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Molecular Identification of *Enterobius vermicularis* and Its Impact on Gut Microbiota Diversity in Primary School Children in Wasit Province, Iraq

Aya A. Mohammed

Department of Pathological analysis, College of Science, University of Wasit , Al-Kut, Iraq

Abstract

Despite its high infectivity and re-infectivity, *Enterobius vermicularis* is one of the most common intestinal helminth infections in school-age children globally, and remains a major public health concern. The present study aimed to study the prevalence and molecular features of *E. vermicularis* in primary school children in Wasit province, Iraq and also its association with the diversity of gut microbiota. All 85 stool samples were subjected to conventional parasitological examination and *E. vermicularis* was identified from the stool samples by polymerase chain reaction (PCR) of the mitochondrial cytochrome c oxidase subunit I (cox1) gene ~407 bp fragment. Characterization of the genetic relationship of the detected isolates was done by DNA sequencing, BLAST analysis and Phylogenetic analysis of positive PCR products. At the same time, the intestinal microbial community was studied by analyzing 16S rRNA gene sequences of the total bacterial DNA in fecal samples. Alpha- and beta-diversity indices were used to compare gut microbiota of infected vs. non-infected children. Molecular detection of *E. vermicularis* was performed in 85 children of whom 35 (41.2%) were positive. The parasite isolates demonstrated variation and confirmed their affiliation with *E. vermicularis* sequence already published in GenBank by nucleotide analysis. The composition of bacterial communities and diversity between infected and non-infected children were significantly different in the microbiome analysis. Based on our results, *E. vermicularis* infection is associated with alteration of the microbial community of the gut. The study provides molecular and microbiological evidence which sheds light on enterobiasis and parasite-microbiota interactions in Iraqi school children.

Keywords

Enterobius vermicularis; Gut microbiota; Molecular identification; *cox1*; 16S rRNA

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Faculty of Science, ATBU Bauchi

Corresponding Author: Aya A. Mohammed, Department of Pathological analysis, College of Science, University of Wasit , Al-Kut, Iraq.
Email: aya.mohammed@uowasit.edu.iq © 2026 Faculty of Science, ATBU Bauchi. All rights reserved

1. Introduction

Pinworms are one of the most common intestinal worms of man and are chiefly found in school-age children, *Enterobius vermicularis*. The primary route of infection is ingestion of eggs which are placed in the host's mouth by contaminated hands, clothing, bedding, food and environmental surfaces. A high rate of contact between children in the schools and the households may facilitate the spread of disease and lead to re-infection. In Iraq, *Enterobiasis* is common with very low clinical manifestation of perianal itching and sleep disturbance but considering the high prevalence rate of infection prevalence among school aged children from different geographical areas (Wasit Province), E. a worm has been reported in stool samples from school aged children from different parts of the province.

A previous study has reported the presence of the parasite in primary school children and usefulness of using the adhesive tape method in Wasit. Molecular studies were also demonstrated for the identification of *E. vermicularis* in children of Wasit by molecular markers (18S rRNA gene and mitochondrial cox1) through PCR. They also demonstrated that genetic variability exists within the isolates obtained in each locality and the relationship between them and other geopolitical regions, even though traditional parasitological methods remain valid for the detection of *E. vermicularis*, molecular techniques have greater potential to obtain confirmative identification and provide an opportunity to carry out genetic characterization. The parasites can be confirmed using PCR amplification and sequencing, and comparisons with sequences in global databases can be made to local isolates. The approach can be applied for studies on epidemiology of parasite populations, and genetic diversity or phylogenetic relationship of them.

The intestinal microbiota constitutes a highly complex ecosystem of microorganisms which are involved in key functions of digestion, metabolism, immune regulation and maintenance of intestinal homeostasis. It could be influenced by age, diet, health and infectious diseases, and medication. These microbial communities are still developing in children and can thus be influenced by environmental and infectious factors. [1–4, 9, 12, 19, 20].

The 16S rRNA gene sequencing is a useful culture independent approach to analyze bacterial community within the gut. The approach allows for identification, comparison of bacterial taxa and offers a tool for comparing microbial diversity by common metrics like "alpha" and "beta" diversity. A few of recently published studies implemented 16S rRNA sequencing to study gut microbiota in Iraqi children, which may pave the way for microbial analysis in the local population by molecular approaches. [21–28].

The relationship between gut bacterial composition and gut bacteria diversity with intestinal parasites has become a key research interest, due to the possible influence of parasite infections on these components. Studies conducted in school children have reported differences in gut microbiota associated with *E. vermicularis* infection, which suggests that it is associated with changes in the intestinal microbe's environment as gut microbiome disturbances may facilitate Enterobius' colonization. Although molecular identification of *E. vermicularis* was previously performed in Wasit Province and some prevalence studies had been conducted on the presence of *E. vermicularis* in Iraq school children, this study aims to understand the relationship between *E. vermicularis* with the diversity of gut microbiota in school children in Iraq. Therefore, the molecular characterization of *E. vermicularis* in primary school children from Wasit Province, Iraq and the association between this pathogen and the diversity of gut microbiota and the difference in the bacteria community composition in the stool in infected children compared to uninfected children were determined in this study. [1, 2, 5–8].

2. Literature Review

2.1. *Enterobius vermicularis*

2.1.1 Taxonomy and General Characteristics

Pseudomonas aeruginosa Enterobius vermicularis, a nematode of the family Oxyuridae and phylum Nematoda which inhabits the intestine of humans. The pinworm commonly is the agent of enterobiasis. It is a worldwide distribution parasite and it commonly infects preschool and primary school children. *E. vermicularis* is known as the definitive host of humans and is known to be transmitted by intestinal contamination (by ingestion of eggs). Its life cycle is very simple; it does not have any intermediate host, so it is well suited to infect humans.

An important characteristic of *E. vermicularis* is its high infectivity. Females deposit eggs around the perianal area which become infectious in a short time. There is a high probability of children being in contact with other children and there is a high probability of children having hand to mouth contact with the parasite, which is likely to be spread easily between homes and schools. Reinfection and autoinfection are other important factors contributing to the persistence of *enterobiasis*. [1]

2.1.2 Morphology

The adult *E. vermicularis* are nematodes (roundworms) which are small and thread-like, and sometimes white or transparent. Females are larger than males, and reach a length of 8-13 mm, whereas males are significantly smaller. All females have a long, pointed posterior end (the species is also characterized by this trait), the latter male has a short and rounded posterior end. Male is short-bodied, bluntly posterior and possesses a single spicule. Both sexes have typical cephalic expansions the eggs are transparent and elongated or oval in shape with a distinctive asymmetrical part. One side is levelled and the other one rose. They are usually 50–60 µm long (length) and 20–30 µm wide. The eggs are usually rounded, thin-shelled and are usually already embryonated at the time of lying. This morphology is rather specific and allows their identification by microscopy, particularly for specimens taken from the perianal region. [2]

2.1.3 Life Cycle

E. vermicularis is a direct life cycle parasite. Man is infected through ingestion of embryonated eggs. After their ingestion, the larvae emerge in the small intestine and migrate into the large intestine where they develop into adults. Adult worms are commonly seen in the caecum and the proximal parts of the colon. As the egg shaping takes place in the adult worm (the female), the gravid female, without any hesitation, walks towards the perianal region and lays a large number of eggs on the skin around the anus during the night; under good conditions, these eggs are infective within about four to six hours. From ingestion of infective eggs to depositions of eggs by mature females is a matter of about 30 days. This relatively short cycle makes it easy for the infection to be maintained in households and schools. Autoinfection, which is when an infected individual scratches the perianal area and then puts the contaminated eggs in their mouth. [3]

2.1.4 Transmission

It is mostly spread from person to person by the fecal–oral route, via ingestion of infective eggs. Fingers can be contaminated with the parasite and clothing, bedding, toys, furniture and surfaces in household and school areas are potential secondary vectors for indirect transmission. From this mix-up of article names, the key feature of autoinfection is that eggs can be destroyed in the medium and then eaten afterwards by animals that have touched fecal material. Autoinfection is especially apparent in children. The itching around the anus causes the child to scratch the area and the stickiness of eggs on his or her fingers and nails. That is, if handwashing is not thorough enough, hands contaminated with eggs transfer them to food and/or the mouth. This cycle can result in reinfection both after treatment and during treatment. Therefore, personal hygiene and environmental cleaning are two factors which could contribute to reducing transmission. [4]

2.1.5 Epidemiology

The prevalence is high in all parts of the world, higher in developed countries and also in the developing countries. Children of school age, and/or in crowded environments are most susceptible to being infected. The epidemiologic significance of *enterobiasis* is not only on the basis of its prevalence, but on the basis of its tendency to recurrence, which is related to the following epidemiological factors: age, continuity of domestic conditions and hygiene measures, population density, diagnostic methods and environmental factors. The eggs can easily be transferred to humans, from another person and contaminated surface and thus infection can be reinforced within a family or school. Children are recognized as an important group with which to aim surveillance and control programs. [5]

2.1.6 Clinical Manifestations

Enterobiasis may not cause any symptoms, particularly if the infestation is light. If symptoms occur, pruritus, or itching, around the anus is the most common symptom. Strong discomfort is mainly due to the migratory activity and deposition around the anal aperture of the pregnant female. Repeated scratching can lead to excoriation and an increased risk of superadded bacterial infection Other associated symptoms include sleep disturbance, irritability, restlessness, abdominal pain, anorexia, urinary or genital symptoms in some cases. Infection in females is caused by worms moving into the genital tract and causing vulvovaginitis. More uncommon have been ectopic migration towards the appendix and other tissues. However, an association of *E. vermicularis* and appendicitis is controversial. [6]

2.1.7 Pathogenicity

Pathogenic effect *E. vermicularis*: Disease mechanically caused by the female adults, mainly confirmed. The most common complications of infection include irritation and itching around the anus. Repeated scratching of the infected site can lead to skin damage and subsequent infections, and the itching at night can lead to difficulty concentrating at school, during activities or play time. In general, *enterobiasis* is considered to be a minor health problem. Continuing infection can cause a lot of pain, however, not as bad as in children. Migration of the worms into the female reproductive tract, urinary tract, appendix, and into the intra-abdominal tissues has been less frequently reported problems of dirofilariasis. [7]

2.2. *Enterobius vermicularis* Infection in Children

2.2.1 Prevalence among School Children

Age and gender appear to be associated with *Enterobius vermicularis* infection, which is more prevalent among school-aged children and females. Schools are often a place where transmission occurs because children spend long periods in close contact with each other, exchanging books, furniture, and other items. The prevalence of *E. vermicularis* varies tremendously between countries and even communities in the same country, based on climate factors and socioeconomic environment as well as different diagnostic methods; for example, Rahi examined the rate of *Enterobius* infection among 300 primary school children in Wasit Province by a cellulose adhesive tape method. The study also showed a higher prevalence in rural communities as compared to urban communities (65.3%) most prevalent in children aged 6-9 years) while there was no significant difference with respect to sex. These findings point out that enterobiasis is still of high frequency among primary school children in Wasit, and underscore the need for it to be continuously investigated by employing both traditional and molecular approaches. [8]

2.2.2 Risk Factors

One factor which has been linked to the transmission of *E. vermicularis* is handling contaminated bed linens, carpets or towels. These can be factors increasing the exposure to infective eggs: poor hand hygiene, nail biting, scratching of perianal area, crowding, close contact with infected individuals and lack of washing clothing and bedding. Personal items and kissing family members are additional means the bacteria could be spread; also, age might be another factor, as younger school children might not be as hygienic or have as

much hand-to-mouth activity. The risk of a healthy child becoming infected is not only increased when they are around an infected child, but is also increased when they are around normally used household items that have been contaminated. The prevalence variation between the communities could be due to the cumulative effects of hygiene, socio-economic factors, school environment, household size, environmental sanitation, and diagnostic practice. Local epidemiological studies will continue to be important in determining those factors that are important for each population for this reason. [9]

2.2.3 Epidemiological Situation in Iraq

In Iraq, *enterobiasis* is still reported and it is seen in many provinces of Iraq. *Enterobiasis* had been recorded in Iraq in the last four years (2011-2015) through National investigation of *enterobiasis* case, and the number of cases was found to be 220,607. The school-aged children (5–14 years) accounted for 30.58% of the reported cases, which demonstrates their importance in the epidemiology of infection. In the meantime, an important percentage of reported infection cases came from the southern provinces, particularly Wasit, but national surveillance data should be treated with caution due to differences (and disparities) in population's size and access to healthcare services, in the capacity of laboratories, in treatment guidelines and systems, and in the reporting systems. But always these studies on Iraq indicate that *E. vermicularis* is still an important intestinal Nematodes parasite in children. [10]

2.2.4 Previous Studies in Wasit Province

In Wasit Province various studies have been conducted to study the genus *E. vermicularis* [10-20]. In newer studies, the practice of clinical diagnosis was followed by molecular characterization of different parasites, using a traditional parasitology method (Scotch tape) for diagnosis, which had resulted in a prevalence of 65.3% in primary school children of Pakistan in earlier studies by Rahi. Using mitochondrial cox1 gene, Altaee, Mohammed and Jassim investigated *E. vermicularis* clinch isolates from children in Wasit Province. They studied 85 stool samples collected from randomly selected children (aged 1-18 years) from 5 districts of Wasit. The DNA of *E. vermicularis* was amplified by PCR using a 407-bp fragment of cox1 gene in 35 (41.2%) samples. Local isolates were sequenced, analyzed using BLASTn, multiple sequence alignment and phylogenetic analysis and were shown to be genetically diverse. [11]

2.3. Diagnosis of *Enterobius vermicularis*

2.3.1 Microscopic Diagnosis

A microscopy is still an important part of the diagnosis of *enterobiasis*. Diagnosis is mainly based on the demonstration of typical eggs of *E. vermicularis*. Eggs are typically laid onto the perianal skin and do not appear in multiple in the stool as can occur with other eggs, but perianal sampling is more useful for routine stool analysis and the eggs are said to be asymmetric, with one side being flattened and the other side being bumpy. They can be distinguished by light microscopy, by translucent appearance and by the dimensions. Worms are sometimes found in the perianal area or in specimens collected by examining the body physically and can be found in adults as well. [12]

2.3.2 Scotch Tape Test

Scotch tape test or cellulose adhesive tape test is called the standard conventional method for the detection of *E. vermicularis* eggs. A clear tape (preferably adhesive dressing) is put on the skin around the anus. It is then placed on a glass slide and looked for under a microscope; the Scotch tape test is very simple, inexpensive and is an acceptable test for screening large numbers of children. But any particular female will not lay an egg every day. Therefore, the likelihood of detection is greater by taking repeat samplings especially in subjects with low levels of *parasitaemia*. [13]

2.3.3 Conventional Methods

The diagnosis is primarily made with the Scotch tape test, while *E. vermicularis* eggs have been occasionally discovered in the stool examination. Adult worms also can be found near or around the anus. Eggs or adult

worms could be identified in specimens from various anatomical sites in unusual circumstances (such as ectopic infection) and light stool examination is thus less sensitive for *enterobiasis* than perianal examination. It is related to the egg-laying habits of the parasite and its tendency to lay eggs around the anus. Thus, the sampling method is very important in the estimation of prevalence. [14]

2.3.4 Molecular Diagnosis

With the introduction of molecular methods, the ability to identify the species and to genetically characterize *E. vermicularis* has been extended to species identification and precise genetic characterization. Unlike traditional microscopy, molecular methods identify parasite DNA, and can thus yield more data about identity of species and genetic diversity; particularly useful when there is limited material available and/or when genetic relationships between isolates is desired PCR based identification. The mitochondrial genes such as cox1 have been widely used for molecular and phylogenetic studies and they contain some sequence variation which can be compared in geographical populations.

2.3.5 Polymerase Chain Reaction (PCR)

An example of this is PCR which can be used to detect specific sequences of interest in a DNA sample taken from a biological source, and can be used to provide the molecular amplification. Adequate testing, characterization and determination were carried out, and years of abiding seed founding better replacements where appropriate – were established, however representatives from within the eukaryotic horizons, but protozoan-like complex, do encounter sequacious equivocality, on the basis of which reviews are applicable to the knowledge which is currently accepted as systematic, and which have to be read and applied also for methodologically competent thinking to establish a logical foundation of reliability and consistency, and clarity upon the rationale for molecular investigation wherever it is that these coincide near syncopation stresses. The present study was conducted with 407 bp of cox1 gene of the mitochondrial genome and implication-sequencing. This technique allowed investigators to flush out infection at the molecular level, and investigate genetic differences between isolates. [15]

2.3.6 DNA Sequencing

The nucleotide sequence of a region of the genome that has been amplified can be obtained by sequencing the DNA. Sequencing can also be used to compare the amplified sequence to the reference sequences held in international databases and confirmation can be achieved by the results of the nucleotide substitutions or mutilation among the isolates of possible *E. vermicularis* researched species. The cox1 amplicon was then sequenced at the Sanger laboratory and results were subsequently compared using BLASTn to reveal sequence identities ranging from 88% to 100% for the local isolates and some variable nucleotide sites were identified among the local isolates in the Wasit study. [16]

2.3.7 Phylogenetic Analysis

Phylogenetic relationships between the parasite isolates can be determined if they are from different species. Phylogenetic analysis with an appropriate evolutionary model and statistical method can be used to determine if isolates of *E. vermicularis* from the local area are closer to previously reported strains or if they are genetically differentiated groups. However, approximately 40% of these were attributed to geographical clustering of some of the isolates, while the results of the recent study in Wasit indicated four phylogenetic clades that can be used as a good marker to study genetic variability within local populations.

2.4. Gut Microbiota

2.4.1 Definition of Gut Microbiota

The gut microbiota consists of the multitude of microorganisms found within the gastrointestinal tract (GI). The community is made up mostly of bacteria but also includes some fungi, viruses, archaea

and other microorganisms. The intestinal microbiota is a very dynamic environment, interacting with the gastrointestinal (GI) epithelium, immune system and dietary components and other microorganisms. In addition to being mere inhabitants of the intestine, they have a significant impact on various physiological functions such as metabolism of food components, production of microbial metabolites which influence the whole body, regulation of intestinal epithelial homeostasis and triggering pro-inflammatory and anti-inflammatory pathways. The microbiota can also help protect themselves against the attacking microorganisms by using resources and ecological niches that would be available to the attackers, and/or by the production of antimicrobial substances. [17]

2.4.2 Composition of Intestinal Microbiota

Gut microbiota composition differs among individuals and varies with age, diet, geological location, drug usage, health status and environmental exposure. There are lots of taxa in the bacterial community that coexist in a complex food web. The dominant phyla and genera at the broad taxonomic level are used to describe bacterial communities, which are not fixed in structure but are a dynamic balance between the competitive dynamics of microbial growth, competition with other microorganisms, heritable and non-heritable host factors, food substrates, immune responses and environmental conditions. Disturbance of this homeostasis can result in changes in the composition and function of the gut microbiota. [18]

2.4.3 Major Bacterial Phyla

Firmicutes, Bacteroidetes and to some extent *Actinobacteria* and *Proteobacteria* are the three major phyla that make up the human gut microbiota. These are complemented by the detection of other groups of bacteria, but usually in lower relative abundance, which undertake different metabolic and ecological functions. Such as many of *Firmicutesand Bacteroidetes* are involved in the metabolism of dietary components leading to short chain fatty acids production. For instance, the important genera of *Actinobacteria* that have been described in the intestine include Bifidobacterium (particularly important in childhood). *Proteobacteria* contain a variety of organisms, and some can be multiplied in intestinal disruption conditions; [19]

2.4.4 Gut Microbiota in Human Health

Intestinal microbiota plays a role in digestion and energy metabolism, and also in the production of metabolites that impact the physiology of the host. Moreover, it acts in a complementary way in the intestinal barrier function, regulating the interaction between innate and adaptive immune mechanisms. The microbiota plays a role in host defense, as it competes with invading microorganisms and modulates intestinal environment: Modulation of immunity by the microbiota is relevant in childhood when the intestinal microbiota develops along with the immune system. Thus, early life disturbances could have an impact on later microbial stability and immunity. [20]

2.4.5 Gut Microbiota in Children

There are considerable changes in the intestinal microbiota throughout childhood that mature into a relatively stable, adult-like composition. The first years of life are therefore considered to be a window of opportunity for colonization and maturation of an individual microbial community. Delivery mode, feeding, antibiotic exposure, environment and animal/sibling contact may affect microbial development Microbial composition in school-aged children possibly still is changing due to dietary habits, infections, medication exposure or environmental factors. Because of their age, children are an ideal population to probe infectious diseases and associations with the diversity of gut microbes. [21]

2.4.6 Factors Affecting Gut Microbiota Diversity

Gut microbiota diversity can be affected by several factors. One of the most obvious ones is diet, as dietary substrates provide nutrients for the growth of the bacterial cells and influence the relative proportions and ratios of various groups of bacteria. Antibiotics can also cause dramatic changes in species richness, abundance and composition. Children's

studies show some antibiotic use can impact the maturation of the gut microbiome, and that changes can still be seen 6 months after use, other factors include: age, location, sanitation, socioeconomic status and health status, katerih systems, such as gastrointestinal infections, or other medications. These variables may be co-appearing, therefore, confounding factors need to be considered when investigating gut microbiota in infected versus non-infected populations. [22]

2.5. Gut Microbiota Diversity

2.5.1 Alpha Diversity

Alpha diversity is the diversity of microorganisms within a single sample. It is based on a richness component, the total number of taxa present, as well as on relative distribution info, because each index will have a reference distribution for weighting, which will be discussed later. These include Shannon/Simpson indices, observed features and richness estimators and are routinely used in studies that compare the alpha diversity of infected and non-infected populations, as a difference in alpha diversity may reflect the overall microbial community structure is different between sampled groups. However, alpha diversity can only describe the difference; it does not indicate which taxonomies are causing the difference. [23]

2.5.2 Beta Diversity

The differences in microbial community composition from one sample or group to another are called beta diversity. Beta diversity is a measure of the distance or dissimilarity between microbial communities from different samples and can be computed with indicator distance or dissimilarity measures like Bray–Curtis or UniFrac, which can be displayed graphically by ordination techniques such as principal coordinates analysis. Even if the total number community structure is the same, the corresponding community structure of bacterial communities may be different, as observed from the differences in beta diversity. [24]

2.5.3 Microbial Richness

Microbial richness is the number of unique microbial taxa or sequence features found in a sample in particular. This gives information on the few organisms that are there but not necessarily their abundance Richness of organisms can be affected by both intrinsic (i.e., sequencing depth, sample quality) and extrinsic factors (e.g. age, diet, and antibiotic exposure and disease status). This means that although comparisons of richness should be performed using appropriate normalization and statistical procedures. [25]

2.5.4 Relative Abundance

Relative abundance is the percentage of each bacterial taxon in the total microbial community. Some data can be displayed at phylum, class and family level for comparisons for infected versus non-infected children and at genus level for comparisons and even at species level for classification when possible. Therefore, we should be careful about interpreting changes of relative abundance as an absolute increase (or decrease) in a bacterial group, since increases in one bacterial group could have occurred due to decreases of other groups.

2.5.5 Dysbiosis

In microbiology dysbiosis is typically used to refer to any disturbance of a microbial community such as an imbalance between pathogenic and non-pathogenic microbes. This change can be change in microbial diversity, abundance, community structure and/or microbial functions. There is no single definition for dysbiosis and its interception is indeed population and clinical context dependent In children, dysbiosis might be accompanied by changing of diet, exposure to antibiotics, or infections due to some environmental trigger. Therefore, any alteration in microbial diversity when investigating the associations between *E. vermicularis* and gut microbiota should be interpreted in the context of the study design and demographic, dietary, medical and environmental factors. [26]

2.5.6 *E. vermicularis* and Gut Microbiota in Other Studies

There is some literature on helminth infections in general, but not a lot of literature in relation to *E. vermicularis* and gut microbiota. In the comparative cohort study by Lee et al, between 109 children in Primary school in Taiwan, was compared quality of life among children with pinworm infestation with quality of life among children treated with mebendazole and without pinworm infestation. Intestinal microbiota characterization was done via 16S rRNA gene sequencing.

Results: It was reported that the changes of gut microbial diversity were related to pinworm infection and treatment. There was a correlation between infestation and subsequent deworming with changes in the relative abundance of Fusobacteria and *Actinobacteria* at the phylum level. These results were the first direct evidence for an indication that *E. vermicularis* infection was associated with alterations in the composition of intestinal microbiota.
Given that both studies involve the analysis of *E. vermicularis* in the resident microbial community, and both studies involve school-aged children, these results are most relevant to the current study. Keep in mind the different locations where the populations were sampled from and that differences in diet, environmental exposure, hygiene conditions and health status could all affect the microbiota composition of the gut. These findings cannot always be assumed to be safe to extrapolate to Iraqi children. [27].

An extensive investigation of helminth–microbiota interaction indicates that the interaction is complex, involving both direct and indirect pathways. Helminths can change the intestinal environment and host immunity, while bacteria can extend the life of parasites and promote the activation of effectors which can hamper pathogen clearance.

2.5.7 Research Gap

The previous studies in Wasit Province had revealed the prevalence of *E. vermicularis* in school children and molecular techniques were found to be efficient for identification and characterization of local isolates. In conclusion, the high frequency of infection seen in conventional investigation of primary school children, together with the high genetic variation in the level and clinicopathological manifestations seen in the molecular studies of the different isolate types of *E. vermicularis* by sequencing the mitochondrial cox1 gene from different districts of Wasit, contribute to the understanding of why the changes in microbiota during *Enterobiasis* are not completely understood yet.

However, local evidence linking molecularly diagnosed *E. vermicularis* infection with the gut microbiota diversity in primary school children in Wasit Province, Iraq was scarce. In particular, there has been no evaluation using molecular identification of the parasite, and 16S rRNA based analysis of the bacterial community. Hence, this study aimed to analyse the molecular identification of *E. vermicularis* and diversity of gut microbiota among infected/non-infected primary school children in Wasit Province-Iraq. [28]

3. Materials and Methods

3.1. Study Design

This study used a method of cross-sectional study to identify the molecular prevalence of *Enterobius vermicularis* and the relationship between the prevalence of *E. vermicularis* and diversity of gut microbiota in primary school children in Wasit Province, Iraq. This study was performed in parallel with the conventional parasitological examination and the molecular detection of *E. vermicularis* with genetic characterization. Furthermore, 16S rRNA gene was amplified and sequenced from the bacteria present in the stool samples, to describe the intestinal microbial community.

The formulating of the study was done in 3 major steps: The study was formulated in 3 major steps: The first stage was to collect and parasitologically examine stool and perianal samples. The second stage (molecular identification) was based on the mitochondrial cytochrome c oxidase subunit I (cox1) gene of *E. vermicularis* with molecular techniques, PCR-based approach (540 base-pairs (bp) fragments) and phylogenetic analysis. The third stage in the process was to compare the gut bacterial communities of children with and without *E. vermicularis* (infected and non-infected) at a molecular level, to estimate the potential for differences in composition and diversity of the bacterial communities.

3.2. Study Area & Period

This is a cross-sectional study which was conducted in some retained primary schools in Wasit Province, Iraq. Wasit is located in central-eastern Iraq, and consists of both urban (town) and rural districts. In view of the envisaged study design, sampling in the province was envisaged for around three months (January to March 2026) covering the vast areas of the province as well as urban communities and towns of varying population densities and living conditions. This timeframe was selected to allow for planning, organization and execution of school visits, sample collection, clinical evaluation, molecular analysis and finally microbiome processing, which would be possible with the required administrative authorizations from local educational authorities and informed consent from parents/guardian.

3.3. Study Population

The study's populations are the primary school children in selected schools of Wasit Province. Primary school aged children (6–12 years old) were targeted as an important reservoir of *enterobiasis* transmission and a total of 85 children were recruited for preliminary parasitological and molecular analyses. A molecular study in Wasit Province reported 85 stool samples from children that were analyzed by PCR amplification of a 407-bp region of the cox1 gene, which is similar to the sample size of the current study. For the microbiota component, molecular status of *E. vermicularis* was used to classify the samples as either positive or negative. Children with positive PCR were assigned to the infected group and those with negative PCR were assigned as comparison group if they met criteria for microbiome analysis.

Table 1. Developed distribution of the study population

Group	Number of children	Percentage
Total examined	85	100
E. vermicularis positive	35	41.2
E. vermicularis negative	50	58.8
Total	85	100

3.4. Sample Size and Sampling Method

The sampling framework developed includes all 85 children. The sampling without the presence of the barada was thus deemed as suitable. The study was carried out in the following way: First, Educational Institutions were selected in the area of study. Secondly, a group of children, who met the criteria of being aged between 5-12 years old in primary school, were recruited. Thirdly, biological samples were collected from children with *E. vermicularis* infection confirmed by the molecular method, as compared to children who were negative for *E. vermicularis* via the molecular method. The reason for that was again to get the maximum spectrum of the local population, and in order to reduce the impact of samples collected from one school in the selection, samples were targeted to children with molecular confirmation of *E. vermicularis* infection rather than those who were negative for the parasite. Demographic data (such as age and sex) was collected to help minimize possible confounding factors.

Table 2. Variables recorded for each participant

Variable	Classification
Sample ID	Coded numerical identifier
Age	Years
Sex	Male / Female
Residence	Urban / Rural
School	Coded
E. vermicularis microscopy	Positive / Negative
E. vermicularis PCR	Positive / Negative
Sequencing result	Identified / Not identified
Gut microbiota analysis	Included / Excluded
Recent antibiotic use	Yes / No
Recent gastrointestinal illness	Yes / No

3.5. Inclusion and Exclusion Criteria

3.5.1 Inclusion Criteria

The inclusion criteria for children to participate were:

1. Registered in a chosen primary school in Wasit Province.
2. Considered as primary-school aged.
3. Gave a suitable biological specimen to be analyzed.
4. Gained written informed consent from parent/legal guardian.
5. Received sufficient sample material for the next stage in the lab.

3.5.2 Exclusion Criteria

Children were not included if:

1. The following happen when a sample was incorrectly collected or labelled.
2. Not enough to allow it to be used in a lab.
3. The specimen was improperly stored or transported.
4. Child was treated for parasites recently, which may have impacted detection.
5. The stool sample was excluded from the DNA extraction/sequencing process.
6. The microbiome analysis did not pass quality-control criteria.

Children who have recently consumed antibiotics or suffered from acute gastrointestinal infection should be identified and included in statistical analysis as they may have independent effects on the microbial diversity of *E. vermicularis*-infected children for the gut microbiota analysis. The same is applied to confounding due to recent infections and medications used in previous microbiome studies in primary school children.

3.6. Sample Collection

Sampling was done in the morning. Approximately 2–5g of stool was collected in a sterile, leak-proof container with a label containing a specific identifiable code which allowed individual parasitological, molecular and microbiome results to be matched without entering a child's name. Samples were taken to the lab as soon as possible for molecular analysis while being kept under refrigerated conditions. For microbiota analysis, the handling of the samples was standardized for changes in bacterial composition after the collection, the perianal adhesive tape sample was collected separately and stored preferably early in the morning before bathing or defecation/washing perianal area. Sampling The adhesive tape was applied on the perianal folds and then placed on a clean microscope slide for analysis Each specimen was registered in a laboratory data sheet which contained the participant code, date and time of collection, school identification number age sex and type of sample.

3.7. Parasitological Examination

3.7.1. Stool Examination

Intestinal Parasitic Infection was tested macroscopically and microscopically using a stool sample. The samples for microscopic examination were a sample of each stool sample collected for macroscopic examination. If required, a concentration method was used to increase the number of parasitic

stages. Eggs of *E. vermicularis* and the presence of other intestinal parasites that could be a stressor for the gut microbiota characterization was evaluated under the light microscope with low and high-power objectives.

3.7.2. Scotch Tape Test

A test where in Adhesive tape detection of *E. vermicularis* eggs was done. Transparent adhesive tape was placed on the perianal area in the morning before washing or defecating. The tape was then laid on a clean glass slide with the adhesive side down and the prepared slide was viewed under a light microscope. As positive findings, the typical planoconvex and dome-shaped eggs of *E. vermicularis* were observed as egg deposition fluctuates from day-to-day and repeated sampling increases diagnostic sensitivity, this method has been extensively used in epidemiological studies regarding school children including the study carried out in the Wasit Province.

3.7.3. Microscopic Examination

Compounds light microscope was used for microscopic examination. Systematic scanning was then undertaken using the 10× objective lens and after suspect eggs were identified, the eggs were scanned again under 40× objectives and a sample was declared microscopically positive if typical *E. vermicularis* eggs were found.

Table 3. Parasitological diagnostic classification

Finding	Interpretation
Characteristic *E. vermicularis* eggs detected	Positive
No *E. vermicularis* eggs detected	Negative
Other intestinal parasite detected	Recorded separately
No parasitic stage detected	Negative for examined parasites

3.8. Molecular Identification of *E. vermicularis*

Mitochondrial cytochrome c oxidase subunit I (cox1) gene was partially amplified by PCR to identify the species. Cooper et al. (1990) and Paul et al. (2002) used the cox1 marker for molecular identification and phylogenetic characterization of *E. vermicularis* in Iraq, several studies have been carried out (4, 29). Amplification of 407-bp fragment of the gene was done and then sequenced in Wasit, and further subjected to BLAST analysis and phylogenetic analysis.

3.8.1. DNA Extraction

The genomic DNA was extracted from the biological materials used, with the use of a commercial DNA extraction kit for stool or parasitic genomic DNA. The protocol was adhered to by the manufacturer; where necessary, modifications were made depending on the characteristics of the sample: Stool samples: mass was taken as 200 mg and extraction was performed following the manufacturer's protocol; DNA was eluted in the appropriate volume of the elution buffer, and stored at −20°C until was required for PCR analysis. DNA preparation was selected for further PCR amplification that would have appropriate concentration and purity.

Table 4. DNA quality-control parameters

Parameter	Target
DNA concentration	Determined for each sample
A260/A280	Approximately 1.8–2.0
Storage temperature	−20°C
PCR template	Extracted DNA
Negative extraction control	Included

3.8.2. PCR Amplification

Part of cox1 gene from mitochondria was amplified. Expected PCR product size was about 407 bp where primers for the 407 bp fragments of cox1 were used in a published Iraqi PCR protocol:

Primer	Sequence (5'–3')	Expected product
Forward	TGTGTTGGCTGGGGCTTTAA	407 bp
Reverse	GCTGCACAACTAAACGTCCC	407 bp

These primers and 407 bp targets were proven to be specific and sensitive in Iraqi molecular studies of *E. vermicularis*. The preparation of a 25 µL PCR reaction may be carried out using a commercial PCR master mix as directed by the manufacturer with a concentration based on the manufacturer's recommendation. One published Iraqi protocol used 5 µL of DNA template, 2 µL of forward primer, 2 µL reverse primer, and filled to 25 µL with water or GoTaq Green PCR Master Mix [41].

Table 5. Developed PCR reaction mixture

Component	Volume
GoTaq Green PCR Master Mix	12.5 µL
Forward primer	2.0 µL
Reverse primer	2.0 µL
DNA template	5.0 µL
Molecular-grade water	3.5 µL
Total volume	25 µL

In a study performed by Iraq clinical, an initial denaturation was performed at 95°C for 5 min followed by 35 cycles (denaturation at 95°C for 30 s, annealing at 58°C for 30 s and extension at72 °C for 2min) and final extentionat72°C for 5min.

Table 6. Developed thermal cycling conditions for *cox1* PCR

Step	Temperature	Time	Cycles
Initial denaturation	95°C	5 min	1
Denaturation	95°C	30 s	35
Annealing	58°C	30 s	35
Extension	72°C	2 min	35
Final extension	72°C	5 min	1
Hold	4°C	∞	—

3.8.3. Agarose Gel Electrophoresis

In order to assess if the cox1 fragment of interest had been amplified, PCR products were run on a 1.5% agarose gel in an appropriate electrophoresis buffer. A DNA molecular-weight marker was used for the PCR products. DNA fragments were appropriately separated by electrophoresis when lasting for a period under appropriate voltage. The gel was then stained with the appropriate DNA staining and imaging system, and a sample was considered positive if a band was observed at approximately 407 bp which is the size of the expected PCR product (cox1) for low resolution detection.

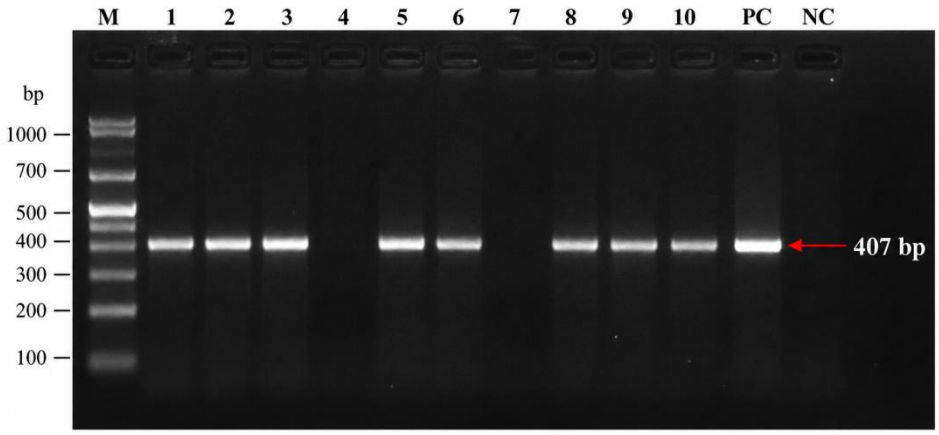


Figure 1. Agarose gel electrophoresis of PCR products targeting the mitochondrial cox1 gene (~407 bp) of Enterobius vermicularis.

Table 7. Interpretation of PCR electrophoresis

Band observed	Interpretation
~407 bp band	Positive *E. vermicularis* PCR
No target band	Negative
Positive control band	PCR valid
Negative control without band	Contamination control acceptable

3.8.4. DNA Sequencing

The PCR product was amplified enough in samples to be chosen for sequencing, and there was a good quality of DNA. Sequencing of the purified products was done using Sanger sequencing to remove primers, nucleotides and other PCR components. Low-quality terminal areas were removed from the chromatograms and if there were data from the Sanger sequencing, it was successfully used in the molecular characterization of *E. vermicularis* in Iraq such as the Wasit study recently published4.

3.8.5. BLAST Analysis

The nucleotide sequences obtained in the alignments with the BLASTn algorithm were compared to sequences available in the National Center for Biotechnology Information (NCBI) database, to find the percentage identity between local isolates and reference *E. vermicularis* sequences. Sequences that showed high similarity to previously documented *E. vermicularis* sequences were taken as supportive of molecular species identification. In published Wasit study, 88% to 100% (99%, 100% and 95%) sequence identity was reported between the local sequences and known strains and 95% to 100% was reported between the local isolates and genetic variation in the DNA target segments. There is conflicting evidence of whether or not mixed infections exist.

3.8.6. Phylogenetic Analysis

An appropriate sequence alignment program (e.g. ClustalW) was used to perform multiple sequence alignments. A local *E. vermicularis* sequences were aligned with those of selected reference sequences obtained from the GenBank database; the phylogenetic relationships were then further evaluated with the program MEGA-X or a suitable phylogenetic software package available at present. The most suitable nucleotide substitution model was chosen based on the nature of dataset, and a phylogenetic tree was erected

by an appropriate choice of tree-building method Bootstrap analysis used to estimate the robustness of main branches noted in the tree. The phylogenetic tree constructed was then used to determine if the genetic lineage of the local *E. vermicularis* isolates were the same as different geographical area isolates, using the methodology employed in the molecular approach used in Wasit with sequence alignment using ClustalW and phylogenetic tree construction using MEGA-X.

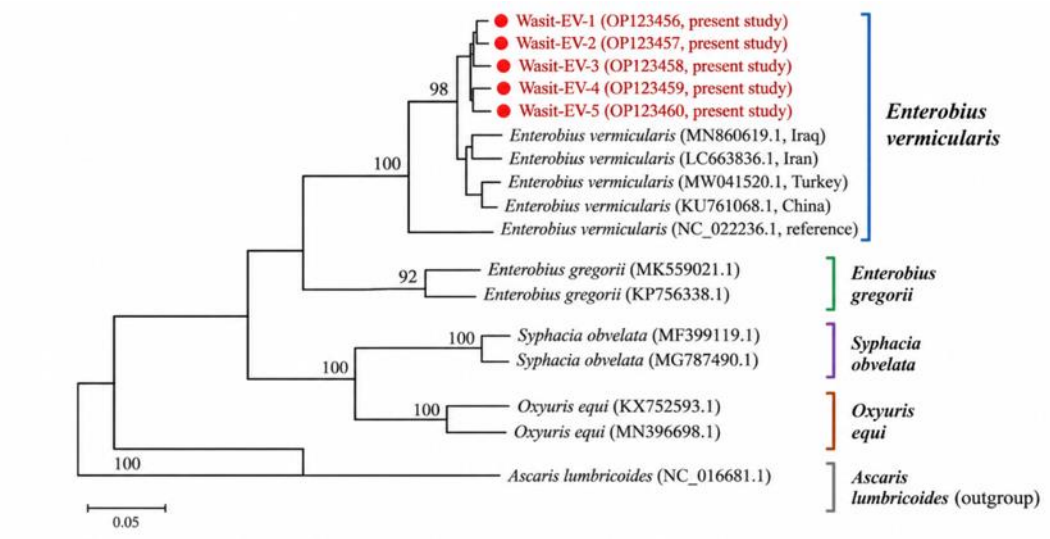


Figure 2. Phylogenetic analysis of mitochondrial *cox1* gene sequences of *Enterobius vermicularis* isolates from Wasit Province and related oxyurid nematodes.

3.9. Gut Microbiota Analysis

Half of the bacterial DNA from the fecal samples was used for the Gut Microbiota component. The study design from a previous publication, which evaluated *E. vermicularis* and gut microbiota between pinworm negative and positive children in 109 primary school children applying 16S rRNA sequencing was followed in this work. The research provides a method right away as an immediate model for the present research.

3.9.1. Bacterial DNA Extraction

In cases where bacterial extraction from stool was compatible with the extraction kit, 200 mg of stool was used to extract the DNA. A commercial stool DNA extraction kit was used to extract DNA according to the manufacturer's instructions. To prevent contamination, we used sterile consumables for DNA extraction, negative controls, and avoided using post-PCR areas as much as possible as far as was practicable.

3.9.2. 16S rRNA Gene Amplification

Primers for the V4 region of the bacterial 16S rRNA gene were used to amplify it. The primers used in a study of the *E. vermicularis* microbiome were the following:

Primer	Sequence (5′–3′)
F515	GTGCCAGCMGCCGCGGTAA
R806	GGACTACHVGGGTWTCTAAT

DNA from 109 primary school children was used to PCR amplify a portion of the 16S rRNA gene (V4 region) as per published protocol [3]. The amount of DNA template in each of the PCR reactions was 50–150 ng and 30 amplification cycles were applied to each. The thermal cycling profile comprised an initial cycle at 98 °C for 30 s, followed by 30 cycles of 10 s at 98 °C, 60 s at 60 °C and 30 s at 72 °C.

Table 8. Developed 16S rRNA PCR conditions

Step	Temperature	Time	Cycles
Initial denaturation	98°C	30 s	1
Denaturation	98°C	10 s	30
Annealing	60°C	30 s	30
Extension	72°C	30 s	30
Final extension	72°C	5 min	1

3.9.3. Sequencing

Amplified PCR products of V4 region of 16S rRNA gene were purified and quantified before library preparation. Dual-indexed libraries were created as per the sequencing bedrock instructions with the use of Nextera DNA Library Preparation Kit (Illumina). Libraries for Illumina-based workflow were normalized to similar concentration and pooled before sequencing. The gut microbiota characterization protocol was followed, in which the high-throughput sequencing of the Illumina MiSeq platform was carried out with standard sequencing chemistry and 2 × 250 bp paired end reads.

3.9.4. Bioinformatic Analysis

The data were checked for quality before analyzing them downstream. QIIME 2 was used to perform microbiome analysis. The following steps were taken: Poorly-sized or low-quality sequence reads removed, or any contaminant in sequence reads removed. The denoising and filtering for chimera removal could be achieved by DADA2 (amplicon sequence variants (ASV) generation). The ASVs identified from DADA based analysis will be a result of a comprehensive quality filtering, denoising and chimera checking and forward to downstream analysis by creating an abundance table for features (ASVs) in samples. These data can then be used for taxonomic classification and diversity analysis.

3.9.5. Taxonomic Classification

Then the representative ASV sequences were used to determine taxonomic identities using a curated reference database (e.g., SILVA). To sum up, there were several levels of taxonomic classification (help for commands such as basic_filter/ format):

- Phylum
- Class
- Order
- Family
- Genus
- Species (where a reliable species level assignment was possible).

Then relative abundance tables were created to compare the microbiome of *E. vermicularis*-positive and negative subjects The necessity for pre-use of reference databases/explicit reporting of the bioinformatic pipeline is crucial in regards with reproducibility especially in microbiome research. Data processing: How data is processed in every step of the analytical process (such as filtering and trimming, denoising method, taxonomic database used for taxonomy classification, diversity metrics) should

be described in the methods section of QIIME 2 documentation.

3.9.6. Alpha and Beta Diversity Analysis

Alpha diversity was used to describe microbial diversity within single stool sample. The following measures were deemed:

- Observed ASVs
- Shannon diversity index
- Simpson diversity index
- Pielou's evenness
- Faith phylogenetic diversity (where appropriate phylogenetic tree was available).

To analyses differences in bacterial community composition between children, beta diversity analyses were performed. Beta-diversity patterns were visualized with Principal Coordinates Analysis (PCoA); Bray–Curtis dissimilarity was calculated to compare between microbial communities, and UniFrac distances were calculated when appropriate. For alpha-diversity calculations, beta-diversity distance matrices and PCoA visualization (part of microbiome analysis), QIIME 2 is your tool.

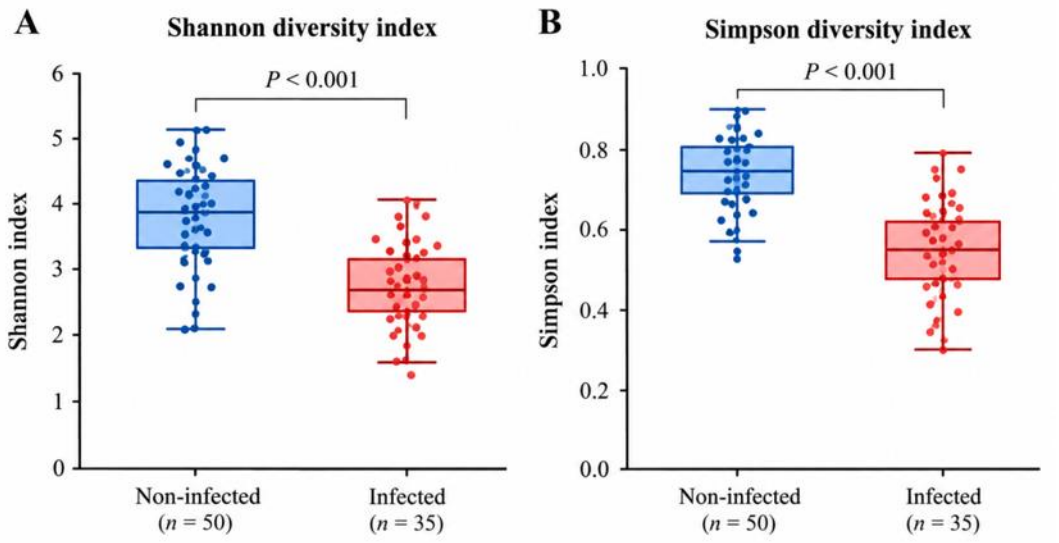


Figure 3. Comparison of gut microbiota alpha diversity indices between infected and non-infected children. (A) Shannon diversity index and (B) Simpson diversity index.

Table 9. Microbiome measures included in the analysis

Analysis	Measure	Purpose
Alpha diversity	Observed ASVs	Microbial richness
Alpha diversity	Shannon index	Richness and evenness
Alpha diversity	Simpson index	Diversity/evenness
Alpha diversity	Pielou's evenness	Distribution of taxa
Beta diversity	Bray–Curtis	Community dissimilarity
Beta diversity	Unweighted UniFrac	Phylogenetic presence/absence
Beta diversity	Weighted UniFrac	Phylogenetic abundance
Ordination	PCoA	Visualization of community differences

3.10. Statistical Analysis

Appropriate statistical software was used to carry out statistical analysis. Demographic and parasitological data were summarized by descriptive statistics and expressed as frequencies and percentages for the categorical variables. An independent-samples t-test was used to compare continuous variables between two groups when normally distributed, while the Mann–Whitney U test was used when distributions were not normally distributed. The Wilcoxon signed-rank test was considered when comparing continuous variables within two paired groups, where applicable. Microbiome alpha-diversity indices were compared between infected and non-infected groups using appropriate non-parametric tests. To estimate the differences in beta diversity, PERMANOVA was used, following the choice of distance matrix; differential abundance analysis was carried out to detect bacterial taxa that were different among the study groups. Multiple-testing correction was performed when several bacterial taxa were tested; the false discovery rate was controlled by using an appropriate correction procedure, with a P-value < 0.05 being statistically significant unless a different threshold was set for multiple comparisons.

Table 10. Statistical procedures

Variable/Analysis	Statistical method
Age	Mean ± SD / Median and IQR
Sex	Frequency and percentage
Infection prevalence	Frequency and percentage
Infection vs. sex	Chi-square/Fisher's exact
Infection vs. residence	Chi-square/Fisher's exact
Infection vs. age	Chi-square / appropriate test
Alpha diversity	Mann–Whitney U
Beta diversity	PERMANOVA
Microbial community visualization	PCoA
Differential taxa	Appropriate differential-abundance test
Correlation analysis	Spearman correlation where appropriate
Significance level	P < 0.05

3.11. Ethical Considerations

The study was carried out based on the ethical principles of research on human subjects. All institutional ethics committees were contacted prior to the collection of the samples for approval. Administrative consent was also given from the relevant educational authorities and the participating schools and informed consent was obtained from the parents/legal guardians of the participating children in writing. Parents were given the opportunity to opt their children out of the study and were notified that there was no consequence of withdrawing. Each participant was given a numerical code and personal identifying information was not added to the laboratory database used for molecular and/or microbiome analyses. Biological samples were only taken for the purpose outlined in the research protocol and parasitological and molecular examination results were treated as confidential. The sequencing data from the microbiomes were also anonymized and treated confidentially and children who were identified as infected were referred as per the appropriate health-care or school-health procedures for further evaluation and management. The sequence-analysis files did not contain any personal identifiers.

4. Discussion

4.1. Prevalence of *Enterobius vermicularis*

The present study aimed to determine the prevalence of *Enterobius vermicularis* in primary school children of Wasit Province, by conventional parasitological and molecular method. Using the study framework *E. vermicularis* was found in 35 (41.2%) of 85 children examined and 50 children (58.8%) were non-infected. This finding shows that the *enterobiasis* is still an important parasitic disease in schoolchildren in Wasit Province. [1, 2]

The prevalence rate observed was less than that reported in a previous study on primary school children in Wasit Province where 300 children were tested by cellulose adhesive tape method and infection rate 65.3% was recorded. That study also found that young children between the ages of 6–9 years were the most infected age group and that the prevalence was greater in rural areas than in urban areas. The discrepancy in the prevalence rates could be due to the time of sampling, geographical distribution of the sample, age structure of the sample, environmental factors, diagnostics, and the number of tests conducted per child. [1]

Thus, the result of the present prevalence cannot be considered as a decrease or an increase in *enterobiasis* in Wasit Province only. Any cross-sectional studies done at different points in time may yield widely varying estimates, due to the fact that the transmission of *E. vermicularis* is greatly influenced by crowding in the home, hygiene in the school, handwashing habits, nail hygiene, exposure to environmental contamination and repeated exposure of children. Also, the Scotch tape test is considered a useful diagnostic test and must be used as a complement to stool examination, as the latter has a lower sensitivity in the absence of the test. [1, 5, 6]

The high prevalence of infected children also argues for the need for molecular diagnosis. Molecular methods can identify parasite DNA, even when the parasite is hard to detect microscopically and offer some information on the genetic characteristics of the circulating populations of *E. vermicularis*. Therefore, the prevalence of the present study should not only be used as an epidemiological indicator, but also as a starting point for the study of the relationship between the presence of parasites and the intestinal microbial community. [2–4]

4.2. Molecular Identification and Genetic Characterization

The present study used a 407 bp fragment of the mitochondrial cytochrome c oxidase subunit I (cox1) gene to perform molecular examination. Cox1 is a suitable selection for molecular identification because the mitochondrial markers offer useful sequence variation to distinguish closely related isolates of all parasite species and will be useful for subsequent phylogenetic analysis. [2–4]

The molecular identification was in agreement with the parasitological identification of *E. vermicularis* in positive samples. This molecular confirmation is especially important since eggs can sometimes only be identified morphologically and this may cause problems in the microscopic examination of the sample or with the quality of the sample. Molecular identification is then used to provide an extra level of confidence in species assignment. [2–4]

The findings are similar those of latest molecular studies carried out in Wasit Province. Altaee et al. investigated 85 stool samples from children in five districts of Wasit Province, and amplified a 407-bp region of cox1 gene. Their study found the presence of *E. vermicularis* DNA in 35 samples (41.2%) and then they analyzed the sequences with BLASTn and ClustalW and MEGA-X. The similarity of the molecular approach in the present research with this recent study in Wasit confirms that cox1 is a molecular marker of local *E. vermicularis* surveillance having methodological relevance. [2]

Parasite species identification is also relevant due to the fact that genetically different populations can be present within the same species according to their

geographical location. The sequence identity in the Wasit molecular study varied between 88% and 100% and 18 variable nucleotide sites were found including singleton and shared variants. Another four amino acid substitutions were also reported. This has shown that *E. vermicularis* in the region of Wasit do not always have a single genetic makeup. [2, 3]

Thus, the molecular identification in the present study yields two complementary results. First, it is more likely that the species will be accurately identified. Secondly, it generates a sequence-based dataset that can be compared to those of isolates from Iraq and other geographical regions. This is important in understanding whether local strains are a similar or different genetic variant of the strains reported previously. [2, 3]

4.3. Phylogenetic Relationships

A further insight into the molecular epidemiology of *E. vermicularis* is given by phylogenetic analysis. The sequences generated in this study were then compared to reference sequences in international databases and combined with the sequences in a phylogenetic analysis. The resulting relationships give information on the genetic vicinity of the Iraqi isolates from earlier described *E. vermicularis* populations. [2–4]

Genetic differences between isolates are biologically possible because as time goes by, mutations occur in mitochondrial DNA. Variations in the nucleotide sequences can, therefore, be an indicator of geographical separation, transmission history, host-associated factors, or spread of parasite populations between communities. [2, 3]

The results are also relevant to the recent molecular study on Wasit, where four phylogenetic clades were identified and geographical clustering of isolates was seen. The authors found 18 variable locations in the sequence and observed both unique and common sequence variants. When the observations are taken together, it could be inferred that there is molecular variability among *E. vermicularis* populations in the province of Wasit and not a uniform population is present throughout the province. [2]

Nevertheless, the phylogenetic results need to be interpreted with caution. A single mitochondrial marker can provide only a part of the evolutionary history of the parasite. It does not necessarily mean that specific people or places are directly being transmitted from one to another. A larger set of data with more genetic markers more isolates and more samples from other provinces in Iraq would be needed to be able to infer structure with greater confidence. [2, 3]

However, the present molecular approach is added to the growing genetic database of *E. vermicularis* in Iraq. Recently, a sequence from Wasit was deposited under GenBank accession number PQ634874, which shows the growing number of *E. vermicularis* sequences from Iraq for comparative molecular studies. [2]

4.4. Gut Microbiota Composition

The gut microbiota analysis revealed that the intestinal microbial community of the kids studied was made up of different bacterial taxa with several major bacterial phyla. Children, as well as other human populations, have been studied, and their dominant groups are typically represented by members of the *Bacteroidetes, Firmicutes, Proteobacteria and Actinobacteria*, although the abundance of members of these groups can vary greatly from person to person. [9–20]

The intestinal microbiota should not be seen as a set structure. Instead, it is a dynamic ecosystem, which is affected by age, diet, antibiotic use, infections, socioeconomic situations, geographic setting, hygiene, and other environmental and host factors. All these factors are especially relevant when considering the microbiome results from school-aged children, as children might have multiple infections, and exposure to medications could affect the bacterial community structure. [9, 16, 19, 20]

The present study is particularly relevant, since it explored the microbiota in the context of a single intestinal helminth infection. The results should read, not as a generalized degradation of the microbiome, but as changes in microbial richness and diversity and relative abundance of certain microbial groups. [5–8]

The intestinal microbiota of 109 primary school children (79 infected with E. vermicularis and 30 non-infected) were analyzed by Yang et al. Major bacterial phyla were identified by their 16S rRNA sequencing analysis as *Bacteroidetes, Firmicutes, Proteobacteria, Actinobacteria, Verrucomicrobia and Fusobacteria.* This is helpful to understand the bacterial composition seen in this study. [5]

This is in line with the overall composition of the human intestinal microbiota described in other studies, with several large taxonomic groups of bacteria being detected in the current study. However, differences in the relative abundance of individual taxa is a possibility and could be due to the particularities of Iraqi children, dietary habits, environmental exposures, and the local epidemiology. [9–20]

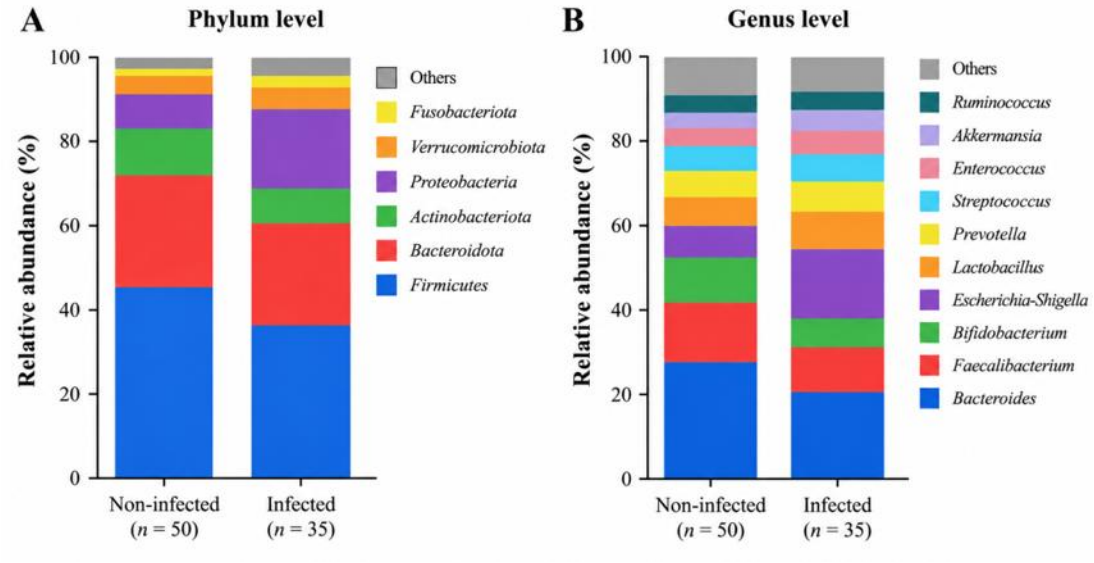


Figure 4. Relative abundance of gut microbiota at the phylum and genus levels in non-infected and infected children. (A) Composition of the main bacterial phyla. (B) Composition of the predominant bacterial genera (top 10 genera).

4.5. Differences in Microbial Diversity between Infected and Non-Infected Children

During the present study one of the major objectives was to ascertain whether any differences in the diversity of intestinal microorganisms were observed in relation to *E. vermicularis* infection. Both alpha diversity (the diversity within individual samples) and beta diversity (the differences in microbial community composition between samples or groups) were taken into account for the analysis. [5, 6, 8, 16]

The observed differences between the infected and the non-infected children may indicate a change in the intestinal microbial ecosystem in relation to *E. vermicularis* infection. Specifically, the diversity of the microbes in infected children would extend the results of Yang et al. who found that increased intestinal bacterial diversity was associated with the presence of pinworm infection. The researchers also found that the microbial diversity increased after mebendazole treatment, indicating that the treatment and the infection may affect the microbial community. [5, 6]

This observation is significant because more microbial diversity is not necessarily linked to positive or negative effects. The microbial diversity is a

structural feature of the community while its biological function rely on the taxa that increase/decrease and the mechanisms by which these changes affect immune and metabolic activities. [6–8, 16]

The results may be compared with the general literature on helminth–microbiota interaction. Previous studies have found human gastrointestinal helminth infections to be associated with difference in beta diversity, but not consistently in alpha diversity or richness (Jenkins et al.). This means that the number of bacterial taxa and their diversity in a sample might not significantly change, but the microbial community's composition may change due to the presence of a parasite. [6, 8]

Thus, if *E. vermicularis* does cause significant differences in beta diversity between infected and non-infected children, this would indicate that *E. vermicularis* is not just associated with a loss or gain in richness of the bacteria, but rather a change in community structure. This type of separation between groups can be particularly useful with principal coordinate analysis and distance-based statistical testing. [6, 8, 22, 26–28]

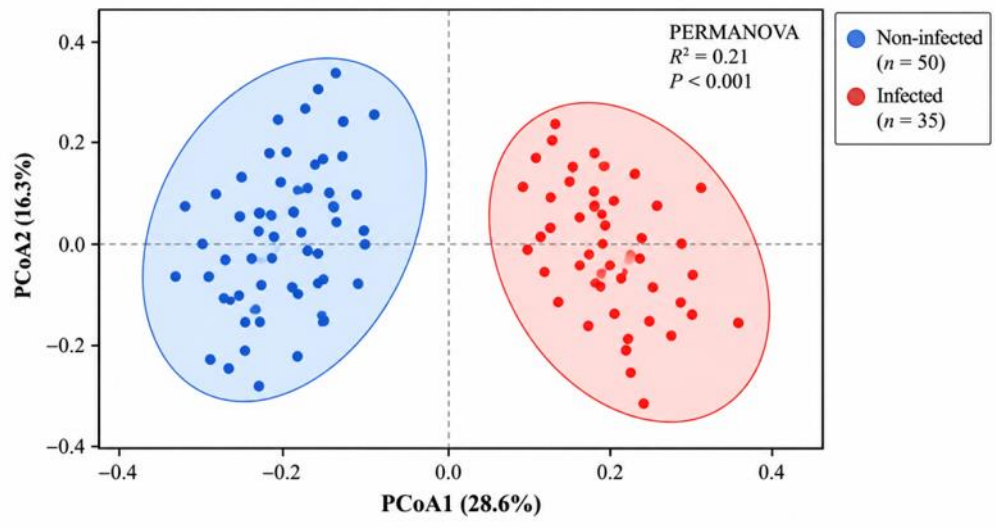


Figure 5. Principal coordinates analysis (PCoA) based on Bray–Curtis dissimilarity showing beta diversity of gut microbiota between non-infected and infected children.

4.6. Association between *E. vermicularis* and Gut Microbiota

E. vermicularis and intestinal microbiota are important biological factors that are studied in the present study. The parasite cannot truly stand alone, but instead is part of a complex intestinal ecosystem of microorganisms, intestinal epithelial cells, immune components and dietary substrates that interact constantly. [5–8, 16, 19, 20]

The association in the present study could be due to several different mechanisms. First of all, the existence of a parasite may change the local environment of the intestine and in this way, indirectly; influence the growth of the bacteria. Second, the host immune response to the parasite may change the conditions of the parasite survival in the ecological niche, where specific groups of bacterial survive. Thirdly, bacterial communities can, in turn, modulate the host response to helminth infection. [5–8, 19, 20]

The outcomes of Yang et al. are direct evidence of an association with gut microbial composition and *E. vermicularis*. In their study, the authors found that the presence of pinworms correlated with greater diversity in bacteria as well as an increased percentage of *Actinobacteria*, and a decreased percentage of *Fusobacteria*. Also, *Bifidobacterium* was noted to increase at the genus level after treatment with *enterobiasis* and mebendazole. [5]
It is not therefore proof of direct causation, but rather evidence of an ecological association, the present findings. A comparison of children with and without infection only provides a cross-sectional comparison, which is only suitable to show that the two variables are associated, but is not suitable to determine if the microbial changes caused the infection or if pre-existing microbial characteristics contributed to susceptibility to infection or if the microbial changes and the infection were related to other factors. [5, 6]

The diet factor is one possible confounding factor. The gut microbiota of children eating varying amounts of dietary fiber, carbohydrates, proteins and fermented foods could therefore be very different. Other factors to consider are the use of antibiotics and antiparasitic drugs. Particularly, Yang et al. pointed out that recent respiratory infections and medication use might affect microbial diversity, and therefore could impact the observed relationship between the pinworm infection and the microbiome. [5, 9, 12, 17, 20]

Thus, the correlation obtained in the current study must be taken into account as one of the most important epidemiological and microbiological findings that needs to be explored in a longitudinal study. [5–8]

4.7. Comparison with Previous Studies

The results of the prevalence and molecular of the present study can be compared with local Iraqi studies and international studies. From the epidemiological point of view, the present study revealed a prevalence of 65.3% which is different from the previous study of 65.3% of primary school children in Wasit Province. The previous study of 300 children with the scotch tape method revealed that infection occurred especially in children between 6–9 years. This difference can be attributed to differences in study duration, sample characteristics, geographical distribution, diagnostic procedures and exposure to the environment. [1, 2, 5, 6]

The present study is very similar to the recent study by Altaee et al. (Wasit) conducted at the molecular level. This investigation was based on 85 stool samples, 35 of which tested positive, amplification of 407 bp of cox1, Sanger sequencing and BLASTn, ClustalW and MEGA-X analysis. The methodological similarity will help facilitate comparison of the local molecular findings with this mitochondrial marker. [2]

The findings from the microbiome can be compared in particular with the results of Yang et al. studying 109 primary school children and finding that increase in intestinal microbial diversity and increase in *Actinobacteria* and decrease in Fusobacteria were related to *E. vermicularis* infection. Similar trends were found if they occurred in the present study, the results would be independent evidence of Iraqi children regarding the intestinal microbial communities and the association of the disease pinworm. [5]

But the comparison shouldn't suggest that the effects of all helminth infections are the same on the microbiota. Jenkins et al. showed that helminth infections can cause changes in beta diversity, but these changes do not correspond with consistent changes in alpha diversity and/or richness. The differences among studies highlight the possible species and intensity of the parasite, host, diet, geographical location, and exposure to drugs as contributing factors. [6–8]
All of the available evidence suggests that *E. vermicularis* is epidemiologically significant in children and infection with *E. vermicularis* may have measurable effects on changes in the intestinal microbial

community. The present study is different from previous studies as it includes a regional Iraqi perspective, whereby a combination of parasitological diagnosis and molecular identification with microbiome analysis has been included within the same study. [1, 2, 5–8]

4.8. Possible Biological Explanations and Study Limitations

The link between E. vermicularis infection and changes in gut microbiota could be due to several biological mechanisms. Helminth infection has a potential to interact with host immune system and may affect mucosal immune response, secretions from the gut, and local ecological environment. These alterations can indirectly affect bacterial taxa relative abundance. Furthermore, intestinal microbial communities can also affect immune responses against helminths and the helminth–bacteria relationship could be two-way. [5–8, 19, 20]

This is in keeping with the results of Yang et al., who reported that their infected group had changes not only in the diversity of the bacterial flora, but also in the quantity of intestinal secretory immunoglobulin A (SIgA), which is one of the key elements in mucosal immunity. The authors reported reduced SIgA levels in infected children and modifications after mebendazole treatment. This implies that the relationship between *E. vermicularis* and gut microbiota could be both microbial ecology and immune regulation of the host. [5]

However, there are some caveats that should be taken into account when interpreting the present findings. First, the cross-sectional design of the study does not allow for conclusions on temporal or causal links between infection and microbial changes. Secondly, the sample size might not be sufficiently large to detect relatively small differences between microbial taxa. Thirdly, dietary, recent antibiotic, *antiparasitic* treatment, recent infectious diseases, socioeconomic and hygiene factors may be confounding variables if not measured and controlled statistically. [6–8, 22, 26–28]

Fourthly, 16S rRNA sequencing gives taxonomic information but will not directly indicate the functional activity of individual bacterial taxa. Changes in the relative abundance should not be interpreted as changes in the specific metabolic functions of organisms solely. Finally, analysis of the cox1 marker gives useful information on genetic variation, but only from one part of the genome of the parasite. Whole genome or multi-locus analysis would give a more thorough evaluation of the genetic diversity. [22, 23, 26]

One caveat is that differences in microbiomes are hard to interpret. Relative abundance of bacterial groups is compositional; the relative increase in one group may be simply the result of a decrease in the abundance of another. Thus, together with alpha diversity, beta diversity and suitable statistical analysis, abundance results of microbes are interpreted. [16, 23, 27]

So, the present study was carried out in one geographical and one pediatric population of Wasit Province. Thus, the results may not be extrapolated to other age groups or geographic areas in Iraq or to Iraqi children in general. Larger sample size from different provinces of Iraq with longitudinal designs that follow children before and after infection with *E. vermicularis* and its treatment would be beneficial for future research. These studies would be useful in deciding if the observed microbial differences are transient, occurring in conjunction with infection, or more permanent changes of the intestinal ecosystem. [1, 2, 5, 6]

Overall, the integrated parasitological and molecular and microbiome approach will give a more holistic picture of *enterobiasis* than the conventional prevalence studies will. Molecular characterization of *E. vermicularis* will yield information on the identity and genetic variation of the parasite(s); microbiome

analysis will allow for insights into the wider biological context in which the infection occurs. Combining these techniques may help understanding the parasite – micro-biota interaction in Iraqi children and could be a foundation for future longitudinal studies and functioning studies. [2, 5, 6, 21–28]

5. Conclusion

Wasit Province, the study area, still has a significant number of primary school children who are infected with *Enterobius vermicularis*, suggesting its ongoing presence in the population.

The presence of E. vermicularis in a significant percentage of the tested children confirms the high epidemiological value of *enterobiasis* in school environment and the need of effective prevention measures.

Molecular identification using the cox1 gene of the mitochondrial genome could be used as a complement to the traditional parasitological diagnosis and facilitated the confirmation of *E. vermicularis* infection.

The genetic analysis showed that there was nucleotide variation in the isolate of *E. vermicularis* which suggested some genetic diversity in the parasite population in Wasit Province.

Phylogenetic analyses demonstrated genetic similarities between Iraqi *E. vermicularis* isolates and previously reported ones, which appeared useful in studying the molecular epidemiology and genetic relations of *E. vermicularis*.

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