Prevalence and screening of exon 4 of the Interleukin-4 gene in *Ascaris lumbricoides* infected patients from district Mardan, Pakistan

By

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M. Phil Parasitology

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A dissertation submitted in partial fulfillment of the requirement for the degree of Master of Philosophy

In

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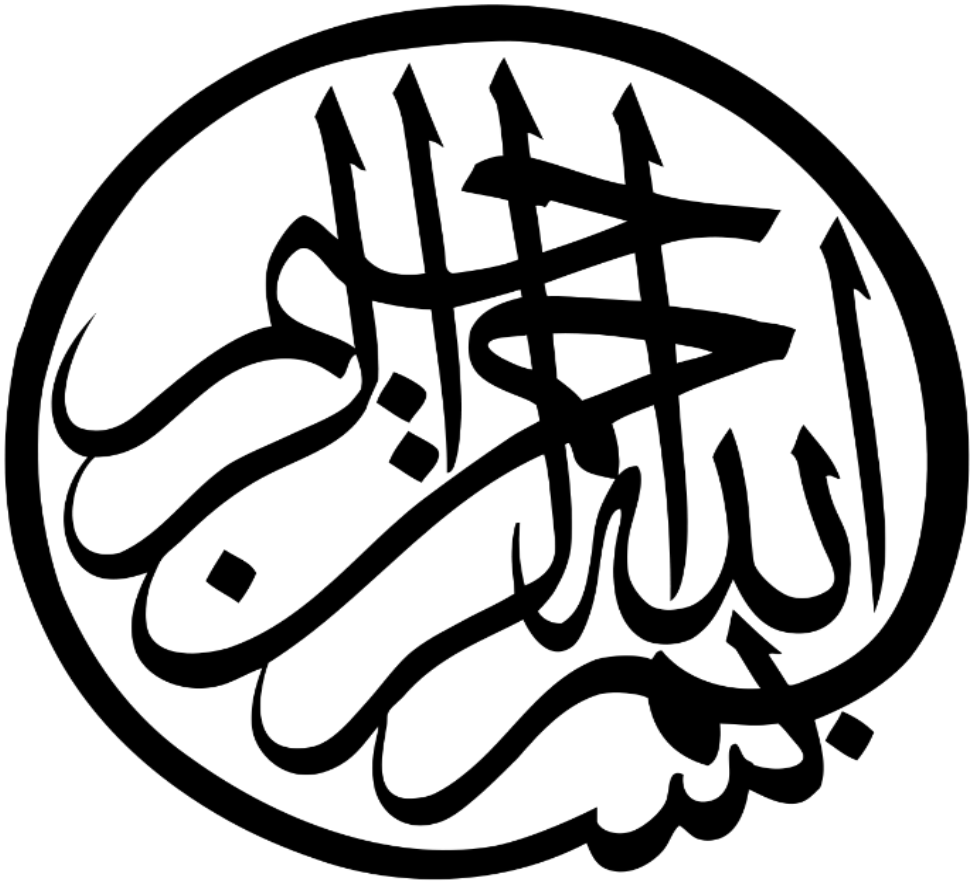

CERTIFICATE

This dissertation "**Prevalence and screening of exon 4 of the Interleukin-4 gene in *Ascaris lumbricoides* infected patients from district Mardan, Pakistan**" submitted by Mr. Ali Said is accepted in its present form by the Department of Zoology, Faculty of Biological Sciences, Quaid-i-Azam University Islamabad, as satisfying the thesis requirement for the degree of Master of Philosophy in parasitology.

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Chairperson

Date: _____

DECLARATION

I Ali Said, student of M. Phil. Parasitology, Session 2021-2023, hereby declare that the material and information contained in this thesis titled "**Prevalence and screening of exon-4 of the Interleukin-4 gene in *Ascaris lumbricoides* infected patients from district Mardan, Pakistan"** is my own work and has not been printed, published or submitted as research work, thesis or publication in any University or Research Institute in Pakistan or abroad.

_____

Ali Said

DEDICATION

MY BELOVED PARRENTS, BROTHER AND FRIENDS WHO HAVE BEEN PILLARS OF SUPPORT, GUIDANCE AND LOVE IN MY LIFE.

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LIST OF ABBREVATIONS

Abbreviated form	Full form
ABA-1	Abscisic acid 1
AHR	Airway hyperresponsiveness
Cm	Centimeter
CT-Scan	Computed tomography-scan
DALYs	Disability-adjusted life years
EDTA	Ethylenediamine tetra acetic acid
GBD	Global Burden of Disease
IgE	Immunoglobulin E
IL-3	Interleukin 3
IL-4	Interleukin 4
KPK	Khyber Pakhtunkhwa
M & HI	Moderate-to-heavy severity
Mm	Millimeter
NPA	Nematode polyprotein allergens
PCR	Polymerase chain reaction
PK	Proteinase Kinase
SDS	Sodium Dodecyl Sulphate
STH	Soil-transmitted helminth
TH2	The T helper type 2
UAI	Unique Anonymous Identification
UV	Ultraviolet
WBCs	White blood cells
WHO	World Health Organization
µl	Microliter

ACKNOWLEDGEGEMENT

In the name of **Almighty Allah**, the most gracious and merciful, all praise to the Almighty Allah (the most merciful and the most benevolent) and all respect to His Holy Prophet Hazrat Muhammad (peace be upon him) for bestowing upon me the courage and energy to accomplish this task. I experienced so much during this process, not only from the academic aspects but also from the aspect of personality. My humble gratitude to the **Holy Prophet Muhammad (S.A.W.),** whose way of life has been continuous guidance for me.

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ALI SAID

ABSTRECT

Background: *A. lumbricoides,* commonly known as the large intestinal roundworm, is one of the most prevalent parasitic worms infecting humans worldwide. These pale, cylindrical worms can grow up to 35 centimeters in length and primarily infest the human small intestine. This nematode is thought to affect 25 percent of the world's population. The purpose of this study is to investigate the prevalence, morphology and screening of exon 4 of the interleukin-4 gene in district Mardan, Khyber Pakhtunkhwa, Pakistan.

Methodology: The cross-sectional study involved the collection of prevalence data, worms and blood samples from *Ascaris*-infected patients. Worm marphometry was performed using a ruler, balance scale, and vernier caliper. DNA was extracted from blood samples using the phenol-chloroform method. Exon-4 of the IL-4 gene was amplified using PCR. Agarose gel electrophoresis was used to validate the PCR results. Chi-square was used to find the association of infection with demographic, environmental and clinical risk factors.

Results: The diameter of the worm ranges from 3–6.25 mm, the length is 12.9–32.8 cm, and the weight of the worm ranges from 0.47 g to 4.485 g. Among 265 individuals, the prevalence rate was 20%%. The prevalence rate in males was 37 (13.96%), while in females it was 16 (6.03%). Among age groups, the highest prevalence was seen in the age group 1–10 years, which accounts for 33 (12.45%) ($p = 0.052$). This result showed no significant association with infection. People living in village 50 (18.86%), lower class 42 (15.84%; $p = 0.009$*), poor status of living 44 (16.60%; ($p = 0.001$*), and people working as labor had 26 (9.81%; $p = 0.013$*) significantly associated with infection. People with no or primary education have the highest recorded prevalence rate, which is 21 (7.92%) ($P = 0.128$). The risk factors associated with the prevalence were wearing footwear 44 (16.60%) ($p = 0.04$*), standing water ponds 36 (13.58%) ($p = 0.002$), having no washroom 27 (10.18%) ($p = 0.000$*), and people defecating in open fields 42 (15.84%) ($p = 0.011$) were significantly associated with infection. Washing hands before meals, the presence of grass and bushes, drinking water, using waste as fertilizer, sewage water merging with running water, condition of living and washing hands after defecation ($p > 0.05$) did not show any significant relationship with the risk of infection. People who had never been treated with anti-helminthic drugs have the

highest prevalence, which is 43 (16.22%), as in those who were previously dewormed ($p = 0.007$). The most effective drug during this study was Albendazole, whose effectiveness was the highest at 12.07%, followed by piperazine which has 3.01%. Other anti-helminthic drugs show less effect against the *Ascaris* worm. DNA samples from people with *A. lumbricoides* infections were amplified using the Polymerase Chain Reaction (PCR). The IL-4 gene (exon 4) 559 bp long PCR amplified product was obtained.

Conclusion:

The study illustrates a number of factors associated with *A. lumbricoides* transmission, including poor living, environmental conditions, a lack of washrooms, and open-field defecation, which are major contributors to prevalence. Above all, stress and reaffirm that acute poverty and *A. lumbricoides* infection are inevitably linked. Improving hygiene conditions and habits is essential to reduce the risk of *A. lumbricoides.*

INTRODUCTION

According to the World Health Organization (WHO), if at least 1% of school children have moderate-to-heavy degree of severity (M & HI)) of soil-transmitted helminth (STH) infections, consider this a public health issue. One-quarter of the world's population is impacted by STHs that include Ascaris, Trichuris, and the hookworms which result in a loss of more than three million disability-adjusted life years (DALYs) **(**Levecke *et al*., 2020)**.** *Ascaris* of pigs, *Toxocara canis, Trichuris* of pigs and lower primates, *T. vulpis, Capillaria hepatica, Ancylostoma ceylanicum, A. braziliense, Ternidens spp.,* and *Trichostrongylus spp.* are additional pathogens that may significantly contribute to the poor health of communities (WHO, 1964). Due to their same diagnostic requirements and therapeutic retorts, these STH species are usually treated together (Report of the WHO, 2023). More than 3.5 billion individuals worldwide have intestinal worm infections due to the widespread distribution of these helminths. They number 1.47 billion in total and suffer from roundworms, 1.3 billion from hookworms, and 1.05 bln from whipworms. These helminth parasites are thought to affect 400 million school-aged children worldwide. Hookworm infection is common in children between the ages of 5 and 15, but the risk appears to increase with age (Khan and Khan, 2017). Since the 1990s, the WHO has advocated a diagnostic technique for estimating the number of eggs in feces and then categorizing infections as light, moderate, or heavy based on the fecal egg counts (FECs, expressed as eggs per gram of stool (EPG)) collected (Levecke *et al*., 2020). Children are most severely affected by STH, which affects almost 2 bln people worldwide. W.H.O estimates that 870 million children reside in areas with a high frequency of STH. The most severely impacted regions are in Africa, South Asia, and South America, and India alone accounts for about 1/4 of all cases worldwide, with 220.6 million children in need of preventative treatment (Salam and Azam, 2017). Nearly 70% of STH infections are found in Asia, according to a recent study of the disease's global impact. According to the same study, there was at least one STH species present in one-fourth (26.4%) of the Asian sample population. The high prevalence of STH in Asia is likely a result of the region's humid, tropical climate, inadequate sanitation and lack of easy access to clean drinking water, and unhygienic behaviors, all of which promote worm survival and transmission (Silver

et al., 2018). In the current study, our focus is on only Ascaris lumbricoides, as their prevalence is rapidly increasing in the study area.

1. Ascaris lumbricoides

Ascaris lumbricoides (*A.lumbricoides*) One of the biggest nematodes (roundworms) parasitizes the human gut. This nematode is thought to affect 25 percent of the world's population. The small intestine is where the mature worms reside (Kilic *et al*., 2003). Two of the most common species of *Ascaris*, which are *A.suum* and *A.lumbricoides*, have been found and previously believed to infect humans and pigs, respectively; nevertheless, they are believed to cross-infect. For instance, *Parascaris univalens*, formerly known as *Ascaris megalocephala*, is a significant horse parasite. *Toxocara spp.* are parasites of dogs and cats, and their larval stages are harmful to humans as well (Wang and Davis, 2020). These species have been accepted as legitimately identified since Linnaeus first described and named *A.lumbricoides* from humans in 1758, and Goeze subsequently named *A. suum* from pigs in 1782 (Leles *et al*., 2012).

1.1 Taxonomic classification

Kingdom: Animalia

Subkingdom: Hielminthes

Phylum: Nematoda

Class: Chromadorea

Order: Ascaridida

Family: Ascarididae

Genus: *Ascaris*

Species: *A.lumbricoides*

(Al-Kahfaji and Alsaadi, 2021)

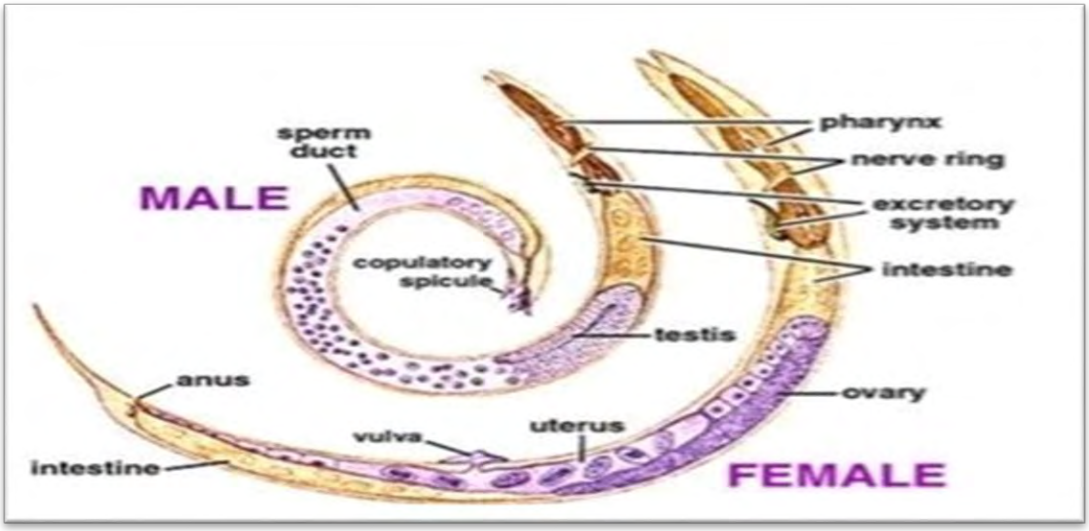
1.2 Historical background

There may be no earlier human helminth than ascariasis, which is mentioned in manuscripts from Mesopotamia, Greece, Rome, and China. The Greeks and Romans mistook the worm for an earthworm and gave it that name. Linnaeus initially defined the genus *Ascaris* in 1758; the name comes from the Greek word askaris, which means "worm. In 1758, Goeze gave a description of the pig's roundworm, *A. summ*. Later researchers such as Grassi (1877), Davaine (1877), and Epstain (1892) demonstrated that ascaris infection happens when the eggs are ingested and then develop into mature form in the intestines (Khuroo, 1996).

1.3 Morphology

A.lumbricoides, the adult, is a largest, pink, cylindrical, elongated creature that tapers at both ends. In males, the ventrally curving tail end tapers. Additionally, the male possesses multiple pre-anal and post-anal papillae groups. Male and female sexual dimorphism is well evident. Male have a length of 15 to 30 cm and a diameter of 2 to 4 mm, while female lengthen from 20 to 49 cm and 3 to 6 mm diameter. The outer chitinous layer, is composed of a transversely striated, non-nucleated cuticle that is secreted from the underlying epithelium. The mature worm uses its muscular activity to keep itself in the gut's lumen. The somatic muscular bands in the worm are made up of a single layer of longitudinal, non-striated muscle cells with sarcoplasmic processes reaching into the body cavity; the worm lacks circular muscle. The worm's hypodermis, which in turn extends into the body cavity as four lateral cords, is bordered by a thick cuticle in cross section. The reproductive system, neurological system, excretory system, and digestive system are just a few of the viscera that are hanging in the bodily cavity, also known as the pseudocoelom. The vascular system is absent in *A.lumbricoides*. The mouth, pharyngeal cavity, esophagus, midgut, rectum, and cloaca are all parts of the alimentary canal, which is a tubular, longitudinal structure. The excretory system of the worm is comprised of simple channels that run along its two narrow lateral streaks. The testis, vas deferens, seminal vesicle, and ejaculatory duct are all differentiated from a single fragile tubule that makes up the male genital organ.

The cloaca is entered by the ejaculatory duct. A double thread-like tube that divides into the ovary, oviduct, seminal receptacle, uterus, oviduct, and vagina makes up the female genital organ. The intersection of the anterior and middle thirds of the body is where the vulva1 entrance is placed ventrally, and this is where there is a constriction (Pawlowski, 1990; Sun, 1982). The mature worm has a lifespan of 6 to 18 months on average. A daily production of 240,000 eggs per worm is thought to be released into the fecal stream by the female (Khuroo, 1996).



Wikipedia

Figure 1.1 Morphology of *Ascaris lumbricoides.*

1.4 Epidemiology

Since the turn of the century, nearly each aspect of parasitic illness has been better understood, which has helped many societies experience less parasitic infection. But regrettably, the bulk of the world's poorest populations who live in tropical and subtropical climates continue to experience these illnesses on a regular basis. *A.lumbricoides T.trichiura*, and hookworm, three prevalent soil-transmitted intestinal nematodes that are thought to infect 1.4, 1, and 1.2 bln people, respectively, or nearly 1/4% of the global population, are in the spotlight (Scott, 2008). The estimates of the illness burden, which are 10.5, 6.4, and 22.1 million DALYs, respectively, are maybe more significant than the reported infection rates (Hotez *et al*., 2006). The Americas, East Asia, China and sub-Saharan Africa are the regions with the highest recorded

infection rates. Individuals carrying substantial loads show related morbidity, Moreover, the frequency distribution of mature worms in their hosts is overdispersed. (Dold and Holland, 2011). About 60,000 fatalities per year are associated with acute *Ascaris* infections, most of which are caused by intestinal blockage in children. Children are more likely infected than adults, especially between the ages of 3 and 8 (Scott, 2008). This is primarily seen in regions with warm, humid temperatures. At least 150 nations on the planet suffer from ascariasis. According to the distribution of cases of ascariasis, Africa and the Middle East accounted for 16.7% of cases, 8.3% of cases were in South America, Central America, and the Caribbean, and 75% were in Central and Southeast Asia and the Oceania area (Asaolu Ofoezie, 2018). As *A.lumbricoides* infection is quite common in Pakistan, yet its prevalence varies greatly across the nation. The prevalence has been reported as follows in several surveys: Gilgit-Biltistan: 22.8%; rural: 45%; Peshawar Swat District: 39.8%; Peshawar: 38.8%; Pakistan: 11.9% Peshawar 4,2% Quetta City 1.8%, Dir District of K.P.K. 14.9%, Hazara Division 7.3% Chitral 68.7% 15.0% Larkana-Sindh Kashmir Azad 3.8% of Swat district, 39.8% of the country as a whole, and other regions (Wali *et al*., 2016).

1.5 Life cycle

Ingestion of infected fruits, vegetables, and greens is linked to the transmission of *Ascaris* eggs. Accidental contact with soil is also a risk factor. According to Al-Tameemi and Kabakli (2020), the illness can also happen in areas where human feces are utilized as fertilizer for producing vegetables. Both fertilized and infertile eggs, which are contagious, can be found in soil. Rather than the earth, the hosts' intestines are where the eggs hatch. The eggs in the soil can remain infectious and survive for ten years or more. Additionally, they are unaffected by standard chemical water filtering methods. (Kawoosa *et al*., 2018). A second-stage larva measuring 50–70x40–50 mm (infective stage) is found inside the fertilized egg. After ingestion, the eggs begin to hatch into larvae in the jejunum. Within two to eight days, the larvae cross the intestinal mucosa and go through the lymphatic system into the portal vein to reach the liver. They go via the heart to reach the lungs. They are 564x28 μm in size. When they penetrate the capillary walls and enter the lung alveoli. After around ten days in the lungs, they undergo two molts to become fourth-stage larvae, which grow to be between 1700 and 2000 mm long. After that, they go back to the trachea and throat. The larvae

enter the small intestine through the gut and esophagus. When they get to the small intestine, they molt and become immature adults. These worms develop into adult male and female in the 14–20 days that follow mating. Millions of eggs are released into the female's excrement after 70 days of eating contaminated eggs. The egg shell can tolerate a range of climatic conditions and can remain in the soil for up to six years. (Dold and Holland, 2011; Al-Tameemi and Kabakli, 2020; Lima *et al*., 2017). They can infect new human settlements by being transported by the wind in dry dust.

Eggs: The three morphologic phases of an egg are as follows: They are eggs that have been fertilized, decorticated, and left unfertilized (Geenen *et al*., 1999).

Fertilized eggs: These eggs have the standard broad oval form, are brown in color, and range in size from 40 to 70 by 30 to 45 millimeters. An undeveloped embryo is what's within (Sinniah, 1982).

Unfertilized eggs: The unfertilized egg is generally oblong in form, measuring around 85–90 mm by 35–45 mm (Elkins et al., 1988). It is also longer than the fertilized eggs.

Decorticated eggs: Eggs that have been decorated, fertilized, or unfertilized may occasionally be transparent and lack color due to a mammalated or missing outer shell (Haswell-Elkins *et al*., 1989).

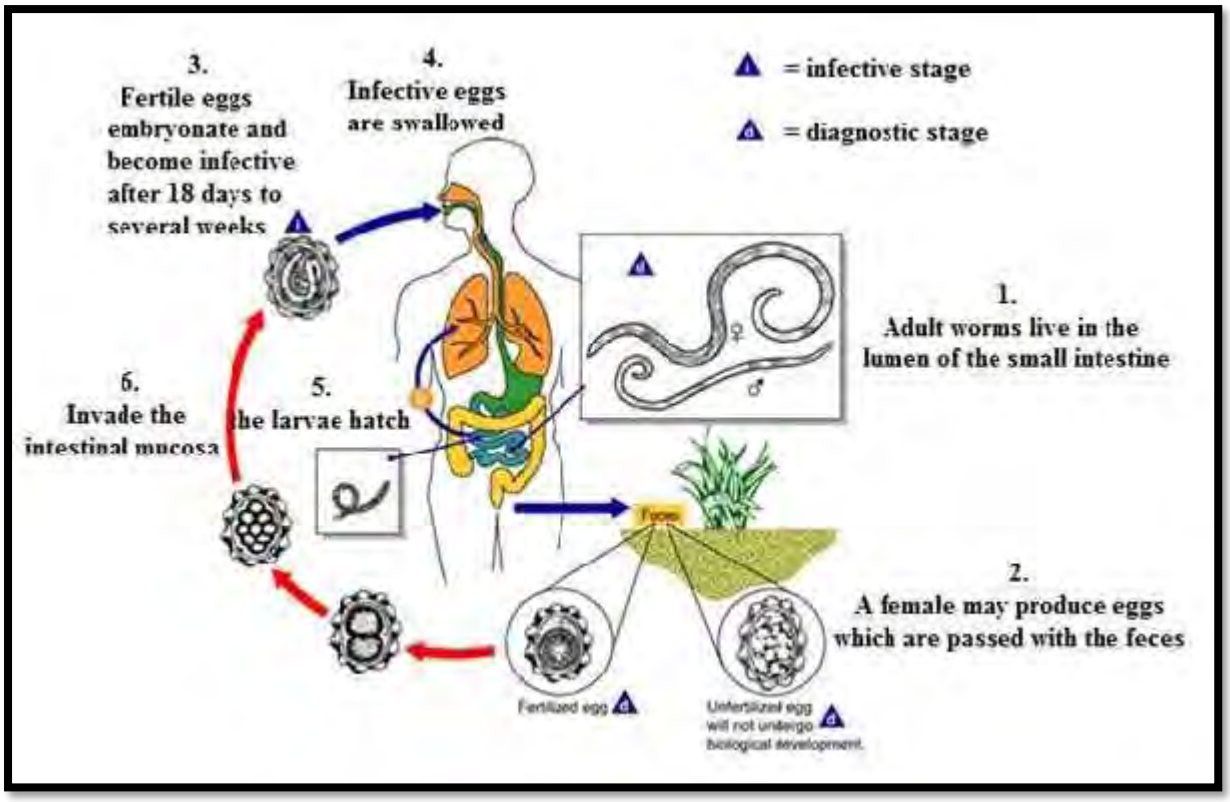
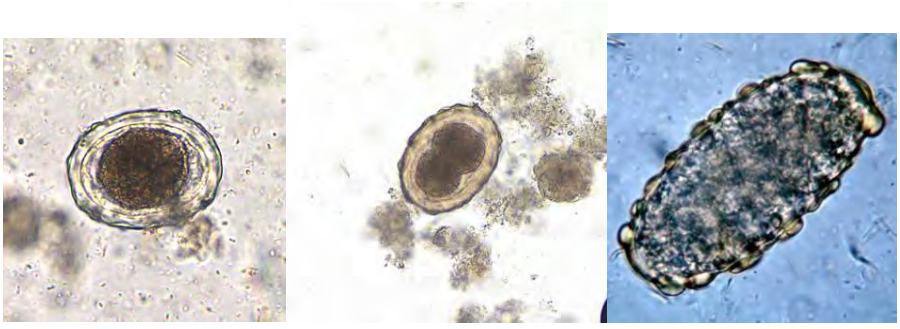


Figure 1.2 .Life cycle of *A.lumbricoides* (http://www.cdc.gov/) (Al-Tameemi and Kabakli, 2020).



(Decorticated fertile egg) (Fertilized egg) (Unfertilized egg)

Figure 1.3. Egg Morphology (https://www.cdc.gov/dpdx/ascariasis/index.html)

1.6 Pathogenesis

The most prevalent helminth infection in the world is *A.lumbricoides* (a roundworm), which can induce allergic airway disease, a phenotype of asthma, and is a lifelong illness. We propose that despite the remission of an infection that resembles allergic airway illness, Ascaris larvae migration across the lungs results in chronic airway hyperresponsiveness (AHR) and type 2 inflammatory lung pathology. Children in endemic areas frequently contract the disease shortly after birth, go through recurring infections as they grow, and achieve peak worm load by preschool and school age (Weatherhead *et al*., 2018). (Collaborators on GBD 2015 Mortality and Causes of Death) Ascariasis is linked to a high degree of morbidity worldwide, resulting in 1.46 million DALYs. Growth retardation, cognitive delays, malnutrition (vitamin A deficiency), stomach discomfort, and blockage are all symptoms of ascariasis caused by adult worms present in the gut (Mahalanabis *et al*., 1976). Beyond the intestinal type of ascariasis caused by adult worms, Loeffler's syndrome (pneumonia), a temporary inflammatory lung condition, has been clinically associated with larval migration through lung tissue after oral consumption of Ascaris eggs (Akuthota and Weller, 2012). According to Sinniah *et al*., 2009 Loeffler's syndrome symptoms include breathing problems, coughing, fever, bloody sputum, asthma, and an abundance of eosinophil cells. Additionally, some researchers have hypothesized that larval *Ascaris* infection is a significant environmental factor in the development of allergic rhinitis, an asthma phenotype, and inflammatory lung disease in countries with few resources (Buenda *et al*., 2015). These epidemiological studies imply that allergic airway illness in children caused by Ascaris is more severe (Hagel *et al*., 2007). Ascaris lumbricoides infection is an overlooked risk factor for the development of asthma, and some studies have demonstrated an association between elevated IgE levels from *A.lumbricoides* infection and the onset and severity of asthma (Taghipour *et al*., 2020). The second stage is the adult-related intestinal phase. Even though there are few adult worms present in the intestinal lumen, they can occasionally induce colic and stomach pain, especially in young children. Malnutrition may develop from a severe adult worm infection. Large, mature worms can perforate the walls of the intestine and mechanically obstruct the common bile duct or appendices canal. As a result, the illness may have side effects such as pancreatitis, appendicitis, intestinal blockage, biliary ascariasis, intestine perforation, and peritonitis (AL-Kahfaji and Alsaadi, 2021). Appendicular ascariasis, Hepatobiliary and pancreatic ascariasis, and peritoneal ascariasis are further clinical conditions (Khuroo, 1996).

1.7 Symptoms

When there are few worms present in the samples, the early stages of infection are often asymptomatic (Dold and Holland, 2011). Depending on which portion of the body is impacted, moderate and chronic infections present with a variety of symptoms? For a few days, the migratory larvae in the lungs, for instance, might produce skin rashes, fever, and coughing (Kanneganti *et al*., 2013). Hemorrhage occurs as a result of the larvae moving from the blood in capillaries into the lung alveoli, and the alveolar sacs fill with serous exudate. Vomiting, stomach discomfort, and mechanical blockage of the pancreatic, bile, and intestinal ducts are all symptoms of an abundance of adult worms in the intestines. Bowel perforation and peritonitis are caused by worm migration. Ascaris releases a variety of digestive enzymes into the intestinal canal, including amylase, protease, and lipase, which causes nutrient malabsorption in some cases where the adult worms passing from the anal or mouth (Carroll *et al*., 2016; Crompton *et al*., 1985) cause diarrhea or bloody stools. Invading worms can result in abscesses in the peritoneum, pancreatic duct, liver, bile duct, and surgical incisions (Wani *et al*., 2010).

1.8 Transmission

A.lumbricoides is a parasite that exists in the small intestine and spreads through the feces of infected people. Humans who are sick occasionally urinate outside, such as in bushes, fields, or on the ground. This feces may be utilized as fertilizer. Ascaris eggs are therefore released into the ground, where they grow into mature embryonated eggs, serving as a kind of infection. Humans contract ascariasis when they swallow eggs, typically through food like green, raw vegetables or fruit that has been tainted with mature Ascaris lumbricoides eggs. Inhaling contaminated dust is one way that transmission may happen. Direct disease transmission from hand to mouth after coming into contact with contaminated soil is possible. (Thein-Hlaing *et al*., 1984; Hotez *et al*., 2006). According to several studies, pressures to reduce the use of artificial fertilizers and conserve water may be indirectly encouraging the use of wastewater for hothouse gardens and field crops, as well as as an organic fertilizer, which might raise the danger of transmission from contaminated food (Adams *et al*., 2005).

1.9 Factors related to prevalence Lack of access to clean water, overcrowding, open defecation, poverty, low nutritional status, using human waste as fertilizer and

irrigation, not cleaning hands before eating, owning or breeding pigs, eating raw pork and raw water plants, and poor education of mothers are among the environmental, social, and behavioral predictors of elevated Ascaris egg output that are frequently reported, depending on the population communities (Fortes *et al., 2004;* Corrales *et al.,* 2006; Scott, 2008).

1.10 Immunity and *Ascaris lumbricoides*

Numerous epidemiological and experimental studies, both in humans and animals, have suggested that ascariasis alters the development of allergic disorders like asthma (Cooper, 2009). These studies have found that nematode infections may either cause severe immunosuppression or an increase in TH2 responses, depending on a variety of parameters, including the kind of parasite, the host, the time of exposure, the strength of the infection, and the environment. IgE responses to environmental allergens and allergies are boosted by ascariasis. Immunomodulation and IgE hyperresponsiveness are combined by nematode infections, but in varying amounts; the latter is a characteristic of allergic reactions and is influenced by the host's genetic make-up. However, because domestic mites and Ascaris do not have the same clinical significance, it is equally crucial to understand the antigenic and allergenic content of each in order to comprehend the processes driving this specific phenotype (Yan Chua *et al*., 2007; Thomas *et al*., 2010). Research on the significance of *A.lumbricoides* as an asthma risk factor has produced mixed results, despite the fact that it has been linked to a considerably increased risk of asthma in a systematic review and meta-analysis (Leonardi-Bee *et al.,* 2006). Infection can either protect against IgE sensitization and asthma in some population surveys or function as a risk factor in others. It is generally agreed upon that intestinal parasites, such as nematodes, are managed by a T-cell-dependent adaptive immune response in which IL-4 and IL-13, as well as certain antibodies, play a crucial role (Acevedo and Caraballo, 2011).

1.11 Interleukins gene

According to the "hygiene hypothesis," which contends that lack of exposure to parasites in contemporary settings leads to immune imbalances that increase susceptibility to the emergence of autoimmune and allergic conditions, many human genes have evolved in response to the ongoing threat of exposure to infectious agents.

The prevalence of type 2 cytokine responses to gastrointestinal helminths and the significance of IL-4, IL-5, IL-9, and IL-13-associated immune pathways in mediating parasite expulsion and resistance to infection have both been shown in a number of studies using experimental animal model systems (Seyfizadeh *et al.,* 2015). According to research, helminths have exerted strong selection pressure on a subset of these genes. Five IL genes, including IL7R and IL18RAP, were shown to have been the subject of balancing selection, a process of selection that preserves genetic diversity within a population, according to a population genetics investigation. We looked into the connection between adaptability and illness after finding polymorphisms in several of these sites and their correlation with autoimmune diseases. By looking for IL gene variations resulting from genome-wide association studies. The majority of immunological and inflammatory responses are regulated by ILs (interleukins), which are tiny secreted molecules that work by attaching to certain receptors found on target cells. Different IL genes have been linked to differences in how susceptible an individual is to developing certain infections (Picard *et al.,* 2006; Blackwell *et al.,* 2009), as well as an increased risk of developing autoimmune or allergic/atopic illnesses (Lettre and Rioux, 2008). The IL-4 gene may be located on mouse chromosome 11 and on human chromosome 5's long arm, specifically at 5q23.3-31.2. The IL-4 gene itself is made up of four exons that are 6 kb long in mice and 10 kb long in humans (Paul, 1991). By attaching to receptors expressed on target cells, IL-4 mediates the actions of these cells. Responsive cell types express just 400 or fewer receptors per cell in both mouse and human cells (Park *et al.,* 1987). Freshly generated B and T lymphocytes, macrophages, lymphoid cells, mast cell lines, a number of other hematological cell lines, and fibroblast and stromal cell lines have all been shown to include receptors (Lowenthal *et al.,* 1988). The IL-4 and IL-5 genes are connected. Pulsed field electrophoresis has determined that the two genes in the mouse are separated by around 160 kb. Despite not being physically connected, the "IL-4/IL-5 pair" has been demonstrated by study of restriction fragment length polymorphisms (Paul, 1991).The hygiene hypothesis, first put out by Strachan, primarily explains the historical pattern in the prevalence of allergy illness as a result of decreasing exposure to pathogens (such as helminths and germs) in the environment during childhood. TH2 cytokine production, particularly interleukin (IL) 4 and IL-5, which operate on B cells to trigger IgG and IgE class switching, controls the immune response to helminths (dos Santos Costa *et al.,* 2017). In the USA, it has been demonstrated that genetic markers

within and close to 5q3 1-33 are associated with total serum IgE concentration (Van der Pouw Kraan *et al.*, 1994). They have shown compelling evidence that at least one locus in the 5q31-33 region is directly related to increased serum IgE levels and bronchial hyperresponsiveness. This area contains the gene for interleukin-4, making it a potential candidate for the genetic connection that has been identified. Prior research has connected an SSCP I1-4 marker to asthma (Walley and Cookson, 1996). When the promoter polymorphisms of three distinct interleukins were investigated. The prevalence of this interleukin-4 promoter polymorphism in asthmatics was found to be high. Additionally, a higher serum IgE content was linked to it. According to Rai *et al.,* 1989), the polymorphism is a C to T mutation at position -590 counting from the first ATG codon. All of the previously mentioned regulation components of interleukin-4 are upstream of the polymorphism. Despite this, it may be a component of a previously unidentified element as it has been established that regions up to -3000 are necessary for complete wild type expression levels. It has recently been revealed that nuclear factors bind to this area, and placing the variant promoter in a construct for the production of the luciferase reporter gene has increased the activity of the promoter ((Hijazi and Haider 2000).

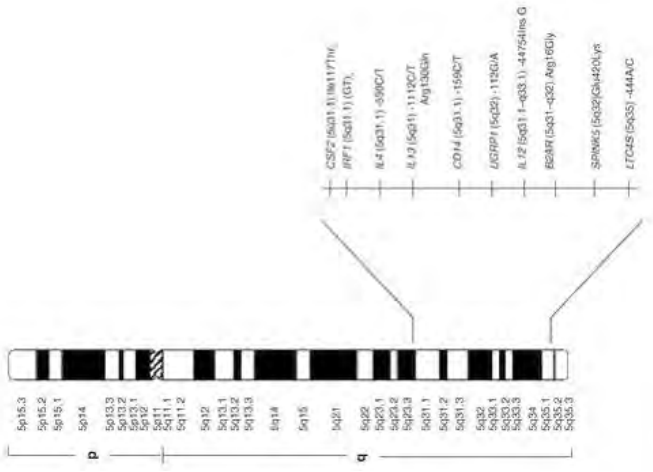


Figure1.4 (IL-4 gene location on chromosomes 5 of Human)

Aim:

- Prevalence and screening of exon 4 of the Interleukin-4 gene in *Ascaris lumbricoides* infected patients from district Mardan, Pakistan

Objectives:

- Phenotyping of *A. lumbricoides* collected from feces of patients from district Mardan, Pakistan.
- To find the prevalence of *A.lumbricoides* and their association with demographic, clinical and environmental risk factors.
- To screen the exon 4 of IL-4 gene of *A.lumbricoides* infected patients.

MATERIALS AND METHODS

2.1 Study Area

The data were collected from September 2022-April 2023 from distract Mardan, Khyber Pakhtunkhwa, Pakistan. The current research data was collected from distract Mardan. The area is located between latitudes 71° 48' and 72° 25' east and longitudes 34° 05' to 34° 32' north (Fig.3.1). Its boundaries are the Malakand protected area and the Buner district to the north; the Nowshera district to the south; the Buner and Swabi districts to the east; and the Charsadda district and the Malakand protected area to the west.

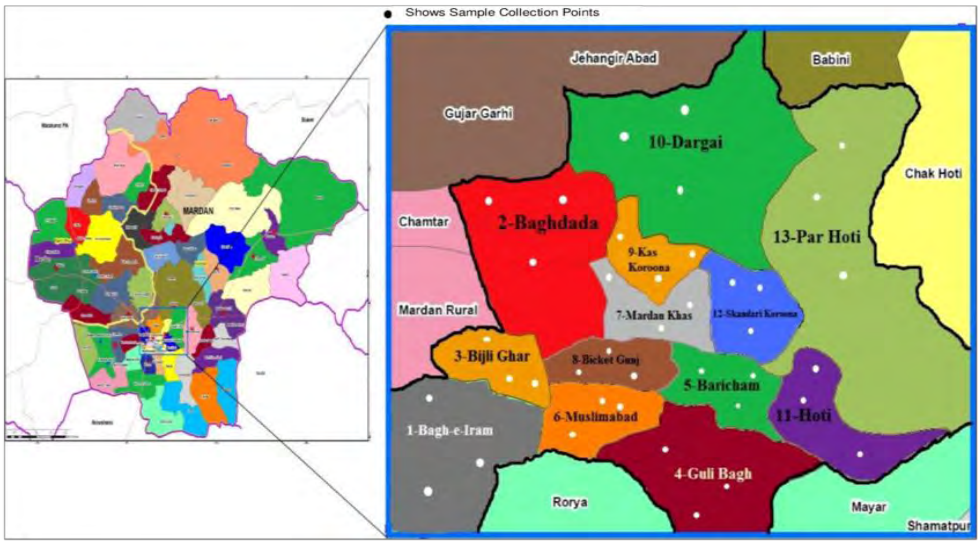


Figure 2.1 Geo-graphical representation of distract Mardan.

2.2. Questionnaire

The present study was conducted under the Department of Zoology Quaid-i-Azam University Islamabad and its consent was attained by the Bioethical review committee of Quaid-i-Azam University. Patients were evaluated using a questionnaire. The questionnaire was originally designed to be applied to patients and was subsequently interviewed. The questionnaire consists of questions related to laboratory diagnosis and symptoms and questions about the personal, social, social status and economic status of the family.

2.3 Collection of worm samples

After the administration of anti-helminthic medicine, the worms were extracted from the stool. Then it was collected and washed three times with fresh water and cleaned properly. The samples were then kept for some time in distilled water to make sure they were properly cleaned from fecal waste. After that, they were put into a sterilized container containing 70% ethanol for storage.

2.3. Phenotyping of *A. lumbricoides*

Identification of worm was done by using identification keys (Pawlowski, 1990; Sun, 1982) (Khuroo: 1996).

2.3.1. Measuring length of worms: The length of the worm was measured using a ruler. The ruler was first set on a plain area. Then the worm was straighter and avoided movement to measure its proper length. The worms were strung and held by two people to find an accurate result. The zero point on the ruler was considered the starting point from where the worm was held by one person. And then gently and carefully straighten the worm toward the measuring side. The end point was measured after the worm reached its maximum size. After that, record the length of the worm and write it on paper along with the date and time. The observation for each worm was performed three times to make the result accurate. After that, the worm was carefully removed from the ruler and re-preserved.

Figure 2.2: Measuring the length of *A. lumbricoides*

2.3.2 Measuring width using a *Vernier caliper*

A vernier caliper was used for measuring the width of worms. As an accuracy protocol, the vernier caliper was first cleaned, especially their jaws, for an accurate and precise result. Before measuring the width of the worm, the jaws were aligned with each other. Check the readings on the main scale and vernier scale, and they were perfectly aligned with each other and showed zero error. After that, the jaws were gently opened, slightly larger than the worm's width. The worm was placed in the jaw parallel and gently contacted the jaws with the worm sample. When it gets contact with the worm, stop further closing the jaws. After that, the reading was taken by counting lines from the main scale, which were 1 2 3... That was called the main scale reading. After that, the reading from the vernier scale (small scale) was taken by looking at the main scale, and that was our reading where the main scale and the vernier scale were aligned. The small-scale reading was then multiplied by the Vernier caliper least count, which was 0.05. The product of the least count and small scale was then added to the main scale, and that was our width measurement. This procedure was performed three times for each worm to ensure an accurate result. The following formula was used:

Width of worm = main scale + (least count x small scale counts)

Figure 2.3: Measuring the width of *A. lumbricoides* using Vernier caliper.

2.3.3 Measuring the weight of the *A. lumbricoides*

The digital balance scale was used for measuring the weight of the worm. The worm was taken in the petri dish and made clean and dry from the preserved solution. After that, the balance scale was switched on, and its reading was brought to zero. After that,

the aluminum foil was placed on the balance scale, and the reading was again deleted by using the delete button. After that, the worm was placed on aluminum foil and closed on all sides of the scale for an accurate result. After that, the result was taken from the screen by performing this procedure three times. The result was noted on the paper. After that, the worm with foil was removed from the balance scale, and the worm was preserved.

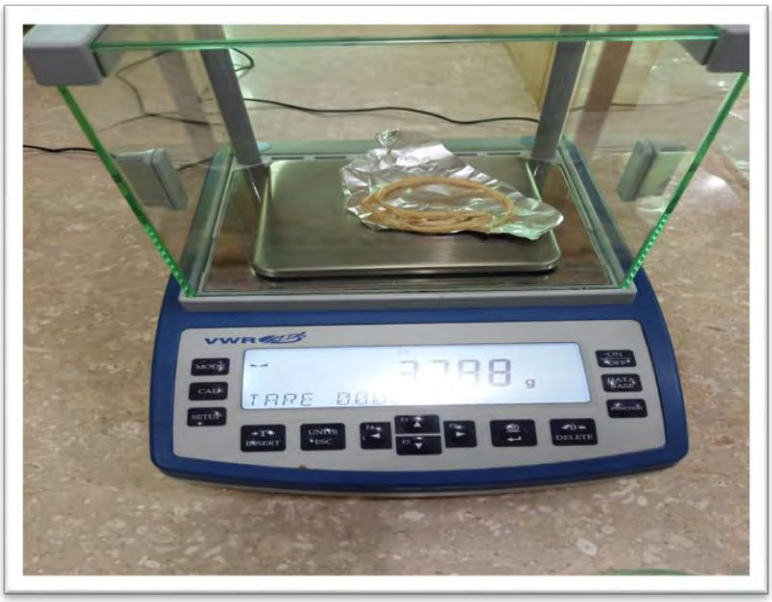


Figure 2.4: Digital balance scale used for measuring weight of *A.lumbricoides.*

2.4 Collection of blood samples

Blood samples were taken from district Mardan, comprised 265 people in which 20% were diagnosed with *Ascariasis,* and blood samples were taken from these patients. From Every single person had 5 mL of venous blood drawn using a sterile syringe. The labels of each EDTA vacutainer tube used to collect blood samples contained the participant's name and a Unique Anonymous Identification (UAI) number, To avoid coagulation, the tubes were gently mixed before being chilled at -4 degrees Celsius.

2.5 Extraction of Genomic DNA

Genomic DNA was extorted from each sample using the Phenol-Chloroform technique, a recognized chemical approach that takes three days.

Protocol: Phenol-Chloroform DNA Extraction Method

Day 1:

Allow the EDTA tubes holding the blood samples to equilibrate at room temperature for around 10-15 minutes before starting the DNA extraction procedure. . The ideal circumstances for the next stages are guaranteed during this equilibration phase. To keep a well-organized workflow and avoid any misunderstanding or misidentification during the extraction process, label each individual Eppendorf tube with the matching name and family ID. In order to accurately represent the original blood sample, carefully transfer 750 l of blood from each sample into the appropriate labeled Eppendorf tubes. Each Eppendorf tube holding the blood samples should have 750 µl of Sol A added. Sol A is an essential chemical for DNA stability and cell lysis. To ensure the best possible DNA extraction, incubate the tubes at room temperature for 20–25 minutes. After the incubation time, spin the Eppendorf tubes for 15 minutes at 13,000 rpm to separate the DNA-containing supernatant from the cellular waste, proteins, and impurities. Make sure to leave the DNA pellet alone while discarding half of the supernatant. This process gets rid of contaminants and makes the subsequent DNA purification better. To continue washing and purifying the remaining DNA pellet in each tube, add 400 µl of Sol A. By tapping the tubes, make sure that sol A and the pellet are well combined. To achieve a clean DNA pellet, repeat the centrifugation stage to pelletize the DNA and wash it with Sol A 3 to 4 times. Once a pure DNA pellet has been produced, each Eppendorf tube should be filled with 400 µl of Solution B, 25 µl of sodium dodecyl sulfate, and 5 µl of proteinase kinase. Vertex the mixture thoroughly to promote DNA precipitation and the elimination of protein impurities. Finish the first day by incubating the Eppendorf tubes at 37°C for the whole time to allow for full protein breakdown and enzymatic reactions, producing the best possible DNA yield and purity. To provide a controlled environment for the next stages, take out the incubated Eppendorf tubes and let them sit at room temperature for a little while. To guarantee precise DNA extraction findings, prepare new Solutions D and C+D the day before usage. To facilitate the selective separation of DNA from other cellular components, 500 µl of Solution C+D should be added to each Eppendorf tube containing the incubated samples.

To pelletize the DNA and isolate it from impurities, centrifuge the tubes at 13,000 rpm for 15 minutes. To ensure accurate sample identification, label fresh Eppendorf tubes

and extract the top DNA layer after centrifugation. To aid DNA precipitation and purification, carefully add 500 µl of Solution D to the freshly-labeled Eppendorf tubes holding the transparent DNA layer. To pelletize the DNA in the new tubes, repeat the centrifugation procedure. In order to collect the freshly separated DNA layer, label fresh Eppendorf tubes. Each tube with a label on it should now have 500 l of isopropyl alcohol and 60 l of sodium acetyl added to help with DNA precipitation and purification. To ensure that any contaminants are eliminated, centrifuge the tubes to separate the DNA pellet from the liquid phase. Keep the DNA pellet while discarding the liquid phase with care. 200 µl of 70% ethanol are added to each tube to wash the DNA pellet, and the tubes are then centrifuged to get rid of the ethanol and any remaining contaminants. To confirm that all of the ethanol has been removed and there are no visible bubbles, let the tubes air dry entirely. At the end of Day 2, each dried tube will receive 200 µl of TE buffer. The tubes should be incubated at 37°C overnight so that the DNA may rehydrate and dissolve in the buffer.

Put the tubes in a water bath set at 70°C for an hour to give the extracted DNA a heat shock treatment. This procedure ensures DNA integrity and prevents denaturation by inactivating nucleases and other enzymes. To reduce thermal stress on the DNA, give the tubes five minutes to equilibrate at room temperature after the heat shock treatment. To ensure that the DNA and buffer are thoroughly mixed, centrifuge the tubes for 2 minutes at 3000 rpm. For long-term stability and upcoming analysis, store the DNA samples in appropriately labeled cryo cartons at -20°C. The chemical composition and concentrations employed in DNA extraction are listed in Table 2.1.

Table 2.1. Chemical concentration and composition used in DNA extraction.

S.No	Chemical	Concentration
1	Sol A	500 µL
2	Solution B	400 µL
3	Sodium Dodecyl Sulphate (SDS)	25 µL
4	Proteinase Kinase (PK)	5 µL
5	Solution C+D	500 µL
6	Solution D	500 µL
7	Isopropyl Alcohol	500 µL

8	Sodium Acetyl	60 µL
9	T.E Buffer	200 µL

2.6 Agarose Gel Electrophoresis (1%)

It is used as a verification step after DNA extraction. The protocol for the procedure is described as follows:

To make a 1% agarose gel, in a conical flask, combine 50 mL of 1X TBE buffer with 0.5 g of agarose powder. To make 1X TBE buffer, dilute 10X TBE buffer in a 1000 mL container with 900 mL of distilled water, and combine 0.5 M EDTA (40 mL), 108 g Tris, 54 g Boric acid, and distilled water to get a final volume of 1000 ml for the 10X TBE buffer. Set the pH to 8 in buffer before adding the created gel to the gel tank. The conical flask should be microwaved for two to three minutes while being wrapped in aluminum foil to produce a clear solution. We wait a few minutes for the flask to drop to room temperature. Ethidium bromide is added to the flask at a volume of 300 microliters. Under UV light, this intercalating chemical is used to detect DNA; however, because it causes cancer, it should not be handled carelessly. A casting tray and combs are put inside a gel mold. A clear solution is poured into the casting tray without creating any bubbles. For 30 to 40 minutes, the polymerization process takes place at room temperature. Before adding the gel, the gel tank is filled with 1 TBE of running buffer. After solidifying, the gel is delicately poured into the gel tank, and the combs are gently removed. Each sample is put into a well along with 3µl of extracted DNA and 3µl of the loading dye, 6X Bromophenol Blue. The gel electrophoresis apparatus is shut down and programmed to operate at 120 volts for 25 minutes. Using the Gel Documentation System, the gel is carefully checked under UV light when the running is over. Table 2.2 summarizes the ingredients of agarose gel and other necessary chemicals.

Table 2.2. Composition of Agarose gel and other required chemicals

S.No	Solutions	Composition
1	1% Agarose Gel (50 mL)	1X TBE Buffer (50 mL)
		Agarose (0.5 g)
		Ethidium Bromide (2 µL)
2	2% Agarose Gel (50 mL)	10X TBE Buffer (5 mL)
		Agarose (1.0 g)
		Ethidium Bromide (5 µL)
		Distilled water (45 mL)
3	Gel Preparation Buffer (10X TBE)	Boric Acid (27.5 g)
		EDTA (3.6 g)
		Tris (54 g)
		Deionized water (500 mL)
4	Gel Running Buffer (1X TBE)	10X TBE Buffer (1 part)
		Distilled water (9 parts)
5	Ethidium Bromide Solution (50 mL)	Autoclaved filter water (50 mL)
		Ethidium Bromide (0.5 g)
6	Loading Dye Solution (25 mL)	Autoclaved filter water (25 mL)
		Bromophenol blue (0.087 g)
		Sucrose (10 g)

2.7 Primer Designing

The Primer-3 program V.0.4.0 (http://bioinfo.ut.ee/primer3-0.4.0) was used to create primers to amplify Exon 4 of the Interleukin-4 gene for this study. To assure the primers' effectiveness, the design procedure included adjusting the amplicon size, salt concentration, primer length, and primer annealing temperature. The Ensemble website (https://asia.ensembl.org/Homo sapiens/Info/Index) provided the reference sequence required for primer creation. The blast-like alignment was used to check the specificity of the chosen primers.

The UCSC genome browser's (https://genome.ucsc.edu/cgi-bin/hgBlat) tool (BLAT) was used. Additionally, the amplicon size for the primers was confirmed using the in-silico PCR tool on the UCSC genome browser (https://genome.ucsc.edu/cgi-bin/hgPer). Table 2.3 provides comprehensive information on each primer, including the melting temperature, product size, and locus of mutation.

Table 2.3.: Primer Information for Amplification of Exon 4 in IL-4 Gene

Gene	Exon	Locus	Primer Type	Primer Sequence (5'-3')	Primer Length (bp)	Product Size (bp)	Melting Temp. (°C)
IL-4	4	Chrom 5	Forward	GAGAGGTTGTTGACAGAGGT	20	559	54.1
			Reverse	TTTAGTGACACGTCCTCAGC	20		56.5

2.7.1 Primer Dilution

Primers were first ordered at a concentration of 100 picomole/µl. PCR water was added to achieve further dilutions, and ultimate concentrations of 10 picomole/µl were produced.

2.7.2 Primer Optimization

To optimize primers, gradient PCR was employed and 68-63°C was best annealing temperature for the primer pair that was used to assess the polymorphism in exon 4 of the IL-4gene.

2.8 Polymerase Chain Reaction (PCR)

All genomic DNA samples taken from individuals who verified positive for ascariasis were amplified using the PCR technique. The PCR reactions were conducted in 200 L-capacity PCR tubes (Axygen USA). Table 2.4 includes a list of the chemical concentration and volume employed in the reaction mixture.

Table 2.4. Chemical concentration and volume used in the reaction mixture.

Chemicals	Concentration	Volume
Taq buffer	10X	2.5 µL
DNTPs	2.5 mM	2.5 µL
MgCl2	2.5 mM	2 µL
Forward Primer	10 pmol/µL	0.5 µL
Reverse Primer	10 pmol/µL	0.5 µL
DNA	>100 ng/µL	2.5 µL
Taq Polymerase	5 U/µL	0.3 µL
PCR Water		14.2 µL

Before being put into the thermal cycler for the PCR reaction, the PCR tubes were given a short spin in a microfuge at 3000 rpm for 1 minute to ensure thorough mixing. The PCR cycle conditions are listed in Table 2.5.

Table 2.5: Conditions of PCR Cycles

Step	Temperature (°C)	Time	Cycle
Initial Denaturation	96°	5 min	1X
Denaturation	95°	45 sec	35X
Annealing	68-63°	1 min	35X
Extension	72°	1 min	35X
Final Extension	72°	10 min	1X
Hold	25°		

2.9 PCR Product Confirmation

In order to authenticate the PCR results, a 2 percent agarose gel was created using 1g of agarose powder, 50 ml of 1X T.E. solution, and 2ul of ethidium bromide. After that, each sample (PCR product) was mixed with 3 l of 6 X fluorescent dye (bromophenol blue). These DNA samples underwent a 40-minute, 120V electrophoresis process on a

2% agarose gel. The gel was then examined using a Gel Documentation System to confirm the amplification of the appropriate IL-4 gene fragment.

2.10 Sanger Sequencing

The PCR products were sent to Microgen Korea for commercial sequencing. And subsequent sequence analysis with chromas and mutation taster will be done once sequence result received.

2.11 Analysis of Demographic Data

The percentage analysis was done using Microsoft Excel and SPSS software constructed for the representations of data. The chi-square analysis was performed to find the most associated risk factors. The level of significance was set at $p \leq 0.05$.

RESULTS

3.1 Baseline features of the study

The demographic features of the participants from whom blood and worms was collected represent different villages and union councils of district Mardan, Khyber Pakhtunkhwa, Pakistan. Out of 265, 53 patients were infected with *A. lumbricoides.*

3.2 Phenotyping of Adult Worms

In 53 positive *Ascaris lumbricoides* patients, 18 worm samples were collected from the patients, of which 7 were male and 11 were female. The color of the worms was transparent to milky white, and brownish to grayish and yellowish as well. Longitudinal lines were present in most of the adult worms, which range from 2-3 in number, while transverse lines were present as well, which look worm-like segmented. Most of the worms haven't appeared yet, as they may not be fully developed. The worms were separated into male and female on the base of the posterior end, as in males it has a coiled posterior end while in females it has a straight posterior end. Vulvar waist: The vulva opens at the junction of the anterior and middle third of the body, and this section is narrower and is called the vulvar waist. This was also noted in some female worms, which is the most prominent part of the worm. The diameter range (from smallest to largest) is 3–6.25 mm. While the length is 12.9–32.8 cm. The vulvar waist that is present only in females has been seen in only 4/11 (36.37%) female worms. The weight of the worm ranged from 0.47 g to 4.485 g (Table 3.1).

Table 3.1: Morphological data of *Ascaris lumbricoides* worms.

S #	Sex	Dimeter (mm)	Length (cm)	Lateral line	Transverse line	Color	Vulvar waist	Weight (g)
1	Male	3.35	17.4	Absent	Absent	White Transparent	Absent	0.78
2	Female	6.25	21.2	Gray lines	Present (segmented)	Grey	Present	3.95
3	Male	4.35	20.9	Lightly present	Absent	Yellowish	Absent	2.079
4	Female	4.25	29.8	Present	Present	Yellowish white	Absent	3.79
5	Female	5.25	32.8	Present	Present	Gray	Present	4.485
6	Female	4.25	23.3	Absent	Present	Gray	Absent	1.91
7	Male	3.25	15.3	Absent	Absent	White	Absent	0.602
8	Male	3	13.5	Absent	Absent	Transparent	Absent	0.664
9	Female	5.40	30.3	Present	Present	Gray	Present	4.31
10	Male	3.25	18.9	Absent	Absent	Milky white	Absent	0.993
11	Female	3	15.4	Absent	Absent	Transparent	Absent	0.47
12	Female	4.075	22.85	Present	Absent	Transparent white	Absent	1.70
13	Female	4.30	24.4	present	Absent	Milky white	Absent	1.94
14	Male	5	19.6	Present	Present	Brownish white	Absent	1.5
15	Male	4.42	12.9	Present	Present	White	Absent	1.0
16	Female	4.25	27.0	Present	Present	Brownish white	Absent	2.64
17	Female	5.075mm	27.7	Present	Present	Brownish white	Present	3.42
18	Female	4.375mm	27.9	Absent	Absent	White	Absent	2.35

3.3 Prevalence of *A. lumbricoides* according to Socio-demographic factor

Table 3.2 presents the sociodemographic characteristics and prevalence of *A. lumbricoides*. Out of a total of 53 positive people who had *A. lumbricoides* infection, 37 patients were male, accounting for 13.96% of cases, and 16 patients were female, accounting for 6.04% of cases (Fig. 3.1). However, the results showed no significant association ($\chi 2$ =0.421, p= 0.316). In the study participants whose age range was 1–10, which accounts for 12.45%, followed by 11–20, which was 3.77% (Fig.3.2), while the difference was not significant ($\chi 2$ =7.74, p= 0.52). People living in villages had account for 18.86%, and 1.14% in the city. However, the results showed no significant association ($\chi 2 = 0.485$, p=0.168). Based on living standard, the highest prevalence was seen in the people who had a lower standard, which is 15.86%; in the middle, it

was 4.16%; and in the upper class, had zero (Fig.3.3). The result showed a significant association with infection ($\chi 2$=0.362 p=0.009). The poor environmental hygiene accounts for 16.60% of infection followed by satisfactory status (1.88%) and good status (1.51%) (Fig. 3.4). However, the association was significant ($\chi 2$ =0.882 p=0.001). People with poor living conditions account for 15.84%, while those with good living standards account for 4.16%. This data showed insignificant relation with infection ($\chi 2$ =0.864 p=0.06). Out of 53 people that had been infected with the ascaris worm, 9.81% were going to school, and 10.19% were at home, while no significant relation($\chi 2$ =0.848 p=0.64) was observed. People with no education have the same infection level as those with primary education (7.92%), high school (2.26%), collage (1.50%), and university (0.37%) The result showed no significant association with infection ($\chi 2$=0.154 p=0.128). Participants engaged in labor had highest infection of 9.81%, followed by farmer 6.03%, and 1.50% was in drivers (Fig.3.5). However, the result showed a significant association with infection ($\chi 2$=0.756 p=0.013).

Table 3.2: Scio-demographic characteristics and prevalence of *A. lumbricoides* among studied participants.

Characteristics	Total n (%)	Positive n (%)	Chi-square Value	P- value
Prevalence over all	265(100)	53(20%)		
Gender			0.421	0.316NS
Male	175(66.0)	37(13.96)		
Female	90(34)	16(6.04)		
Age			7.743	0.52NS
1-10	185(69.8)	33(12.45)		
11-20	56(21.1)	10(3.77)		
21-30	12(4.5)	5(1.88)		
40<	12(4.5)	5(1.88)		
Residence			1.485	0.168NS
Rural	238(89.8)	50(18.86)		
City	27(10.2)	3(1.14)		
Living status			9.362	0.009*
Lower	165(62.3)	42(15.84)		
Middle	86(32.5)	11(4.16)		
Upper	14(5.3)	0(0.0)		
Environmental hygiene			13.882	0.01*
Good	54(20.4)	4(1.51)		
Poor	161(60.8)	44(16.60)		
Satisfactory	50(18.9)	5(1.89)		
Condition of living			6.864	0.06NS
Poor	169(63.8)	42(15.81)		
Good	96(36.2)	11(4.19)		
Are you going to school			2.848	0.64NS
Yes	157(59.2)	26(9.81)		
No	108(40.8)	27(10.19)		
Education			7.154	0.128NS
No education	88(33.2)	21(7.92)		
Primary	126(47.5)	21(7.92)		
High	40(15.1)	6(2.26)		
Collage	8(3.0)	4(1.50)		
Higher	3(1.1)	1(0.37)		
Occupation			17.756	0.013*
Business	41(15.5)	2(0.75)		
Driver	23(8.7)	4(1.50)		
Employee	8(3.0)	1(0.37)		
Farmer	67(25.3)	16(6.03)		
Foreigner	9(3.4)	0(0.0)		
Housewife	4(1.5)	3(1.13)		
Teacher	4(1.5)	1(0.37)		
Labour	109(41.1)	26(9.81)		

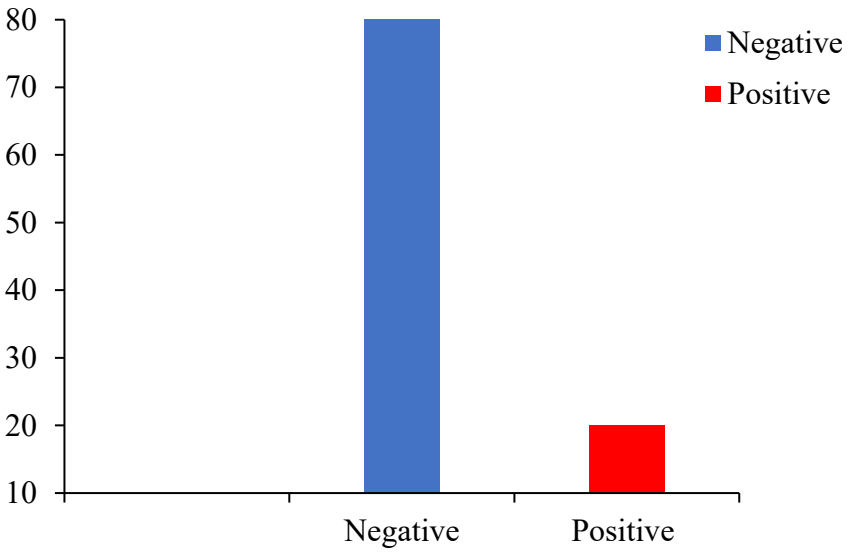


Figure 3.1 prevalence of *Ascaris lumbriciodes* in distract Mardan

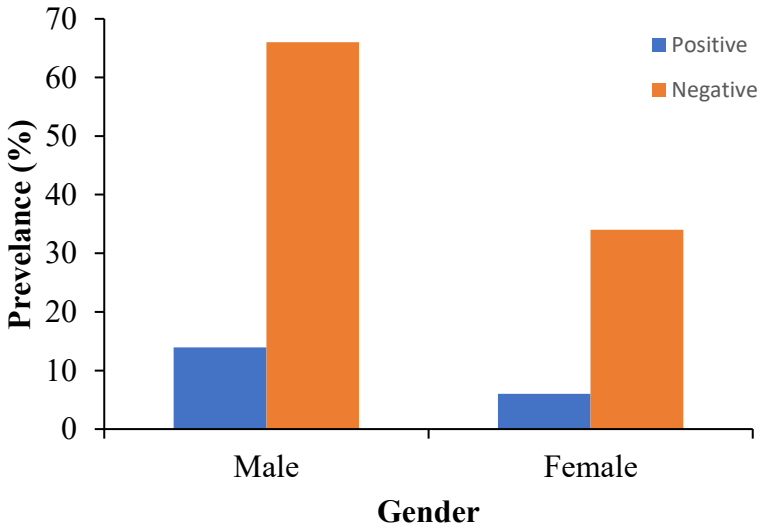


Figure 3.2 Number of infected individuals according to Gender.

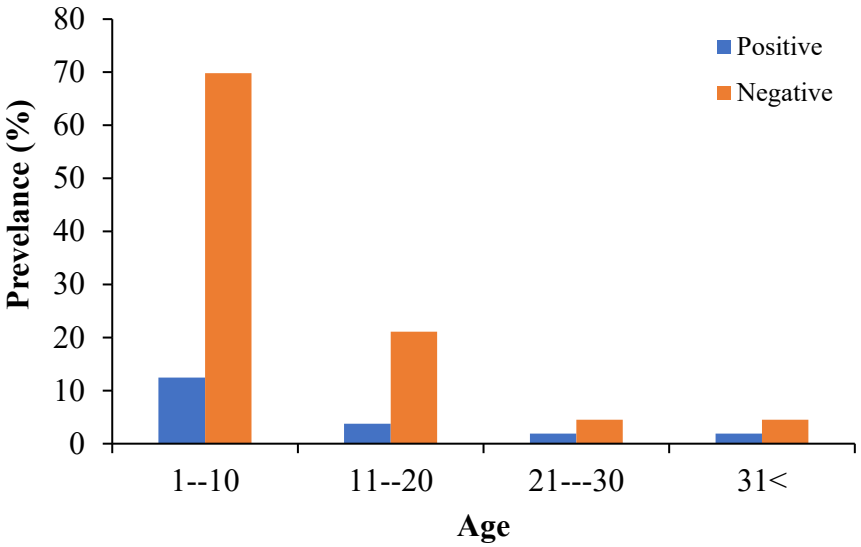


Figure 3.3. Number of infected individuals according to age groups.

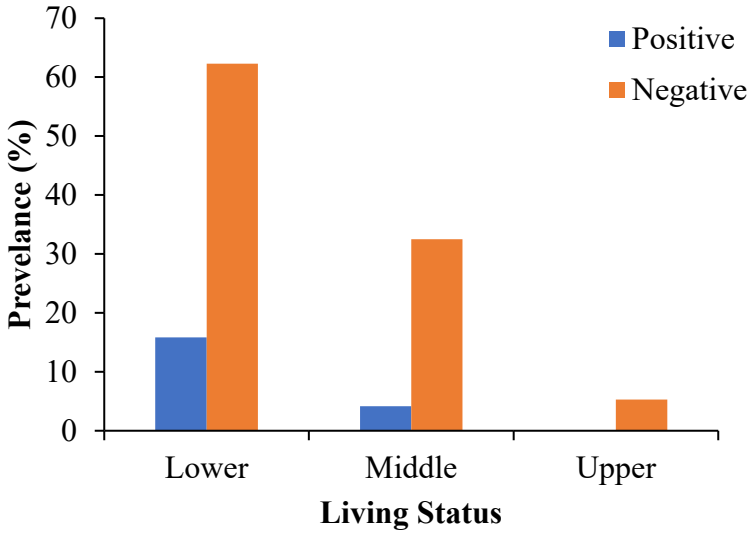


Figure 3.4. Number of infected cases according to living status.

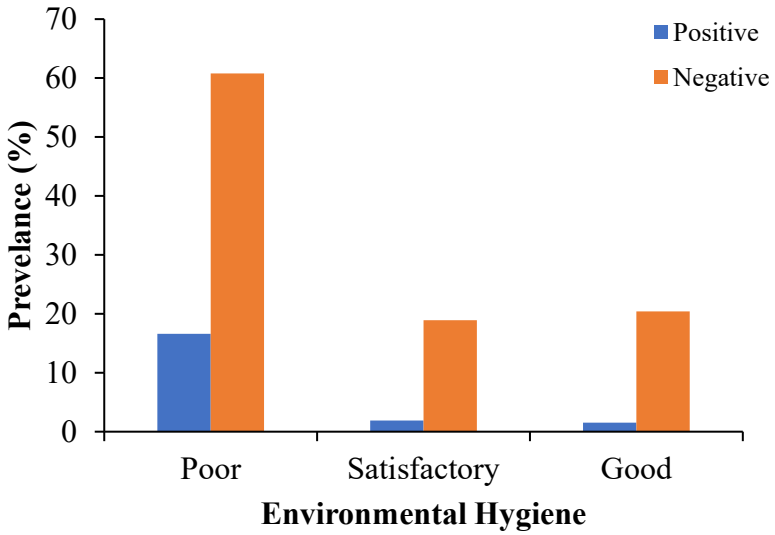


Figure 3.5. Number of infected cases according to environmental hygiene.

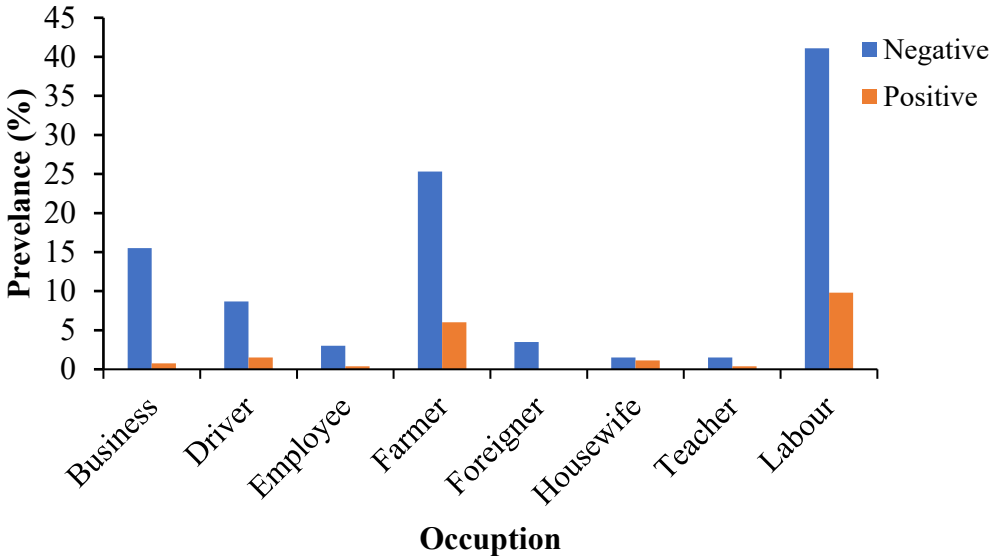


Figure 3.6: Number of infected cases of *A. lumbricoides* according to occupation.

3.4 Prevalence according to Environmental Risk factors

Table 3.3 presents the risk factor associated with prevalence of *A. lumbricoides*. People who wore slippers had prevalence of infection 16.60%, while those who did not wear them had a 3.40% (Fig.3.7). The data showed a significant relationship with infection ($\chi 2$ =0.546, p= 0.004). People who did not wash their hands before taking a meal had a prevalence of 12.45%, while it declined in washing hands to 7.54%. The results did

not show significant relationship ($\chi 2$ =0.523, p= 0.140). Participants having no washrooms at home showed a prevalence of 10.18%, while with washrooms, it was9.81%. This data showed a significant relationship $\chi 2$ =.339, p= 0.00) with infection. People who defecate in open fields have the highest prevalence, which is 15.84%, while those who defecate in washrooms/indoor had 4.16% (Fig.3.8 and 3.9). The result showed a significant relationship($\chi 2$ =0.698, p= 0.011) with infection. The standing water reservoir had a prevalence of 13.58%, while the region without a standing water reservoir had 6.41% (Fig.3.10). The result showed a significant relationship (($\chi 2$ =0.437, p= 0.002) with infection. Other risk factor like presence of grasses and bushes had 16.41% infection, while areas without bushes and grasses had 3.39%, but the results showed no significant relation ($\chi 2$ =0.588, p= 0.372). The highest risk based on drinking water was accounted for people drinking hand pump water, which was 13.58%, followed by motor pump water 1.13%. The data showed no significant relation with infection (x2 =0.917, p= 0.405). Areas where people use waste as fertilizer which was 17.37%, while those not using it had 2.63%. The data showed no significant relation with infection ($x2$ =0.104, p= 0.211). Additionally, an area where sewage water merges with running water had the highest infection rate, which is 16.22%, while in an area where there is no merging, it is 3.78%. The data showed no significant relation with infection ($\chi 2$ =0.453, p= 0.314).For people who washed their hands after defecation, the prevalence of infection was less than for people who did not wash their hands after defecation, which was 12.83% and 7.17%, respectively. Data have no significant relation with it ($X2$ =0.020, p= 0.102. Within the infection rate, 13.96% of people are not even aware of this worm, while 6.04% of infected individuals have awareness of this worm. The result showed no significant association ($x2$ =0.169 p= 0.400).

Table 3.3 Environmental risk factor associated with the prevalence of *A. lumbricoides* among studied participants.

Characteristics	Total n (%)	Positive n (%)	Chi-square value	P value
Wearing footwear			7.546	0.04*
No	87(32.8)	9(3.40)		
Yes	178(67.2)	44(16.60)		
Standing water ponds			9.437	0.002*
No	135(50.9)	17(6.41)		
Yes	130(49.1)	36(13.59)		
Washing hand before meal			1.523	0.140NS
No	145(54.7)	33(12.46)		
Yes	120(45.3)	20(7.54)		
Do you have washroom			211.339	0.00*
No	69(26.0)	27(10.19)		
Yes	196(74.0)	26(9.81)		
Where mostly people defecate			5.698	0.011*
Outsider	173(65.3)	42(15.84)		
Washroom	92(34.7)	11(4.16)		
Presence of grass and bushes			0.588	0.372NS
No	52(19.6)	9(3.39)		
yes	213(80.4)	44(16.41)		
Drinking water			2.917	0.405NS
Hand pump	184(69.4)	36(13.58)		
Motor pump	28(10.6)	3(1.13)		
Tape water	7(2.6)	2(0.75)		
Well	46(17.4)	12(4.53)		
Use waste as a fertilizer			1.104	0.211NS
Yes	240(90.6)	46(17.37)		
No	25(9.4)	7(2.63)		
Sewage water merge with running water			0.453	0.314NS
Yes	233(84.2)	43(16.22)		
No	42(15.8)	10(3.78)		
Washing hand after defecation			2.020	0.102NS
No	147(55.5)	34(12.83)		
Yes	118(44.5)	19(7.17)		
Awareness regards this worm			0.169	0.400NS
No	191(72.1)	37(13.96)		
Yes	74(27.9)	16(6.04)		

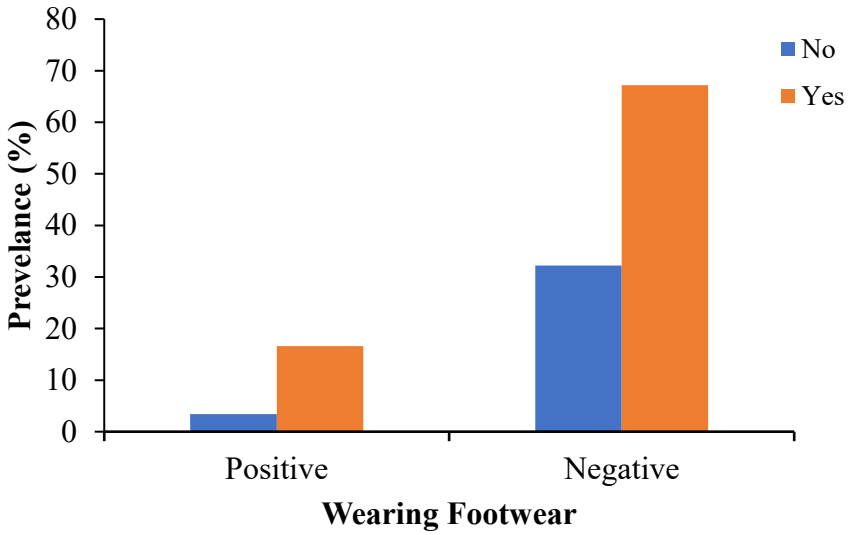


Figure 3.7 Number of infected cases according to wearing footwear.

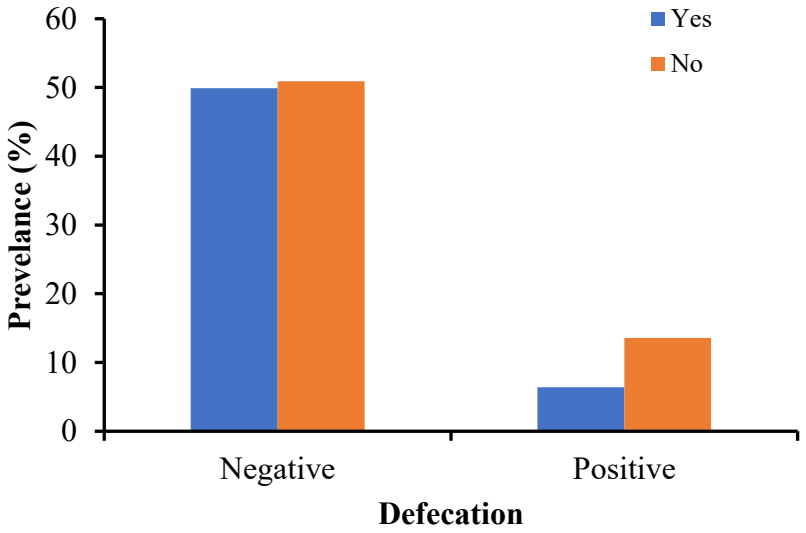


Figure 3.8 Number of infected cases according to defecation place (indoor/outdoor)

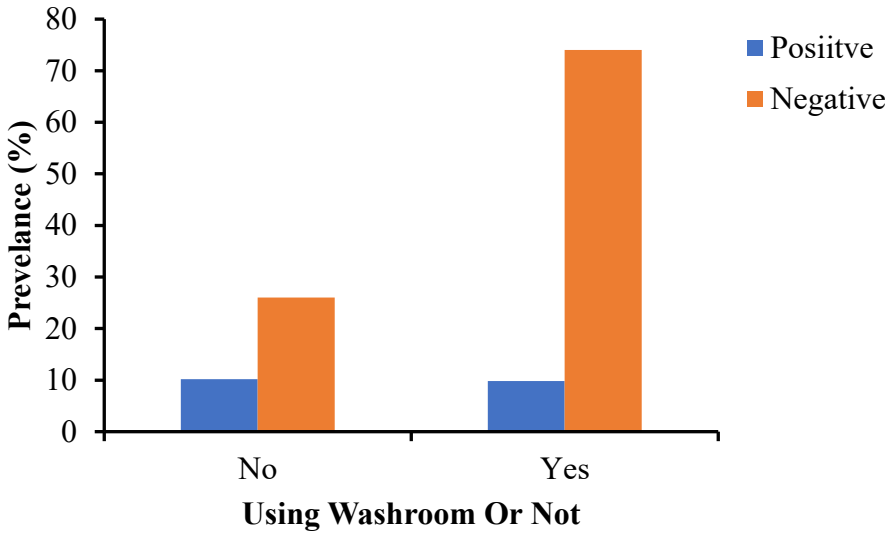


Figure 3.9 Number of infected cases according to having washroom at home.

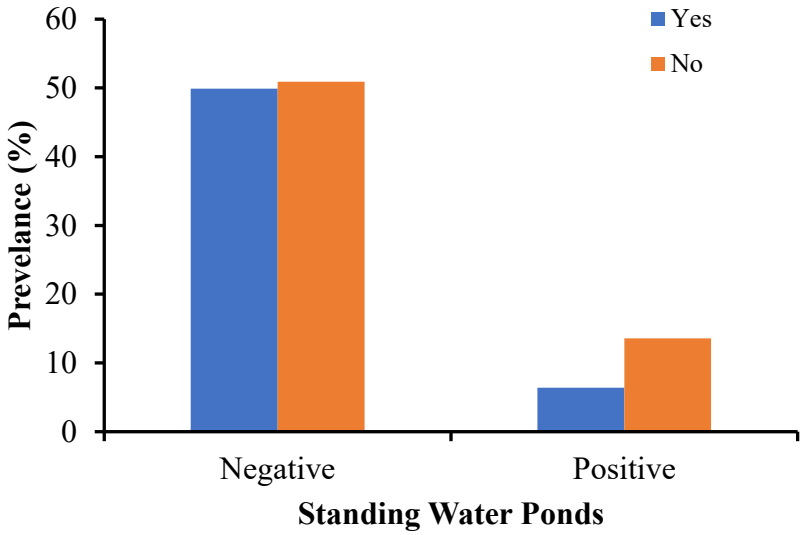


Figure 3.10 Number of infected cases according to standing water pond.

3.4 Prevalence base on clinical factor

Table 3.4 presents the association of clinical factors with *A. lumbricoides* infection**.** The patients who were not treated with anti-helminthic drugs had prevalence of 24.4%, while in treated was 11.2% (Fig.3.11). The result showed a significant relation with infection ($x2$ =0.433, p= 0.007). The people who were directly administered with anti-helminthic for worm collection showed 19.2% prevalence, while people who were

considered negative by stool microscopy had 6.2% (Fig. 3.12), however the difference was significant ($\chi 2$ =0.490, p= 0.000). Four different drugs were used, of which the most effective anti-helminthic was pyrantel pamoate, whose effective rate was 25% within infection, followed by albendazol (22.5%), mebandazole, mebandazole and piperazine (17.1% and 14.3%, respectively) (Fig. 3.13). However, the type of drugs administrated did not show significant association ($x2$ =0.808, p= 0.590).

Table 3.4 Clinical factor associated with *A. lumbricoides* among studied participants.

Characteristics	Total n (%)	Positive n (%)	Chi-square value	P value
Do you ever treated with anti-helminthic drugs			6.433	0.007*
No	176(66.4)	43(16.22)		
Yes	89(33.6)	10(3.78)		
Stool microscopy			69.490	0.000*
Negative	80(30.2)	5(1.88)		
Not done	170(64.2)	33(12.45)		
Positive	15(5.7)	15(5.66)		
Drugs administrated			2.808	0.590^{NS}
Albendazole	142(53.6)	32(12.07)		
Mebandazole	41(15.5)	7(2.64)		
Not given	2(0.8	0(0.0)		
Piperazine	56(21.1)	8(3.01)		
Pyrantel pamoate	24(9.1)	6(2.26)		

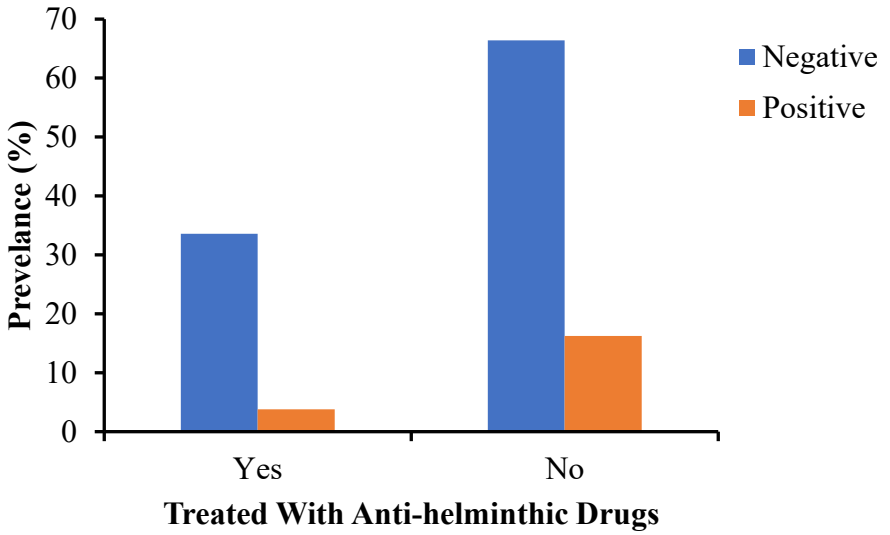
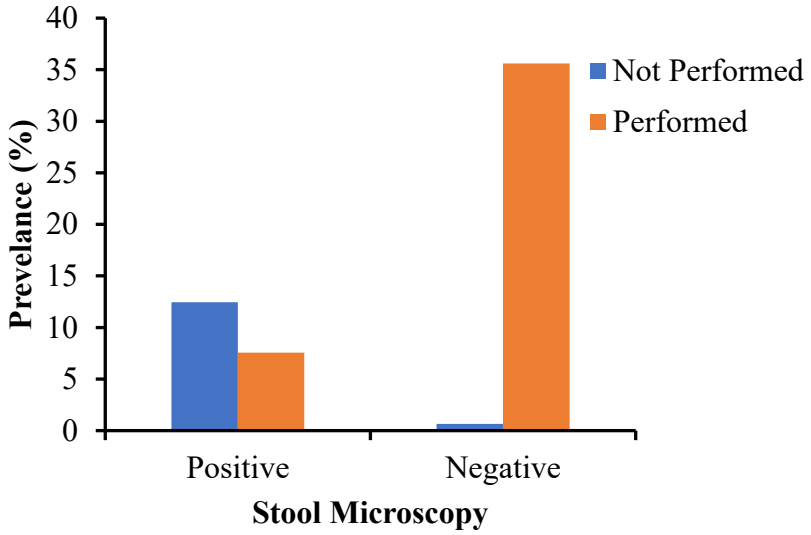


Figure 3.11 Number of infected cases according to treatment with anti-helminthic.



3.12 Graph shows stool microscopy.

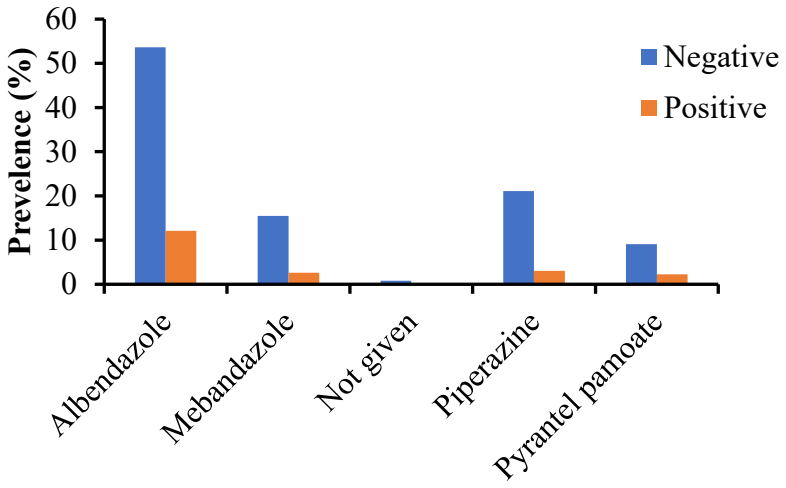


Figure 3.13. Shows the administration of anti-helminthic drugs.

3.6 Molecular Analysis

In this study, DNA extraction was carried out for each *Ascaris*-infected blood sample, resulting in an average DNA concentration of approximately 50 ng/l and the extracted DNA showing a purity value of 1.8 across all samples. Fig. 3.14).

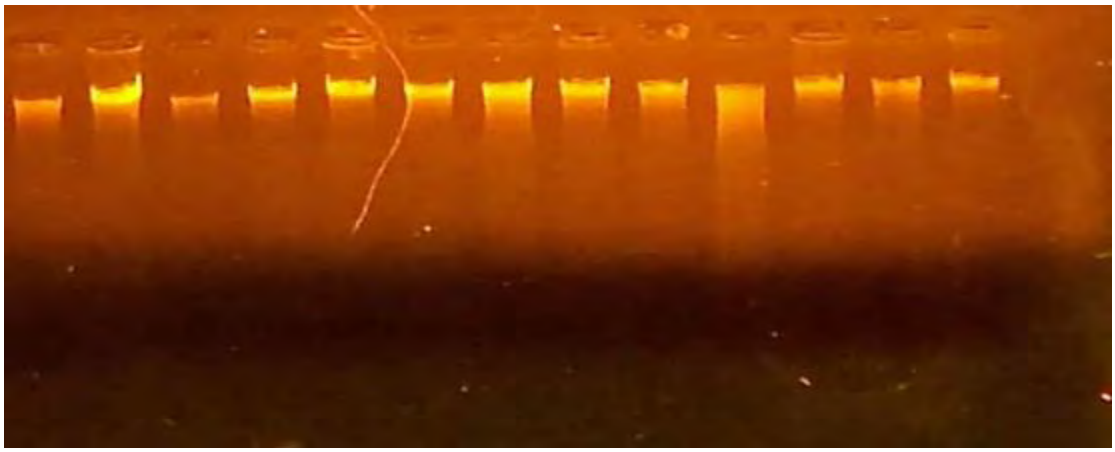


Figure 3.14 Agarose gel electrophoresis of extracted DNA samples

3.7 Polymerase Chain Reaction (PCR)

A. lumbricoides infected individuals DNA samples were amplified using the PCR. The product of 559 bp long of the IL-4 gene exon 4 was obtained. The amplicon conformation was determined by electrophoresis on 2 percent Agarose gel, and the results were recorded in the Gel Documentation system (Fig.3.15).

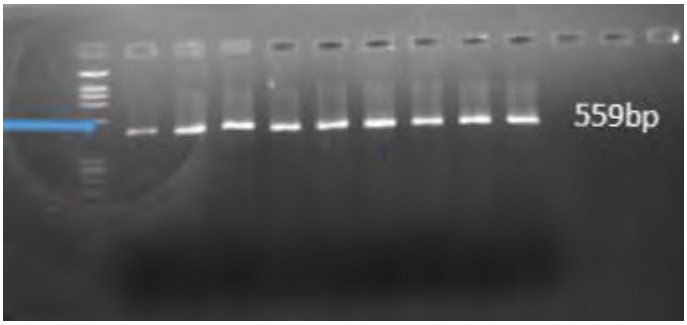


Figure 3.15: PCR product of exon 4 of the IL-4 gene.

3.8. Mutation Analysis

Exon 4 of IL-4 which codes for pleiotropic cytokine with a 559 bp length was used as an assessment for polymorphism and mutations. The IL-4 gene (Afshan *et al.*, 2022) was used as the standard sequence for alignment. The sequencing was not done due to lack of funds.

DISCUSSION

A. lumbricoides one of the biggest nematodes (roundworms), parasitizes the human gut. This nematode is thought to affect 25 percent of the world's population. The small intestine is where the mature worms reside (Kilic *et al*., 2003). According to an estimate 4.5 billion individuals are at risk of contracting one of the three major helminths transmitted by soil: the roundworm, whipworm and the hookworms (Utzinger and Keiser; 2004). The highest prevalence rates of STHs infections are seen in low- and middle-income nations with poor sanitation, access to safe and clean water, and poor hygiene. These factors all contribute to poverty (Utzinger *et al.,* 2010). A number of factors involved in the prevalence of *A. lumbricoides* that includes crowding, inadequate water supply, open defecation, poverty, poor nutritional status, use of human biosolids for fertilizers and irrigation geophagy, not washing hands before eating ((Fortes *et al.,* 2004).

In 53 positive *A. lumbricoides* patients, 18 worm and 30 bloods samples were collected from the patients. 07 were male and 11 were female. The diameter range (from smallest to largest) is 3–6.25 mm. While the length is 12.9–32.8 cm. The weight of the worm ranged from 0.47 g to 4.485 g. previous study reported male are 15 to 30 cm long and 2 to 4 mm in diameter, while female range in length from 20 to 49 cm and in diameter from 3 to 6 mm (Pawlowski, 1990). Male and female adults measure 15–25 cm and 20–35 cm respectively (Sinniah, 1982).

In the current investigation of distract Mardan Khyber Pakhtunkhwa the prevalence rate was recorded 20%, in which male 13.96% and female were 6.04%. Similarly, the prevalence of STHs infection was 12.6%, accounting *Ascaris* as the primary soil-transmitted helminth with a prevalence of 7.8% (Samuel *et al.,* 2017). In our study, the prevalence reported in females was less than that in males. The reason was that the number of females was lower than that of males. Another reason was the lack of interest of females due to religious and cultural factors, which made their contribution slightly less than that of males. Study of Tay *et al.,* The age groups of 1–10 and 11–20 had the largest prevalence, with 81.1% and 67.1%, respectively. The age group of 61-70 years old had the lowest frequency, at 23.3 %(Tay *et al.,* 2010). .

Numerous studies have revealed that Ascaris lumbricoides infection is widespread across the nation, with varying distribution. The prevalence has been recorded in Peshawar 45.5% and 38.8% (Ullah *et al.,* 2009; Shah *et al.,* 2006); Gilgit-Biltistan 22.8% (Khalil *et al.,* 2012), Swat 39.8% (Khan *et al.,* 2012), Karachi 11.9% (Fatima *et al.,* 1982), Hazara Division 7.3% (Wali *et al.,* 2016), and Dir district and Larkana-Sindh was 15.0% (Shaikh *et al.,* 2000), Muzaffarabad-Azad Kashmir 1.8% (Naheed *et al.,* 2009), Chitral 68.7% (Stoddart :1999) and Quetta city was 1.8% (Naheed *et al.,* 2009).

The highest prevalence in study participants whose age ranged 1–10, accounts 12.45%, followed by 11–20 with 3.77%. The results and in agreement with previous study, who recorded children between the ages of 11 and 15 had a slightly greater frequency of STHs than those between 6 and 10 (Samuel *et al.,* 2017). The results of the research revealed that the age of children between the 6 and 15 age groups did not substantially affect the prevalence of STHs infection (Montresor and colleagues, 2002). Taking part in activities that enhance exposure to contaminated soil or water (Bakuza, 2018). Meanwhile, ongoing infection exposure may favor a gradual decline in worm burdens as a partial immunity to new infections develops in adulthood (McManus *et al*., 2018), while low immunity at a younger age may contribute to increased susceptibility to infection in the pediatric inhabitants (Mnkugwe *et al.,* 2020).

In developing nations, open defecation has been a significant issue. Some of the causes were the lack of toilet facilities in homes, particularly in rural regions, and the absence of public restrooms in the majority of human populations (Ajayi and Philip 2018). People who defecate in open fields had the highest prevalence of 15.84% as compared to those who defecate in washrooms. Similar results were recorded in previous study that children who did not have access to latrines at home were more impacted than those who did. Additionally, people who use conventional pit latrines are more likely to contract intestinal helminths than those who use improved latrines with ventilation (Samuel *et al.,* 2017). The presence of a toilet was not linked to the prevalence of STH infection when factors related to sanitation and hygiene were examined, but a link was initially identified based on whether the toilet met the necessary standards. In another research, the prevalence for individuals with standard-compliant toilets was 7.4%, whereas the prevalence for those without standard-compliant toilets was 23.6% (Zacharia *et al.,* 2023). Despite the high coverage of

sanitation facilities in the study area, open defecation is still common; 2.4% of participants do not use a toilet at home, and 20.6% do not use a toilet when going about their daily activities. This increases the risk of co-infection with intestinal parasites that can spread through the mouth and feces.This discovery highlights the necessity of creating and maintaining safe water sources in endemic regions for washing and drinking (Al-Shehri *et al*., 2016). Children who defecated in an open field were statistically associated with A. lumbricoides infection. Children who left the bathroom or went to a public washroom were more likely to become infected with A. lumbricoides (100%) than those who left the bathroom at a neighboring facility (90.0%) (Hajare *et al.,* 2022). While 80.8% of research participants had sanitary toilet facilities in their residences, while 19.2% used open fields for this purpose (Gopalakrishnan *et al.,* 2018). It was discovered that roughly 74.5% of people were using open fields as toilet. The cause of the high rate of open field defecation as determined by a research carried out in the village (Ashok *et al.,* 2013).

The frequent wearing of shoes appears to have a key role in the low prevalence rate of helminth infections; yet, (Tadesse: 2005). People who wore slippers had significantly highest prevalence of infection (16.60%), while those who did not wear them had a 3.40% prevalence. Another study reported a strong ($p = 0.000$) correlation between parasite infection and shoe use. According to these findings not wearing any footwear had the greatest impact on the occurrence of helminthiases (Rahmi *et al.,* 2021). When compared to individuals who did not use shoes, the habit of wearing shoes was associated with a reduced prevalence of infection (8.2% vs. 42.9%), and a higher risk of infection was seen among those who did not wear foot protection (Zacharia *et al*., 2023). children who regularly wore shoes had much lower prevalence rates of hookworm infection (Tadesse: 2005). According to Kidane *et al.,* 17.7% were positive in those who were wearing shoes, while 50% were positive in those who were not, but there is no significant association between wearing shoes or not and STH prevalence (Kidane *et al*., 2014).

Based on occupation significantly highest ratio of prevalence was seen in Labour, 9.81%, followed by farmer, which was 6.03%, and 1.50% in drivers. The results are in agreement with previous study, where they discovered that farmers (11.5%) had much higher prevalence than other occupations (Zacharia *et al.,* 2023). Based on occupation, agricultural workers had the highest rate of intestinal parasite

infections (19.4%), however Suntaravitun and Dokmaikaw found no connection between intestinal parasitic infections and occupation (Suntaravitun and Dokmaikaw 2018). The lowest incidence was identified among civil officials, whereas farmer and jobless had strong intensity (Tay *et al.,* 2010). According to a regression study of possible covariates, pregnant women's occupation (farming) and the source of their drinking water exhibited a statistically significant correlation with intestinal parasite infection (Aschale *et al.,* 2022). The current research also demonstrates similarities to previous findings by Hailu *et al.* and Yesuf *et al.* (Hailu *et al.,* 2020; Yesuf *et al.,* 2019).

According to a recent meta-analysis examining the relationship between water, sanitation, and hygiene and STH infections, having access to piped water and appropriate water treatment was linked to a decreased incidence of infections with A. lumbricoides and T. trichiura (Strunz *et al.,* 2014). In this study the people drinking hand pump water had prevalence of infection 13.58%, followed by drinking well water (1.88%) and tape water and motor pump (0.75% and 1.13%, respectively). Unprotected springs were found to be the primary source of water for domestic use (62.7%), followed by rivers (23.9%), with no discernible difference in the prevalence of infection among participants who fetched water from various water sources. According to water intake, no other significant variation in prevalence was noted (Zacharia *et al.,* 2023). Yang et al. found that utilizing well or river water enhanced the chance of contracting *A. lumbricoides* infection. Wells or rivers, which are both near to populated areas and subject to fecal contamination. In addition, 14.33% of student households drank water from a river or well, and 84.42% of schoolchildren regularly drank unboiled water (Yang *et al.,* 2018).

The prevalence of infection was lower among those who cleaned their hands after defecation (12.45% vs. 7.55%), indicating that this practice reduces the risk of infection. The prevalence among the participants hand washing before meals (always or sometimes) and defecation were statistically significant ($p < 0.001$) (Sitotaw *et al.,* 2019).The study of Khan *et al*, shows that, hand washing after toilet and finger nail status are not statistically (>0.05) associated with the prevalence of these infections(Khan *et al*, 2017). According to Zacharia *et al,* in the beginning, having a hand washing facility was associated with a lower participant infection prevalence and a higher infection risk for those without one. Within the infection rate, 19.4% of people are not even aware of this worm, while 21.6% of infected individuals have awareness of this

worm but data didn't show any significant relationship with infection. Unclean fingers and not washing hands before eating were strongly linked to intestinal parasite infection, which was in line with findings from earlier research. (Hailegebriel, 2017). It is suggested that unclean hands and filthy fingers might harbor parasite eggs and cysts, increasing the chance of infection. This result was in line with previous research (Abossie and Seid, 2014). In contrast to heads of households who consistently wash their hands before eating, individuals who don't always wash their hands before meals have a seven-fold increased risk of developing STHs (Zeynudin *et al.,* 2022). Similar results were seen in studies carried out in the western area of Cameroon, which showed that families that did not wash their hands before meals were more susceptible to STHs. (Igore *et al.,* 2020) and the Bibugn district in northwest Ethiopia (Goshu *et al.,* 2021)

The present results showed a significant association with environmental hygiene and living status. The high frequency of infections among people with low socioeconomic level can be attributed to several factors, including poor living circumstances, a lack of sanitation, the use of contaminated water supplies, and incorrect waste disposal (Siddiqui *et al.,* 2002). Poor personal hygiene and environmental sanitation are widespread, as they are in most developing nations (Yodmani *et al.,* 1982). Environmental contamination, sanitary conditions, and human behavior all play a significant part in the spread of STH infection (Yu *et al.,* (1993). People living in villages had the highest prevalence of 18.86%, while the prevalence declined to 1.14% in the city, results are in line with earlier research (Suntaravitun and Dokmaikaw; 2018). Sanitation, proper toilet use, and environmental and personal cleanliness all greatly reduce the incidence of STHs in a community or institution (Strunz et al., 2014)

Mebendazole and Albendazole, and benzimidazole anthelminthic medications, are the most often used for the treatment of STH. The World Health Organization (WHO) also recommends pyrantel pamoate, levamisole, medications for the treatment of STHs (WHO, 2002).In the present study, four different medications were utilized. Albendazol, with an efficacy rate of 12.07% within infection, was the most effective anti-helminthic, followed by piperazine (3.01%), mebandazole, and pyrantel pamoate (2.64% and 2.26%, respectively). The cure rates for *A. lumbricoides* infection with single-dose oral Albendazole, Mebendazole, and pyrantel pamoate were 88%, 95%, and 88%, respectively (Keiser and Utzinger; 2008). Single-dose Mebendazole and

Albendazole are quite effective against A. lumbricoides, according to a recent meta-analysis, with pooled cure rates of 95.7% and 96.2%, respectively, and egg reduction rates of 98.5% and 98%, respectively (Moser et al., 2017). Anthelmintic medications (like Mebendazole) are known to be very effective against *Ascaris* infection and work on both the juvenile and mature worms in the gut. For *A. lumbricoides*, drug effectiveness is often in the range of 90% or higher (de Silva *et al.,* 1997). Albendazole-ivermectin currently represents the best candidate for inclusion in STH control guidelines to improve treatment of *T. trichiura*, along with field trials (Clarke et al., 2019).

In the present study to understand the role of *IL 4* gene in helminths resistance and susceptibility was investigated by screening the exon 4. IL-4 is an essential immune-regulatory cytokine that controls the development of the TH2 fraction of humorally mediated T helper cells from precursor TH cells (Zhu, 2015). In addition to acting as a key regulator of IgG isotype switching, which is essential for anti-parasitic immunity, it also starts the synthesis of immunoglobulin E (*IgE*) in B-lymphocytes (King and Mohrs, 2009). Eosinophils, a crucial component of the host's defense against various helminths, are produced and activated as a result of IL-4's elevation of immunoglobulin IgG and IgA synthesis (Baqai, 1996). The human *IL-4* gene has been shown to be polymorphic, and variations in this gene have been linked to changed *IgE* and *IgG* levels as well as the balance of the TH-1 and TH-2 ratios, among other cytokine functions (Verra *et al.,* 2004). Numerous variants in the *IL4* gene, including +33C/T (rs2070874), -34C/T (rs2070874), -524C/T, 590C/T (rs2243250), 589C/T, +3437C/G (rs2227282), and 2979G/T (rs2227284), have been linked to various disorders (Rockman *et al.,* 2003). The binding of NFAT, a crucial transcriptional activator of Interleukin 4 (IL4) in T cells, is impacted by a single nucleotide polymorphism in the promoter of this multifunctional cytokine (Weirenga and Messer, 2000). This regulatory polymorphism affects the immune system's equilibrium of cytokine signaling, which can have significant positive and negative effects on human health (Noguchi *et al.,* 2001). The majority of them are located in the promoter region and are connected to *IgE* production regulation (Nasledniková *et al.,* 2007). *IL4* has a number of SNPs that have been found. IL4589C>T (rs2243250), at least one of them, appears to be a functional polymorphism. It has been demonstrated that *IL-4* transcriptional activity is increased by the 589T allele, which is equivalent to the variants 590T, 588T,

523T, and 549T (Rosenwasser *et al.,* 1995). The 589T allele is a component of a shared haplotype, according to a thorough investigation of the sequence variants in the structuralIL4 gene in 23 Caucasians (Nickerson, 2006). In this haplotype, the 33C>T SNP (rs2070874) in the 5′ untranslated region (UTR) of exon 1 is entirely related to 18 additional SNPs. The promoter region of this IL4 haplotype solely contains the 589C>T SNP. Both the 589C>T and the 33C>T SNPs affect intermediate traits like IL4 production (Bittar *et al.,* 2007). Thus, IL-4 polymorphism can affect the severity of a variety of infections, including enteric pathogens (Clough *et al.,* 2011), as well as the progression of a variety of inflammatory and immune disorders, such as asthma (Noguchi *et al.,* 2001), multiple sclerosis (Vandenbroeck and Goris, 2003), and others (Paffen *et al*., 2008). Despite playing a crucial role in the control of parasitic infection and the evolutionary significance of *IL-4* SNPs, empirical evidence of the impact of *IL-4* on the severity of parasitic nematode infections in the human population is still missing. Finding DNA changes called polymorphisms, such as insertions, deletions, and single nucleotide polymorphisms (SNPs), was the major objective of this study. SNPs are the most common type of genetic variation, accounting for over 90% of human genetic variability (Crawford and Nickerson, 2005). The current investigation sought to identify *IL-4* gene in individuals who had contracted *A. lumbricoides* from Pakistan. Numerous research investigated at the functional importance of *IL-4* polymorphisms in various illnesses around the globe, but to the best of our knowledge, this is the first study to look at how *IL-4* variations against STH infection. Therefore, further research on sequencing of *IL-4* gene in Pakistan population is required.

Conclusion and Recommendations

The study illustrates that several risk factors play important roles in the prevalence of *A. lumbricoides.* The area is a diverse region of several helminths and needs a wide-scale investigation to ensure the reason for such a high rate of prevalence. Comprehensive research is required in this area to find a solution for how to reduce the prevalence of STHs in this area. The anti-helminthic campaign shouldn't be limited to schools only but also to local people, as most of the study participants are adults from various age groups. Educating people about environmental and personal hygiene is very important, even if they don't have any awareness about worm transmission.

Further studies on screening of *IL 4* gene are required to understand the genetic basis to treat the helminth infections.

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