most of protein concentrations seem reasonable with respect to the standard curve. Some concentration values are negative. Ideally, the concentration values of the samples to be determined should be within the range of the standards used to plot the standard curve. However, negative values could have arisen because of various reasons. The negative values could have come up because some of the concentrations of the protein samples were below those of the standards used. There could have been something in the protein extract that could have interfered with the assay reagent. The sample concentration could be too dilute such that the protein concentration measurement was distorted. A likely solution to the problem is to concentrate the sample or possibly use a larger volume of the sample in the assay.

Increasing the incubation time increases the sensitivity of the assay. Heating may be stopped earlier than mentioned above. This may prevent the color of the solution from becoming too dark. The BCA assay can be performed at room temperature, but there will be greater variability among proteins. There is no single protein assay method that yields absolutely accurate results. Each method has different advantages and limitation

The Bradford assay can alternatively be used to quantify proteins. The Bradford assay is a popular protein assay. It is simple, fast and inexpensive to execute. The Bradford assay is mainly based on the direct binding of Coomassie blue G-250 dye(CBBG) to proteins such as arginine, tyrosine, histidine and tryptophan. The anionic CBBG binds to arginine residues in an arginine

standard. This produces an absorbance maximum at 470 nm. The assay is usually monitored at 595nm using a spectrophotometer (Bradford *et al.*,1976).

5.4 SDS PAGE

The velocity of a charged particle moving in an electric field is directly proportional to the field strength and the charge on the molecule and is inversely proportional to the size of the molecule and the viscosity of the medium. To increase the resolution of protein separation during SDS-polyacrylamide gel electrophoresis, a discontinuous buffer system is often used. Two gel layers were prepared in the plate: a stacking and a separating gel. The stacking gel contains chloride ions, the leading ions, which migrate more quickly through the gel than the protein sample, while the electrophoresis buffer (Tris buffer) contains glycine ions, the trailing ions, which migrate more slowly. The protein molecules are trapped in a sharp band between these ions. As the protein enters the separating gel, which has a smaller pore size, a higher pH and a higher salt concentration, the glycine is ionized, the voltage gradient is dissipated and the protein is separated based on size.

TEMED was used to stabilize free radicals and to improve polymerization. The rate of polymerisation and the properties of the resulting gel generally depend on the concentrations of free radicals. Increasing the number of free radicals, results in a decrease in the average polymer chain length and in gel elasticity. But it increases gel turbidity. Ammonium persulfate was used as a source of free radicals and as an initiator for gel formation. Ammonium persulfate should be made up fresh or used from a relatively fresh stock (Zeng *et al.*, 2013). It goes bad after a week or two in the refrigerator. TEMED was stored in the refrigerator in glass bottles. It was added last since it causes the polymerization and solidifies polyacrylamide.

Coomassie Brilliant Blue was used to stain the gel. The excess dye incorporated into the gel was removed by destaining with the same solution without the dye. The proteins were detected as blue bands on a clear background. The Bermuda proteins separated were of low MW. The molecular weights varied from 5 to 30 kD. About three proteins of different sizes were separated. The band intensity increased with an increase in the amount of protein loaded on the gel. As SDS is also anionic, it may interfere with staining process. Therefore, large volume of staining solution is recommended, at least ten times the volume of the gel.

A similar technique to SDS-PAGE is Native-PAGE, whereby the separation occurs without denaturing the protein. Because the proteins are not denatured, they are less predictable in the way they move through the gel and take much longer. Different proteins also have different charges and different strengths of charges, so some proteins which may be larger although have a strong negative charge, may travel further than a smaller protein with a slightly positive charge. This can make Native-PAGE unhelpful when trying to differentiate proteins by their size. SDS technique overcomes this problem by imparting a negative charge on the protein relative to its size, giving almost all proteins uniform ratio of mass to charge. Proteins will then be separated incrementally according to their size and not its individual isoelectric point (Suvra and Vikash, 2012).

CHAPTER 6

6.0 CONCLUSION

Our review of 2735 patients showed sensitization to Bermuda grass, grass mix, birch mix and mugwort. The largest proportion of sensitization among pollen allergens was against Bermuda grass (30%).Mugwort and birch had 16% and 15% respectively. After house dust mites, *Dermatophagoides pteronyssinus* and *D. farina*, pollen was found to be the second commonest inhaled allergen source with grass mix as the most allergenic type. In Zimbabwe grasses are the most prevalent allergen sources unlike in the Great Basin (North America) where weeds are the most prevalent allergen sources. The various conditions manifested by allergy patients referred to the Asthma, Allergy and Immune Dysfunction clinic include asthma and rhinitis, conjunctivitis, urticarial and dermatitis. The percentages of sensitization observed were 43,34,16 and 17% respectively. Sensitization was seen to increase from 9-39 years. A sharp decrease was observed from 60 years and above. Bermuda grass contains low molecular weight proteins(5-30kDa).

6.1 Recommendations

Pollen allergies have been routinely investigated at the Asthma Allergy and Immune Dysfunction clinic. The panel of inhalants investigated consists of about 29 allergen sources. However, the shortcomings of the kits used is that they report grass mix and hence do not distinguish individual allergen sources. The relationship between sensitization and symptoms needs to be analyzed and documented. Pollen and food cross reactivities need to be proven biologically. A few reports have been documented on food allergy in Zimbabwe. To date, the most common food allergen is potato. However, in Zimbabwe, unlike in Europe and Australia, peanuts do not cause severe symptoms. There is need to analyze local food sensitization patterns in order to reduce cross reactivity.

There is also a need to carry out epitope mapping of local pollen allergen sources. This will aid in the manufacturing of locally relevant kits and in improving personalized immunotherapy. There is also need to train more allergologists and raise allergy awareness. Raising awareness of the economic and health impacts is an important factor in facilitating the early recognition and control of allergic disease.

CHAPTER 7

7.0 REFERENCES

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CHAPTER 8

8.0 APPENDICES

Appendix 1: BSA Standard curve

1mg/ml standard(μl)	H ₂ O(μl)	Micro-BCA solution(μl)	BSA total(μg)	A ₅₆₂
0	500	500	0	0.149
0	500	500	0	0.150
0	500	500	0	0.151
0.5	499.5	500	0.5	0.167
0.5	499.5	500	0.5	0.169
0.5	499.5	500	0.5	0.168
2.5	497.5	500	2.5	0.423
2.5	497.5	500	2.5	0.422
2.5	497.5	500	2.5	0.423
5	495	500	5	0.778
5	495	500	5	0.767
5	495	500	5	0.770
10	490	500	10	1.220
10	490	500	10	1.228
10	490	500	10	1.208
15	485	500	15	1.687
15	485	500	15	1.684
15	485	500	15	1.670
20	480	500	20	1.987
20	480	500	20	1.915
20	480	500	20	2.100