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TÍTULO: Standardization and Diagnosis of Avian Malaria using Polymerase Chain Reaction (PCR) in Hummingbirds: A Pilot Assay

Trabajo de integración curricular presentado como requisito para la obtención del título de Ingeniera Biomédica

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DEDICATORY

This work is dedicated to my family, my parents, and my brother, who, with a lot of effort, support, and affection, accompanied me throughout my university career. I am enormously grateful for the motivation and dedication that helped me finish this work. Thank you for never leaving me alone and always trusting me. Also, I want to dedicate this work to my friends, who I made at university, those who have become my second family and who have supported me throughout my university career.

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RESUMEN

La malaria es una enfermedad infecciosa tropical que afecta a diferentes especies de mamíferos, reptiles y aves. La malaria aviar es causada por protozoos del género *Plasmodium* y *Hemoproteus* que pueden afectar la reproducción y la vida útil de las aves (Abad et al., 2021). Se puede detectar a través de diferentes métodos de detección, como el examen microscópico de frotis de sangre, métodos serológicos y moleculares.

Los métodos moleculares como la reacción en cadena de la polimerasa (PCR) se utilizan para el diagnóstico de la malaria porque han proporcionado mayor especificidad, confiabilidad y sensibilidad. Una vez optimizada, esta técnica a gran escala es más económica, rápida y confiable en comparación con otras técnicas.

En este estudio, el método utilizado es la reacción en cadena de la polimerasa (PCR), ya que esta técnica ha sido diseñada para mejorar la sensibilidad. y especificidad. Inicialmente, se realizó un procedimiento de estandarización de muestras para establecer los parámetros y reactivos óptimos a utilizar en el procedimiento de PCR.

El objetivo principal de este ensayo fue la estandarización y el diagnóstico de la malaria aviar. En este estudio se examinaron 15 muestras de sangre de colibríes del bosque húmedo tropical del sector de Mindo ubicado en la provincia de Pichincha. Finalmente, los resultados obtenidos del estudio de la prueba PCR se compararon con los datos de un estudio anterior, en el que se realizó un examen microscópico de frotis de sangre con los mismos individuos. En resumen, la técnica PCR presentó menor sensibilidad que el diagnóstico por microscopía óptica.

Palabras clave: Malaria aviar, *Plasmodium*, PCR.

ABSTRACT

Malaria is a tropical infectious disease that affects different species of mammals, reptiles, and birds. Avian malaria is caused by protozoans of the genus *Plasmodium* and *Hemoproteus* that can affect the reproduction and lifespan of birds. It can be detected through different diagnostics methods, such as optical microscopic examination of blood smears, serological and molecular methods.

Molecular methods such as the polymerase chain reaction (PCR) are used for the diagnosis of malaria because they have provided more specificity, reliability, and sensitivity. Once optimized, this large-scale technique is cheaper, faster, and more reliable compared to other techniques.

In this study, the method that was used is PCR since this technique has been designed to improve sensitivity and specificity. Initially, a sample standardization procedure was executed to establish the optimal parameters and reagents to be used in the PCR procedure (Abad et al., 2021).

The main objective of this trial was the standardization and diagnosis of avian malaria in Hummingbirds. In this work, 15 blood samples of hummingbirds from the tropical humid forest of the Mindo sector located in the province of Pichincha were be examined. Finally, the results obtained from the study of the PCR test compared with the data from a previous study, in which a microscopic examination of blood smears was performed with the same individuals. In summary the PCR technique presented less sensitivity that optical microscopy diagnosis.

Keywords: Avian malaria, *Plasmodium*, Polymerase Chain Reaction.

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1. TITLE

Standardization and Diagnosis of Avian Malaria using Polymerase Chain Reaction (PCR) in Hummingbirds: A Pilot Assay.

2. INTRODUCTION

Malaria is one of the most prevalent infectious diseases caused by blood-borne protozoan parasites worldwide. According to the World Health Organization (WHO), until 2021, there were 247 million malaria cases and 619,000 human deaths from this disease worldwide. The regions that are most affected are Africa, Asia, and America (WHO, 2022). The correct diagnosis of this disease would guarantee a more effective treatment. However, the countries with the highest spread rates are also the least investigated (Aschar et al., 2022). In the same way, malaria affects humans; it also affects other vertebrates of different species, such as reptiles and birds (Abad et al., 2021). However, malaria is a species-specific tropical zoonotic disease; for this reason, the protozoa that cause avian malaria cannot infect mammals or reptiles (Abad et al., 2021; Valkiunas & Iezhova, 2018).

Avian malaria is an infection caused by Hemosporites blood parasites transmitted to birds by insect vectors (Gutiérrez et al., 2020). There are more than 50 species of parasites that cause malaria, and they are distinguished from each other due to specific characteristics such as geographic distribution, competent vectors, the range of hosts they can infect, and pathogenicity (Paez et al., 2022). Avian malaria is widespread on all continents except Antarctica, in some areas infecting more than 80% of individuals; it is more prevalent than any other malaria that affects vertebrates (Pigeault et al., 2015). In birds, parasites of the genera *Haemoproteus, Plasmodium,* and *Leucocytozoon* cause avian malaria, which is a severe disease that infects wild and domestic bird species (Gutiérrez et al., 2020). However,

birds that are in captivity have a higher contagion rate because these conditions favor the rapid distribution of infectious agents (Mejia et al., 2020).

Avian Hemosporites of the genera *Haemoproteus* and *Plasmodium* are a diverse group of vector-borne hemoparasites (Zhang et al., 2014). These genera are paraphyletic since they have similar biological characteristics and common ancestor. This biological similarity can lead to confusion with each other (Zhang et al., 2014). The life cycles of these two genera present essential similarities, but they differ in the insects involved in their transmission; the genus *Haemoproteus* is transmitted by midges of the genus *Culicoides* and flat flies (Gutiérrez, 2020) On the other hand, the primary vector of *Plasmodium* is the mosquito of the genus *Culex*; however, there are also other genera, *Anopheles*, *Aedes*, and *Culiseta* (Gutiérrez, 2020). These are capable of completing their life cycle in more than 400 species that encompass 11 orders of birds (Paez et al., 2022).

The life cycle of hemoparasites is divided into two phases: an asexual phase that occurs in birds and the sexual phase that occurs in vector mosquitoes (Valkiunas & Iezhova, 2018). Because the number of host species and insect vectors is large, the probability that avian malaria will have a negative impact on different species is greater (Gutiérrez et al., 2020). This disease not only infects the blood but can also cause damage to various tissues of the host (Abad et al., 2021; Zhang et al., 2014). During the acute phase, it generates symptoms such as loss of appetite, anemia, and lethargy (Abad et al., 2021). These effects can be fatal if new lineages of parasites are unintentionally introduced into populations that have never been infected by this specific lineage (Abad et al., 2021). This can result in the decrease or even the extinction of bird species in ecosystems that have been recently invaded (Paez et al., 2022). The aim of this project was the standardization of a diagnosis of avian

malaria by the nested PCR technique for the genus *Plasmodium* in blood samples collected on filter paper, as a pilot study in the malaria diagnosis in hummingbirds.

2.1. Problem Statement

Malaria is a tropical parasitic disease that causes serious health problems in different species, including humans (Abad et al., 2021; Mbanefo & Kumar, 2020). As previously mentioned, avian malaria is widely distributed worldwide, representing a potential problem for bird ecosystems with endemic or endangered species (Abad et al., 2021). The principal genera of avian malaria are *Plasmodium* and *Haemoproteus,* which have similar life cycles and host symptoms (Zhang et al., 2014). However, the parasites of the genus *Plasmodium* represent greater study interest because this genus, in its distribution, includes species that infect humans. Hence, their relatives serve as an experimental model to study the impact of malaria on human health (Abad et al., 2021; Zhang et al., 2014).

The diagnosis of avian malaria is important to prevent uncontrolled spread (Bensch et al., 2000). The diagnosis of malaria can be made through two methods: the examination of blood smears and through molecular techniques such as Polymerase chain reaction (PCR) (Bensch et al., 2000). PCR is a molecular biology technique used to amplify and replicate specific DNA sequence (Snounou et al., 1993). In the case of avian malaria, PCR is a recently studied resource that allows greater sensitivity in diagnosis. However, it is advisable to combine two or more techniques such as optical microscopic examination of blood smears (Panda et al., 2019).

Despite the fact that Latin America is one of the areas with the highest prevalence of malaria (Abad et al., 2021). There are few studies on avian malaria in this territory, including Ecuador (Abad et al., 2021). For this reason, it is important to draw bases to carry out studies

about this disease, due to the negative impact it represents on a biodiverse ecosystem (Paez et al., 2022). This pilot study focuses on the standardization of diagnostic processes; from the extraction of DNA from the blood of birds, to the amplification and detection of the parasite using the molecular technique of PCR. Also, it was compared the percentages of parasitemia and sensitivity of the previous study of blood smears with the sensitivity of the PCR method. This study represents a guide for the diagnosis of avian malaria using molecular methods, expanding the research on this disease in Ecuador.

2.2. Objectives

2.2.1. General Objective

To optimize the processes to diagnose the avian malaria parasite, specifically to *Plasmodium* genus, using the PCR molecular technique.

2.2.2. Specific Objectives

- ✓ To determine the most optimal protocol for the extraction of DNA from the blood of hummingbirds stored on filter paper.
- ✓ To standardize the most optimal PCR process for the amplification of the DNA of the parasite of the genus Plasmodium.
- ✓ To compare the percentage of parasitemia and sensitivity obtained with the blood smear method and PCR molecular method.
- ✓ To describe the levels of parasitemia in wild birds in the humid forests of Ecuador.

3. THEORETICAL BACKGROUND

3.1. Malaria Disease

Malaria is a serious parasitic disease with a high mortality rate in tropical and subtropical countries; in humans, it is caused by parasites of the genus *Plasmodium* (Mekonnen et al., 2014). Five species of *Plasmodium* parasites affect humans; *Plasmodium falciparum*, *Plasmodium Vivax*, *Plasmodium ovale*, *Plasmodium malariae*, and *Plasmodium knowlesi*, with *Plasmodium falciparum* being the species with the highest incidence in humans (Palmer et al., 1998). Transmission of this parasite occurs by many species of blood-sucking insects, mainly an infective female *Anopheles* mosquito (Saito et al., 2018). Malaria to date represents a public health problem in the tropical areas and is the least studied. The parasite infection produces severe symptoms associated with the rupture of erythrocytes, such as anemia, fever, and highly fatal neurological disorders (Bacerio et al., 2012). In the same way malaria affects humans, it is also a problem for other vertebrate species, such as reptiles and birds (Ribeiro et al., 2005).

Haemoproteus can be identified by the presence of hemozoin pigment in gametocytes and the absence of erythrocyte merogony, while *Plasmodium* can be identified by the absence of erythrocyte merogony, known as malaria pigment (Fecchio et al., 2020). For example, avian *Plasmodium* causes diseases in vertebrate hosts that affect the blood and blood-forming tissues, with exoerythrocytic stages of development causing little or no pathology (Valkiunas & Iezhova, 2018). In addition, genome sequencing of chronically infected avian Haemosporidan is challenging due to very low levels of parasitemia, ranging from 0.0001 to 0.1% of infected red blood cells (Videvall, 2019). The most important research is

Plasmodium in avian malaria, because several subgenres of this species of parasites affect humans (Martins, 2011).

In 1996, researcher Garnham classified malaria parasites into 9 subgenera, of these 3 affect mammals, including humans, which constitute less than 2% of known *Plasmodium*, two affect reptiles and four affect birds (Qari et al., 1996)(Abad et al., 2021). For this reason, malaria is among the most common diseases in birds (Martins et al., 2011). Avian malaria causes physiological damage to both wild and captive birds, affecting their individual condition and survival (Bacerio et al., 2012). Mosquitoes are the most important cause of infection of avian Hemosporidia; which belongs to the genera *Haemoproteus, Plasmodium* and *Leucocytozoon* (Valkiunas & Iezhova, 2018). The most common case studies are avian *Plasmodium* and avian *Haemoproteus*; which are different in ecology, biology and morphology, however, they depend on blood-sucking insects and a bird to finish their life cycles (Videvall, 2019).

3.1.1. Avian Malaria Life Cycle

Malaria-causing parasites are obligate Heteroxene; they have complex life cycles because they need more than one host to complete it (Carlson et al., 2018). The general life cycle of Haemosporidan has similar stages for both *Plasmodium* and *Haemoproteus* (Jarvi et al., 2002). However, the major difference between these genera is that they use different families of vectors from the order Diptera: *Heamaproteus* uses biting mosquitoes and lice, while the genus *Plasmodium* uses blood-sucking mosquitoes (Abad et al., 2021; Gutiérrez-López & Martínez-De La Puente, 2020). The life cycle of avian malaria parasites is quite complex and has two phases, an asexual phase (vertebrate host), followed by a sexual reproduction phase (insect vector) (Gutiérrez, 2020; Valkiunas & Iezhova, 2018).

Hemosporidia undergoes a series of asexual divisions in the intermediate vertebrate host until the development of gametocytes into the sexual stages occur in the definitive dipteran vector host (Valkiunas & Iezhova, 2018). The life cycle begins with the vector feeding on the blood of an infected intermediate host (Clark et al., 2012; Videvall, 2019). In the mosquito intestine, gametocytes carry out gametogenesis and sexual reproduction (Clark et al., 2012). Gametogenesis produces an ookinete, which penetrates the epithelial layer of the midgut to become an encapsulated oocyst (Clark et al., 2012). Later, the oocysts mature and burst, invading the salivary glands of the mosquito vector (Abad et al., 2021; Clark et al., 2012) which, upon feeding, inject saliva infected by sporozoites into the vertebrate host (birds) (Valkiunas & Iezhova, 2018).

In the intermediate vertebrate host, the development of the stages depends on the genus of the parasite that infects it (Pigeault et al., 2015). Still, five main steps are identified: the first occurs when the parasite develops in tissue cells (Cosgrove et al., 2006), the exoerythrocytic schizonts or meronts, which undergo asexual divisions to form merozoites (Clark et al., 2012). The merozoites can produce a new cycle of merogony or re-establish themselves in the bloodstream to become sexual stages (Clark et al., 2012). In the second phase, called the acute period, the first appearance of parasites in the host's blood occurs (Jarvi et al., 2002). In the third phase, it occurs due to when the parasitemia reaches its maximum point (Bensch et al., 2000). During the final two stages, the fourth and fifth, the parasitemia decreases or is eliminated due to the immune response (Zhang et al., 2014).

Avian malaria infection is chronic or latent; that is, once a bird is infected and survives, it retains the disease for many years or even its entire life (Clark et al., 2012). These become the new sources of infection for vector mosquitoes (Carlson et al., 2018).

Environmental factors play an important role in the increase or decrease of parasitemia; for example, an increase in parasitemia can occur in spring and autumn after the reproduction period of the host individuals (Aschar et al., 2022; Valkiunas & Iezhova, 2018). This approach of individuals facilitates the infection of vectors and the transfer of parasites (Bensch et al., 2000).

3.2. Effects of Avian Malaria Infection in Wild Birds

3.2.1. Diversity and Distribution of Haemosporida Parasites in Birds

Birds are affected by different blood parasites. However, the parasites with the highest prevalence in birds are those of Haemosporida (Outlaw et al., 2017). Hemosporidia that causes avian malaria is found on all continents except Antarctic (Inumaru et al., 2021). Neotropical regions are considered hotspots of avian diversity; therefore, many new Haemosporidan species are likely to exist (Chagas et al., 2017). The principal genera of avian malaria are *Plasmodium* and *Haemoproteus* are transmitted by dipterous arthropods. In addition, ecological requirements are relevant to transition, such as temperature, availability and water flow, and in the general distribution of Haemosporidan (Chagas et al., 2017).

Dipterous vectors contribute to the transmission of multiple avian pathogens (Carlson et al., 2018). The functional diversity of the vector is an important consideration (Abad et al., 2021). However, diversity also includes other factors, such as the compatibility of the vector with the host. Also abiotic factors, such as climate and environmental restrictions (Carlson et al., 2018). The distribution and diversity of avian malaria species are related to climatic characteristics (Outlaw et al., 2017). For example, *Plasmodium* parasites can complete the sporogony stage in vectors sensitive to medium or low temperatures, while *Hemaproteus* have a greater distribution than *Plasmodium* except in South America (Abad et al., 2021).

3.2.2. Physiological Impacts of Malaria on Wild Bird Populations

Parasitic infections can cause physiological symptoms in the host and can also reduce reproductive and survival skills (Knowles et al., 2010; Schoenle et al., 2017). The infection percentages vary depending on the species. That is, not all birds are equally affected by malaria parasites (Knowles et al., 2010; Lachish et al., 2011). There are birds that are asymptomatic. On the other hand, birds with a high load of parasites can present fever, diarrhea, and anemia, due to the destruction of red blood cells (Schoenle et al., 2017). These symptoms and the severity of the infection may vary according to the species of parasite and the phase of its life cycle found (Knowles et al., 2010).

In the initial acute phase, the physiological effects are lethargy, loss of appetite, and anemia (Abad et al., 2021; Valkiunas et al., 2018). In this phase, the behavior of the birds is also affected, the reduction of survival rates, the selection of partners, their reproductive cycle, the ability to find food, and the ability to avoid predators (Abad et al., 2021; Schoenle et al., 2017). In the low-level chronic phase, parasitemia indices are reduced, and generally, the affected individual no longer presents symptoms. However, it is an active carrier of avian malaria and can be transmitted to another host (Lachish et al., 2011).

3.3. The Significance of Avian Malaria in the Diagnosis and Treatment of Human Malaria

Malaria or paludism is a chronic infectious disease representing a global public health problem (WHO, 2022). In humans, the condition is caused by protozoan parasites of the genus *Plasmodium* (Spencer et al., 2016). This genus is particularly interesting because some species infect other vertebrates (Spencer et al., 2016). *Plasmodium knowlesis* was reported as a primate zoonosis in humans in Africa (Spencer et al., 2016; Valkiunas et al., 2018). On

the other hand, it is estimated that half of the world's population is at risk of malaria infection; the species *P. falciparum* is responsible for the majority of deaths in humans and is considered the most aggressive about clinical manifestations; there is evidence to support that the cause of its virulence may be a change in host species; from birds to humans (Valkiunas et al., 2018). For this reason, developing diagnostic techniques is essential to develop strategies to control the spread of the disease (Abad et al., 2021).

Avian malaria has been studied for decades because it is essential in understanding human malaria as an experimental model to explain malaria transmission (Abad et al., 2021; Valkiunas et al., 2018). Additionally, avian malaria has been used as an experimental system for developing antimalarial drugs and vaccines (Qari et al., 1996) due to the similarity that bird parasites have with human malaria parasites and that some of these lineages are very close in the phylogenetic tree (Abad et al., 2021; Pacheco et al., 2020). For this reason, diagnosis for treatment in the acute phase of the disease could prevent the uncontrolled spread that endangers the life of humans and wild birds (Snounou et al., 1993).

3.4. Avian Malaria Diagnosis

Birds infected with avian malaria may show physiological symptoms associated with the disease, such as lack of appetite, fever, and anemia (Ribeiro et al., 2005). However, most cases may be asymptomatic or found in the final stages of the infection (Martins et al, 2011) For this reason, it is important to use diagnostic techniques to prevent the spread of the parasite in more birds, improve the quality of life of birds and even guarantee the biodiversity of large ecosystems (Aschar et al., 2022; Panda et al., 2019). In the diagnosis of avian malaria, laboratory techniques are used, such as the blood smear is the oldest method; serological

techniques such as immunochromatography; and molecular biology techniques, such as PCR (Wang et al., 2014).

Parasitological Diagnosis

Avian malaria can be detected through a blood smear, this method consists of staining a drop of blood with Giemsa reagent (Abad et al., 2021). This allows the visualization of the parasites through the optical microscope (Valkiunas & Iezhova, 2018). It allows observing the red blood cells that are infected by the parasite, through a count the level of malaria infection can be determined (Mbanefo & Kumar, 2020). This method has some advantages such as, it is a simple, low-cost technique, it allows us to quantify the level of infection and, depending on the morphological characteristics observed, the parasite species can be identified (Rahim et al., 2020). Although it is one of the most common techniques, currently molecular techniques can complement and even replace this technique (Carlson et al., 2018).

Serological Diagnosis

The detection of malaria by means of serological methods is not applied like that of blood fortis or molecular techniques (Clark et al., 2012). This method recognizes antibodies from the host's immune response to parasites rather than recognizing the parasites themselves (Abad et al., 2021c) One of the most important advantages of this method is that it can detect parasites in stages that parasitological and molecular diagnoses cannot (Cosgrove et al., 2006). For example, indirect immunofluorescence assays use a monoclonal antibody to a protein conserved in all species (Abad et al., 2021; Valkiunas & Iezhova, 2018).

Molecular Diagnosis

Thick Blood Smears (TBS) staining is the gold standard method for the diagnosis of malaria. However, different PCR techniques have shown greater sensitivity for cases with low parasitemia or for mixed infections (Abad et al., 2021; Gama et al., 2007). Accurately detecting the presence or absence of the parasite in the blood of individuals (Zhang et al., 2014). To complement the diagnosis of malaria, it is recommended to implement the different techniques available (Bensch et al., 2000).

Currently, molecular techniques have offered great opportunities for the identification and quantification of avian Haemosporida parasites (Huang, 2021). It also plays an important role in the study of interactions between parasites and their hosts (Freed & Cann, 2006; Huang, 2021). These investigations help to study the adaptation of parasites to different hosts and thus determine the impact of chronic infections on the biodiversity of avian ecosystems (Huang, 2021). The polymerase chain reaction (PCR) is the technique with the highest sensitivity and specificity, which can detect low levels of parasitic infection (Freed & Cann, 2006).

3.5. Polymerase Chain Reaction (PCR) Technique to Detection of Parasitic Infections

Parasitic infections represent a significant disease burden worldwide due to the ease with which they are transmitted (Pomari et al., 2019). For this reason, research on more sensitive and specific diagnostic techniques is necessary (Pomari et al., 2019), which will improve programs for controlling and eliminating parasites that cause chronic infectious diseases (Dacal et al., 2020). Currently, research has been carried out on molecular methods for diagnosing parasitic infections (Pomari et al., 2019). These methods have been shown to have favorable characteristics compared to microscopic methods among which they have

greater sensitivity and specificity (Mahajan et al., 2012). In addition, a simple standardization of diagnostic procedures (Pomari et al., 2019).

In general terms, the technique consists of the enzymatic synthesis of millions of copies of a specific DNA sequence (Rodriguez J.M., 1997). The enzyme responsible for this enzymatic activity is Taq polymerase, through a chain of cycles that includes three stages: denaturation of the template DNA, hybridization of the specific primers for the sought DNA, and finally an extension stage (Rodriguez J.M., 1997). In this way, multiple copies of the target DNA are amplified, making the diagnosis more sensitive even if DNA levels are low (Mahajan et al., 2012; Rodriguez J.M., 1997). Infections such as avian malaria can be detected in the early stages, in which parasitemia is not as high due to the sensitivity of the PCR test (Mahajan et al., 2012). In order to distinguish between species the infection in avian malaria it is necessary to do by the extensive use of molecular methods targeting mitochondrial genes (Pacheco et al., 2018).

3.5.1. Limitations of PCR Test to Diagnostic Malaria

The PCR test has had good results in detecting parasitic diseases such as malaria, except when parasitemia is low (Freed & Cann, 2006). However, the test has limitations; which are the test cannot estimate the level of parasitemia or the stage of infection, false negatives can be obtained because early stages of infection are not detectable, and extreme sensitivity generates false positives, but due precautions are taken in the extraction process (Freed & Cann, 2006). In their study, Freed & Cann, 2006 divided false positive and false negative problems into two categories: genuine and apparent problems. Genuine issues include primer design problems, poor laboratory procedures, variations in polymerase enzymatic activity, and degraded DNA samples. The apparent problems are caused by

problems in the technology and the experience of the microscopy laboratory worker (Freed & Cann, 2006; Gama et al., 2007).

On the other hand, studies have been carried out comparing the sensitivity of PCR tests with microscopy; for example, in the survey by Berzosa et al., 2018 three diagnostic tests for malaria parasites were compared. The microscopic, the rapid RDT test, and the PCR (Berzosa et al., 2018) In this study, a considerable number of samples were analyzed by microscopic examination and then by PCR; also, the rapid test and then PCR. The sensitivity of the combined tests was higher than when only one test was analyzed separately. Therefore, the tests should be done together to improve the sensitivity and specificity of malaria diagnosis in birds and humans (Berzosa et al., 2018).

3.5.2. PCR to Diagnosis of Avian Malaria

Molecular detection methods, mainly polymerase chain reaction (PCR), have allowed detailed molecular investigations of the parasites that cause avian malaria (Jarvi et al., 2002; Feldman, 1995). Several studies suggest that the PCR technique has greater sensitivity and specificity than other methods (Jarvi et al., 2002; Wang et al., 2014). These studies found that PCR improved the detectability of malaria in blood by 67% in birds and up to 6% in reptiles (Feldman, 1995; Wang et al., 2014). Due to the characteristics shown by the PCR technique, DNA fragments can be amplified from a blood aliquot containing as little as 1 malaria parasite per milliliter of blood (Ribeiro et al., 2005). The investigation of DNA amplification by PCR has allowed the development of highly sensitive methods for detecting and identifying parasites (Snounou et al., 1993). Nested PCR efficiently improves sensitivity for better diagnosis of parasites (Snounou et al., 1993).

Several studies have investigated and compared the sensitivity obtained by applying the PCR technique with other techniques, such as blood smears, microscopy, and others (Snounou et al., 1993; Wang et al., 2014). The study carried out by Freed & Cann, 2006 where the PCR assay is compared with the examination of thick blood smears by microscopy, the result was that PCR has a 97.4% sensitivity. Other studies concluded that a single parasite genome can be routinely and reproducibly detected in template DNA purified from 10μL of blood exhibiting 0.000002% parasitemia (Snounou et al., 1993).

On the other hand, several studies recommend a combination of methods to obtain a reliable diagnosis of malarial diseases in birds (Jarvi et al., 2002; Feldman, 1995; Wang et al., 2014). Different methods have been developed to detect parasites, including conventional PCR, real-time PCR, nested PCR, and restriction fragment length polymorphism (PCR-RFLP), among others (Pomari et al., 2019). The PCR technique is widely used for many applications, mainly in diagnosing infectious diseases such as malaria, both in humans and in other vertebrate species such as birds (Freed & Cann, 2006). These methods are successful in the identification of parasites. However, they still have limitations in sensitivity and specificity, especially when the sample is not so abundant (Mahajan et al., 2012).

4. EXPERIMENTAL PART

4.1. Methodology

4.1.1. Reagents

4.1.1.1. Reagents for Extraction Process

DNA extraction from the filter paper using the reagents provided by the GenElute Mammalian Genomic DNA Mini-Prep Kit, Catalog No. G1N70, and for the standardization process of the DNA extraction protocol, the QIAGEN DNeasy Blood & Tissues Kit was used

(QIAGEN, 2020). These kits provide a simple and convenient way to isolate pure genomic DNA from blood from filter paper. The samples are lysed with a solution containing chaotropic salt to completely denature the macromolecules (Sigma-Aldrich Co. LLC., 2017) (QIAGEN, 2020). Tris, a common pH buffer, and EDTA, a molecule that chelates cations (TE buffer). Ethanol 98-100% is then added to bind the DNA as the lysate is spun through a silica membrane in the microcentrifuge tube (Sigma-Aldrich Co. LLC., 2017). The specifications and quantities of the reagents included in the kits are detailed in Appendix 1. Additionally, the protocol suggests the use of other reagents that are not included in the Kits, such as Ethanol (95%-100%) and water molecular biology reagent.

4.1.1.2. Reagents for Diagnostic Process

In the polymerase chain reaction optimization process to diagnose avian malaria, the LightMix Polymerase 1-Step RT-PCR mixture was used, including MgCl2, dNTPs, reaction Buffer, the enzyme TAQ polymerase and reverse transcriptase enzyme. The company LIGO Humanizing Genomics Microgen synthesized the primers as forward Plas-F (5'-GTA ACA GCT TTT ATG GGT TAC-3') and as reverse 4292Rw (5'-TGG AAC AAT ATG TAR AGG AGG AGT-3 ') (Abad et al., 2021c; Martínez et al., 2009), which were reconstituted in elution solution for greater durability. In addition, nuclease-free PCR-grade water was used to complete the volume reaction. In the electrophoresis process, the reagents were used to prepare the 1.8% agarose gel: 1X TAE buffer, agarose, ethidium bromide, the Opti-DNA 100 bp molecular weight marker from the Applied Biological Materials Inc (abmGood, 2023) and loading buffer.

4.1.2. Equipment

Among the equipment used, we have a laminar flow cabinet to maintain the sterility of the process, the thermoblock Thermo Scientific™ Digital Heating Shaking Drybath for the incubation of the samples at different temperatures, Thermo Scientific Pico™ 17 Laboratory Microcentrifuge, and Thermo Scientific LP Vortex Mixer, max speed 3000 rpm. The Thermo Fisher Scientific Nanodrop Microvolume Spectrophotometer was used for the quantification of the DNA obtained. In the PCR process, an Applied Biosystems Veriti 96-Well Fast Thermal Cycler was used, then for the diagnosis by electrophoresis, a Horizontal Electrophoresis Cuvetary Mod. M12 Bioted, a TetraSource™ 300 Power Supply, max voltage 300V, and a Cleaver Scientific microDOC Gel Documentation System with 254/365nm UV Transilluminator were used to visualize the gels.

4.1.3. Standardization Process

4.1.3.1. Standardization of DNA Extraction Protocol

The samples used to standardize the DNA extraction protocol were from Human blood obtained with previously informed consent, and placed on filter paper to simulate the blood samples of birds, which were analyzed once the standardization process was finished. For this, 53 Human blood samples were used on previously sterilized pieces of filter paper (see Appendix 2), stored at -20°C to avoid DNA loss. Several assays were performed following the extraction protocols established by the QIAGEN DNeasy Blood & Tissues Kit (QIAGEN, 2020) and the GenElute Mammalian Genomic DNA Mini-Prep Kit, Catalog No. G1N70 (Sigma-Aldrich Co. LLC., 2017), until obtaining the concentration and purity of DNA necessary to give way to diagnosis by PCR and electrophoresis.

The quantification of the DNA obtained in the samples was carried out using the Thermo Fisher Scientific Nanodrop Microvolume Spectrophotometer, which, based on the absorbance capacity of a compound in a solution at a certain wavelength, determines the concentration and purity of the sample. (Banco Nacional de ADN, 2020; Desjardins & Conklin, 2010). The concentration of the DNA sample is calculated with the absorbance value at a wavelength of 260nm (Banco Nacional de ADN, 2020; Desjardins & Conklin, 2010). The purity of the sample is determined due to the A260/A280 ratio, which is quite stable. An optimum purity is considered a value between 1.8-2.0, an A280/A260 value greater than 1.6 is considered acceptable, while a value less than 1.6 indicates contamination from aromatic compounds such as phenols and proteins. Finally, a value greater than 2.1 can show the presence of RNA (Desjardins et al., 2010; Desjardins & Conklin, 2010). Another purity parameter is the A260/A230 ratio, which is more variable than the A260/A280 ratio, depending on factors such as the DNA concentration or the resuspension cap of the sample. An optimal value is between 1.8-2.2, a value less than 1.8 is related to the presence of contaminants (Desjardins & Conklin, 2010). Based on these already established parameters, the samples were analyzed to determine which of the kits had the best performance for the purification and isolation of DNA.

4.1.3.2. Standardization of PCR and Electrophoresis Protocol

Subsequently, for the optimization of the PCR process for the samples in the first stage, human blood samples were used, performing a pre-PCR assembly process using the primers Beta-1 (5'-TCAACCCTACAGTCACCCAT-3') and Beta_2 (5'-TCA ACCCTACAGTCACCCAT-3') to identify human beta-globin and molecular marker for DNA, after obtained samples were visualized in a 1.8% agarose gel with the weight marker

and under UV light. However, the optimization of the PCR process was carried out mainly using the samples obtained from hummingbird blood; for this optimization it was used the LightMix polymerase, and the Plas-F primers (5'-GTA ACA GCT TTT ATG GGT TAC -3') and 4292Rw (5'-TGG AAC AAT ATG TAR AGG AGG AGT-3') (Abad et al., 2021c; Martínez et al., 2009). The tests were carried out by configuring the annealing temperature following the protocol suggested in the literature (Abad et al., 2021). The samples were run and visualized in an agarose gel with Horizontal Electrophoresis Cuvetary, and a gel documentation system. In which we varied their concentrations between 1.5%, 1.8%, and 2% with two voltages, 120V, and 150V, for an estimated time of 30 minutes.

4.1.4. Extraction of DNA of Blood on Paper Filter

The blood samples of the hummingbirds were collected in a period from October 2018-February 2020, in Mindo Cloudforest Foundation reserves in Pichincha Province, specifically in the Puyucunapi Hacienda located in Nanegalito; the results of the statistical analysis of the parasitemia (see Appendix 3) (Abad et al., 2021). The 15 blood samples of the individuals were collected on filter paper and stored at -20 degrees Celsius.

To start the DNA purification process using the GenElute Mammalian Genomic DNA Miniprep Kit, Catalog No. G1N70, 1) using previously sterilized surgical scissors, filter papers were cut the filter papers as close as possible to the blood sample (see Appendix 4A), and 2) samples were washed with 200 ul of TE buffer and incubated at 52 ° C for 24 hours (see Appendix 4B). 3) TE buffer was discarded preserving the paper, then 20 ul of proteinase K, 200 ul of resuspension solution, and also 200 ul of lysis solution were added; all this solution was passed to the Thermo Scientific LP Vortex Mixer for 10 seconds. 4) The Thermoblock Thermo Scientific™ Digital Heating Shaking Drybath process was used to

incubate the sample with the previous solution for 15 minutes at 56 ° C. 5) Meanwhile, the collection column was assembled with the microcentrifuge tube of GenElute Miniprep Columns and 500 ul of preparation solution was added, later centrifuged at 12000 rpm for 1 minute, discarded the liquid and kept the tubes. 6) In the samples after incubation, 200 ul of ethanol (96%-100%) was placed in the vortex for 15 seconds. 7) The total volume of the solution was transfer to the centrifugation columns which prepared earlier, and centrifuged it at 8000 rpm for 1 minute, discarded the liquid, and kept the collection column. 8) The first wash carries out adding 500 ul of wash solution and centrifuge it at 8000 rpm for 1 minute, discard the liquid, and keep the collection column. 9) In the second wash, added 500 ul of wash solution and centrifuged at 12000 rpm for 3 minutes; throw the liquid and kept the column, and re-centrifuged for 1 minute at 8000 rpm to remove ethanol residues in the sample. 10). Finally, I placed 200 ul of elution solution, incubated at room temperature for 5 minutes, and centrifuged with Thermo Scientific Pico™ 17 Laboratory Microcentrifuge at 8000 rpm for 1 minute. Finally, the quantity and purity of the DNA obtained with the

nanodrop were measured, and stored the samples at -20 °C to proceed with the diagnostic process (see Appendix 4C).

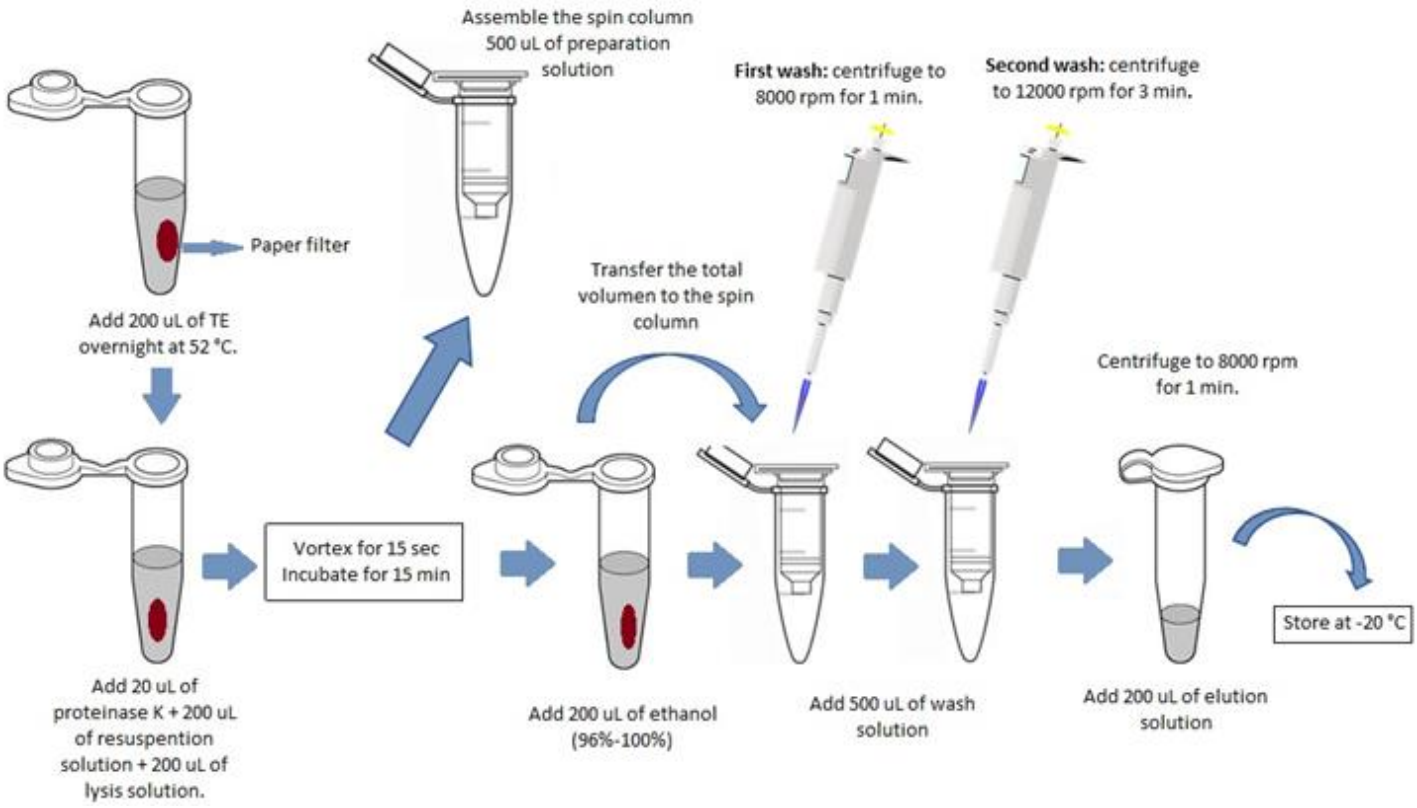

Figure 1. Filter paper DNA extraction protocol, using the sigma GenElute Mammalian Genomic DNA Mini-Prep Kit, Catalog No. G1N70.

4.1.5. DNA Amplification

Following the DNA extraction process, the amplification and detection of the presence of the *Plasmodium* gene in the bird samples were carried out using the PCR technique. In section 4.1.1.1, the reagents used for the assembly of the samples in the pre-PCR are specified. For the detection of the specific fragment, the sequence of primers suggested by the literature was used, which are specific for the *Plasmodium*: Plas-F (5 '-GTA ACA GCT TTT ATG GGT TAC-3 ') and 4292Rw (5' -TGG AAC AAT ATG TAR AGG

AGG AGT-3') (Abad et al., 2021c; Martínez et al., 2009). The primers were dissolved with the manufacturer's recommendations for a volume of 100pmol/ul. The LightMix Polymerase PCR reaction mix contains a reaction buffer for polymerase, dNTPs, and Taq polymerase enzyme. In the table 1 is presented the details the amounts of reagents for a total reaction volume of 20 ul for five samples and the control.

Table 1. Number of reagents for the pre-PCR assembly, the amount calculated for 5 samples and the control. The same quantities are repeated for the following samples until completing 15.

Reagents	1x	7x
Mix	10 ul	70 ul
Primer Forward	0,5 ul	3,5 ul
Primer Reverse	0.5 ul	3,5 ul
Water PCR grade	5 ul	35 ul
DNA	4 ul per reaction tube	
Total volume	20 ul per reaction tube	

In the pre-PCR process, a master mix was prepared to facilitate and reduce errors. The volume that each PCR tube contains is 16 ul of master mix and 4 ul of sample DNA. The graphical representation of this process can be seen in figure 2.

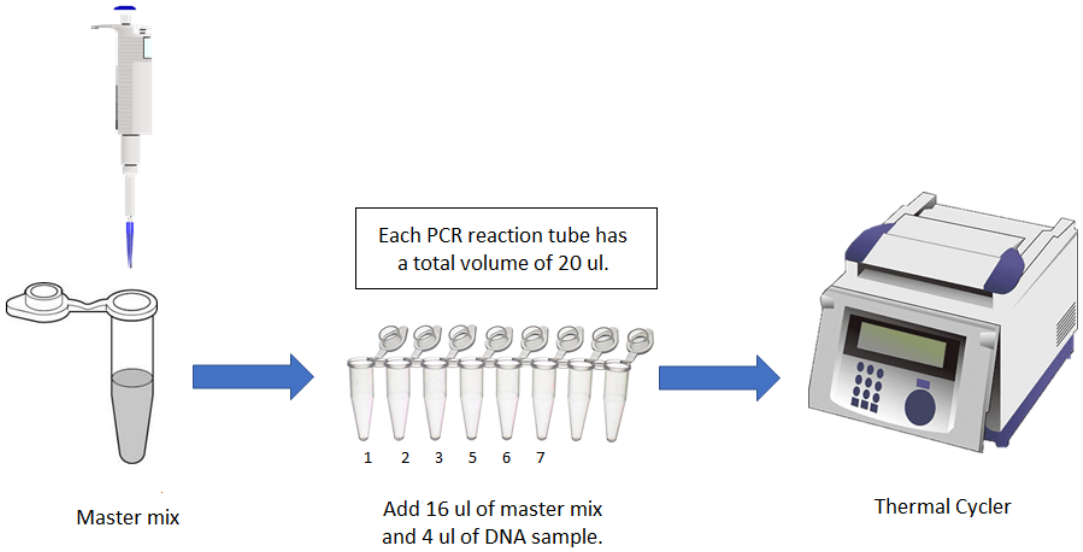

Figure 2. Pre-PCR process specifying the volumes needed for the PCR reaction.

4.1.6. PCR Technique Process

The pre-PCR reaction mixtures are subjected to thermal amplification cycles using equipment Thermo Scientific thermocycler (Ings & Denk, 2022). As we previously read in section 2.3.4, each cycle has three important phases: DNA denaturation, annealing, and extension (Niebuhr & Blasco-Costa, 2016). In this experiment, we followed the PCR phases suggested in Abad et al., 2021 and Martínez et al., 2009 (ref). For the denaturation stage, a cycle was used at a temperature of 94 °C for 10 minutes, then for the tempering stage we programmed 40 cycles at an initial temperature of 95 °C for 40 seconds, a temperature of 57°C for 1 minute and finally 72 °C for 1 minute. For the final stage, we have a cycle at 72 °C for 10 minutes and a standard of 4 °C for an indefinite time (see Appendix 5) (Figure 3). The final volume of the PCR mixtures was 20 ul, then electrophoresis of the 15 samples was performed, separating them into three groups of 5 samples to facilitate the calculations and the experimentation process.

Stage 1 X 1	Stage 2 X 40	Stage 3 X 1	Stage 4 X 1

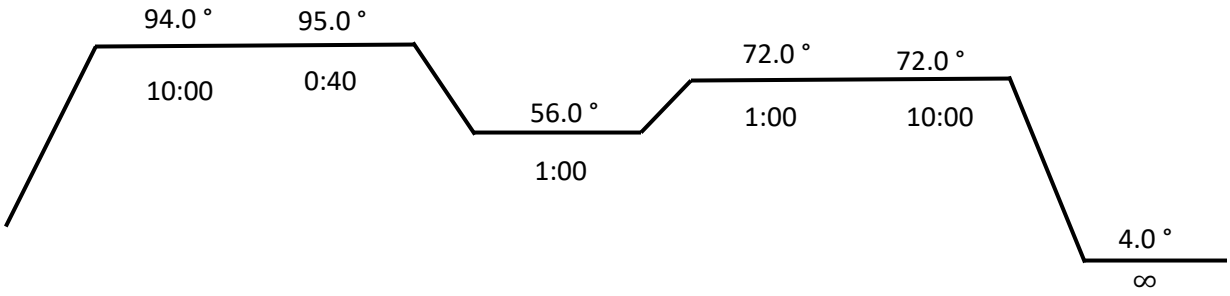

Figure 3. Visualization of the PCR cycle programmed in the Thermo Scientific thermal cycler. Stage 1: one cycle for 10 minutes at 94°C. Stage 2: 40 cycles with sequences of 95°C, 56°C and 72°C at 40 seconds, 1 minute, and 1 minute, respectively. Stage 3: one cycle for 10 minutes at 72°C. Stage 4: one cycle at 4°C for an indeterminate time.

4.1.7. Electrophoresis

After the PCR amplification process, an identification method must be used, in this case, electrophoresis. A 1.8% gel was prepared in this process, calculating the necessary reagents to achieve this concentration. The gel was then stained with 7.5 ul with ethidium bromide. Samples were loaded onto the gel with a 10X concentration loading buffer. The 100bp Opti-DNA Marker molecular weight marker was used as a comparison reference to determine the presence of *Plasmodium*. The gel was run in a system with Horizontal Electrophoresis Cuvetary at a constant voltage of 150 V for 30 minutes. Once the time elapsed, the gels were visualized under ultraviolet light with gel documentation system (see Appendix 7).

5. Results and Discussion

5.1. Results

Once the DNA extraction protocol standardization tests of the total of 53 human blood samples were completed, the samples were quantified using the Nanodrop Microvolume Spectrophotometer to determine concentration and purity (see Appendix 2). The results obtained were analyzed according to what is established in section 4.1.3.1 on the absorbance parameters of the Thermo Fisher Scientific Nanodrop Microvolume Spectrophotometer. Of the total of 53 human blood samples, 48 were processed with the QIAGEN DNeasy Blood & Tissues Kit and the remaining 5 with the GenElute Mammalian Genomic DNA Mini-Prep Kit, Catalog No. G1N70, the number of reagents in the second kit was more limited; for this reason, fewer trials were performed.

In order to simplify the visualization of the data, a fundamental statistical analysis in RStudio was carried out (see Appendix 6), applying the calculation of the measures of central tendency to determine average values that allow us to compare the performance in terms of concentration and purity of the two kits. We know that in the A260/A280 ratio, values between 1.8-2.0 suggest high purity, which is the case with the GenElute Mammalian Genomic DNA Mini-Prep Kit, unlike the 1.57 from the DNeasy Blood & Tissues Kit which is lower but still acceptable based on absorbance parameters of Nanodrop. The most significant differences are in the concentration and the A260/A230 purity ratio, which is lower in the QIAGEN DNeasy Blood & Tissues Kit (Table 2).

Table 2. Mean values of the data calculated with RStudio, to differentiate the performance between the two kits.

Mean values			
Kits name	ng/ul	A260/A280	A260/A230
QIAGEN DNeasy Blood & Tissues Kit	4.06	1.57	0.13
GenElute Mammalian Genomic DNA Mini-Prep Kit	56.22	1.82	1.07

Following the analysis, we proceeded to extract the DNA from the 15 hummingbird blood samples using the GenElute Mammalian Genomic DNA Mini-Prep Kit, which presented the highest performance. The results can be seen in table 3, which details the concentration and purity values measured with the Thermo Fisher Scientific Nanodrop Microvolume Spectrophotometer. The concentration values of DNA in most of the samples are high. However, some samples present concentrations lower than 20 ng/ul. Regarding purity, in the A260/280 ratio, most of the samples have an optimal purity of 1.8-2.0 and an acceptable purity greater than 1.5. Then, in the A260/230 ratio, more varied values are observed between the optimum and those greater than 2.0.

Table 3. Concentration and purity values of the 15 samples of purified hummingbird DNA, measured with the nanodrop spectrophotometer.

Sample name	ng/ul	A260/280	A260/230
A1	54.4	1.79	2.18
A2	55.4	1.68	1.47
A3	44.8	1.82	2.53
A4	55.1	1.75	1.86

A5	53.0	1.78	2.29
A6	19.8	1.77	1.66
A7	12.9	1.53	1.34
A8	41.5	1.72	1.43
A9	28.7	1.76	1.71
A10	34.6	1.87	4.13
A11	20.0	1.72	2.04
A12	46.5	1.76	1.52
A13	16.7	1.94	1.55
A14	5.0	1.58	1.73
A15	37.1	1.84	1.97

In the first PCR diagnostic tests, some human DNA samples were selected. The pre-PCR to detect beta-globulin was prepared with the primers Beta-1 (5'-TCAACCCTACAGTCACCCAT-3') and Beta_2 (5'-TCA ACCCTACAGTCACCCAT-3'), following a suggested protocol for human DNA samples (see Appendix 4). However, the PCR protocol for amplifying human beta-globin is different from *Plasmodium* amplification. The rest of the standardization processes were carried out using previously obtained DNA samples from the birds.

On the other hand, samples A1 and A2 were selected to start the tests, in which the temperatures and time in the annealing stage were varied until optimal visualization of the results was achieved. The temperature range used ranged from 54°C to 60°C. After the tests, the temperatures with the best visualizations were determined at 56°C and 57°C. In the electrophoresis it was verified that a better displacement of the bands in the agarose gel was achieved at a voltage of 150V for 30 minutes (see appendix 7).

Finally, the PCR protocol detailed in section 4.1.6 was used to diagnose avian malaria. The 15 samples of purified DNA were processed in three groups of 5 samples plus a negative control. Once the PCR process was finished, the results were visualized using the previously standardized electrophoresis technique. Three agarose gels with a concentration of 1.8% were prepared; each gel included a total of 7 samples, with the molecular weight indicator and the negative control (Figure 4, Figure 5, Figure 6).

According to Martínez and co-workers 2009, the primers used to detect *Plasmodium* generate a product of 422bp, basing on this weight, the analysis of the electrophoresis images was carried out, giving as result. The visualization of bands with an approximate molecular weight of 422bp, corresponding to the molecular weight of the Plas-F primers (5'-GTA ACA GCT TTT ATG GGT TAC-3') and 4292Rw (5'-TGG AAC AAT ATG TAR AGG AGG AGT-3') used to detect *Plasmodium*. In Figure 4, showed that sample A2 presented a fragment. In Figure 5, samples A8, A9, and A10 also presented bands. Finally, in Figure 6, we observed a band in sample A14, although a sizeable residual sample is also observed.

In figure 4, showed that the band has a molecular weight between 500bp and 400bp, as indicated by the molecular weight marking (100bp). Sample A2 presented a band with an approximate weight of 422bp; this sample had the highest percentage of parasitemia (7%). The remaining samples A1, A3, A4, and A5, do not presented bands that can be visualized.

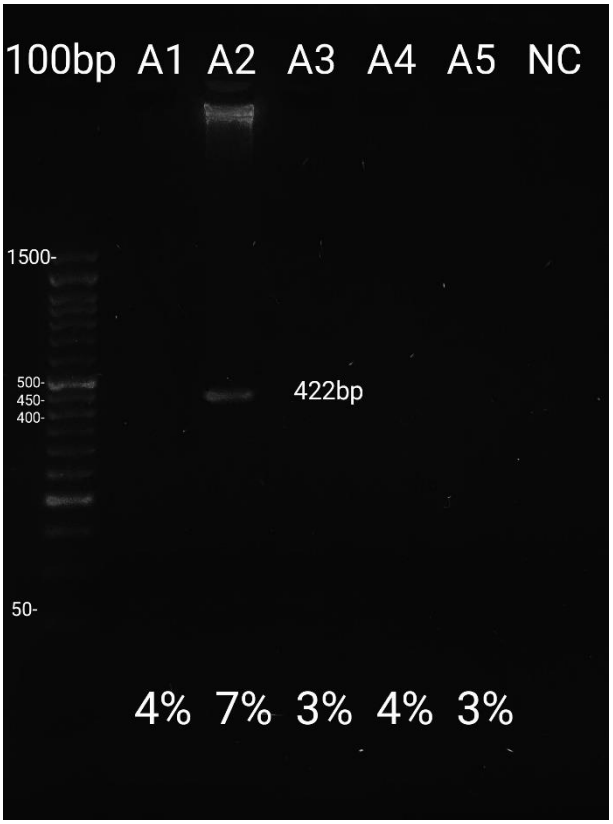

Figure 4. Visualization of the results of the samples named A1, A2, A3, A4, and A5; the negative control (NC) and the 100bp molecular weight marker (MW). Bands indicates the presence of the avian malaria parasite (422bp). Percentages of parasitemia of the previous diagnosis of blood smears (Abad et al., 2021).

In Figure 5, showed that samples A8, A9, and A10 presented bands that are between 500bp and 400bp. This suggested that the samples had the molecular weight value to detecting avian malaria. The parasitemia (%) with the highest percentage presented fragments (422bp), while A1 and A2 had the lowest rates; fragments are not seen in these samples.

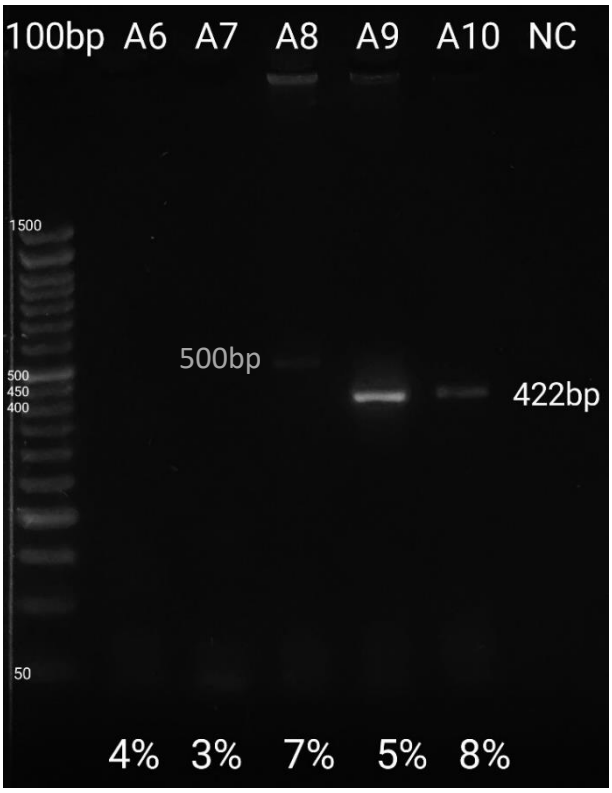

Figure 5. Visualization of the results of the samples named A6, A7, A8, A9, and A10; the negative control (NC) and the 100bp molecular weight marker (MW). Bands indicated the presence of the avian malaria parasite (422bp) (500bp). Percentages of parasitemia from the previous diagnosis of blood smears (Abad et al., 2021).

In Figure 6, a band (422bp) is observed in sample A14, which also has the highest percentage of parasitemia, in contrast to the rates of samples A11, A12, A13, and A15, had the lowest percentages and do not presented bands between 500bp and 400bp.

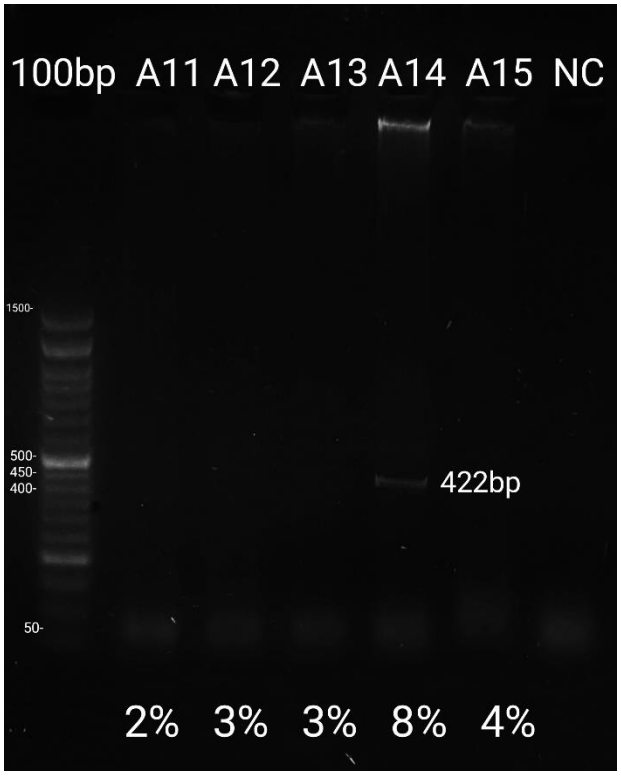

Figure 6. Visualization of the results of the samples named A11, A12, A13, A14, and A15; the negative control (NC) and the 100bp molecular weight marker (MW). Band indicated the presence of the avian malaria parasite (422bp). Percentages of parasitemia from the previous diagnosis of blood smears (Abad et al., 2021).

In general, the 15 samples were processed by PCR, and the results were visualized by electrophoresis. Of the total number of samples processed, five were positive for avian malaria based on the results of Abad and co-workers 2021 by light microscopy were identified as *Plasmodium*, and the remaining ten did not present visible fragments. The five positives have a percentage of parasitemia greater than or equal to 5%, and those that were negative have a parasitemia less than or equal to 4% (Table 4).

Table 4. Results of parasitemia (%) blood smear technique (Abad et al., 2021) and results of PCR technique.

Name Sample	Code Sample	Results Parasitemia (%) blood smear technique (Abad et al., 2021)	Results PCR technique Positive/Negative
A1	**S-27/10/2018-Helrub-2A**	4%	Negative
A2	**S-27/10/2018-Coewil-3A**	7%	Positive
A3	**S-27/10/2018-Helrub-4A**	3%	Negative
A4	**27/10/2018-Helrub-5A**	4%	Negative
A5	**S-28/10/2018-Helrub-6A**	3%	Negative
A6	**S-28/10/2018-Myifas-7B**	4%	Negative
A7	**S-28/10/2018-Aglcoe-8A**	3%	Negative
A8	**S-28/10/2018-Coewil-9B**	7%	Positive
A9	**S-28/10/2018-Coetor-10A**	5%	Positive
A10	**S-28/10/2018-Pacver-11A**	8%	Positive
A11	**S-28/10/2018-Helrub-12A**	2%	Negative
A12	**S-28/10/2018-Helrub-13B**	3%	Negative
A13	**S-28/10/2018-Helrub-14B**	3%	Negative
A14	**S-28/10/2018-Miostr-15B**	8%	Positive
A15	**S-28/10/2018-Coewil-16A**	4%	Negative

The sensitivity of the PCR test was obtained from the results of the samples processed by the PCR Test, using the primers Plas-F (5'-GTA ACA GCT TTT ATG GGT TAC-3') and 4292Rw (5'-TGG AAC AAT ATG TAR AGG AGG AGT-3'). The 15 samples of the individuals are the total population; it was determined by means of a single smear analysis that all the samples present the *Plasmodium* parasite of avian malaria (Abad et al., 2021). However, through the PCR test, it was determined that 5 are true positives (TP) and 10 are false negatives (FN).

Table 5. Sensitivity of the blood smear microscopy and the PCR test.

Avian malaria infection status		Diagnostic tests	
Microscopy Test	PCR Test	Blood Smear Microscopy	Polymerase Chain Reaction (PCR)
+	+	15	5
+	-	0	10
Sensitivity (%)		100	33

$$Sensitivity\ Microscopy\ Test = \frac{TP}{TP + FN} = \frac{15}{15 + 0} = 1 \times 100 = 100\%$$

$$Sensitivity\ PCR\ test = \frac{TP}{TP + FN} = \frac{5}{5 + 10} = 0{,}3333 \times 100 = 33.33\%$$

5.2. Discussion

Standardization and diagnosis

PCR has the potential to estimate the prevalence of parasites; it can also detect the presence of *Plasmodium* in blood samples, except in infections with minimal parasitemia (Freed & Cann, 2006). Its limitations are: 1) extreme sensitivity generates false positives, 2) false negatives if the parasites are in early stages, and 3) deficiency in the estimation of the intensity and the stage of infection (Freed & Cann, 2006). The 15 samples were purified after the standardization process and the standard PCR procedure that was carried out with the primers and protocol suggested by the literature; of the total number of samples, five were positive and ten negatives. According to what has been seen previously, there is the possibility that some samples were false negative, from the correct or incorrect storage of the samples, the design of the primers, common contamination problems in the extraction process, and the state of the buffers involved in the extraction process. These problems can

be solved by improving sample storage and collection protocols, correct sterilization during the extraction process, and using another primer sequence. As shown in Table 2, most of the samples have a concentration and purity between optimal and acceptable values. Previous studies have shown that PCR for human malaria is significantly more accurate compared to PCR for avian malaria (Freed & Cann, 2006).

The positive samples A2, A8, A9, A10, and A14 have similar concentration parameters except for A14, which has a concentration of 5.0 ng/ul of DNA; despite the low concentration, it was possible to visualize a fragment corresponding to *Plasmodium* (422bp). This suggests a high sensitivity of the PCR test to detect avian malaria. Sample A2 has a purity A260/280 of 1.68, which is an acceptable value but may influence the poor run that the sample had on the agarose gel (see Figure 4). The other samples, both positive and negative, showed values between optimal and acceptable, which suggests that DNA extraction process from the samples was carried out correctly. In addition, other parameters, such as the percentage of parasitemia, can influence the negative and positive results obtained from the PCR. Most of the parasitological diagnoses made in previous studies of hummingbirds presented early stages of infection, and this is possibly the reason for the low sensitivity of diagnosis by PCR. This agrees with the reported by Baek and co-workers 2020.

Prevalence and Parasitemia

According to the study carried out by Abad and co-workers 2021, the prevalence of *Plasmodium* in humid forest areas was proportional to the expected 96% for Nanegalito and 100% for Milpe. However, in this study, only blood samples from 15 individuals of seven

different species from the Nanegalito area were analyzed (see Appendix 3). The low number of samples is due to the difficulty of capturing and extracting blood from hummingbirds (personal communication from Professor Lilian Spencer, Ph.D.). The samples that presented the highest percentage of parasitemia with the blood smear (Abad et al., 2021), were positive in the PCR test carried out in this trial; they presented parasitemia between 5% and 8% (Table 2). The most prevalent species were *Coeligena wilson, Coeligena torquata*, *Pachyramphus versicolor*, and *Mionectes striaticollis*. According to Abad and co-workers 2021, the most parasitized species in the humid forest were *Helioxoda imperatrix* and *Pachyramphus versicolor*, with a parasitemia of 9%, which coincides with the PCR results with respect to the last species.

The PCR test presented a sensitivity of 33% in the detection of the *Plasmodium* parasite, which was detectable by PCR from a percentage of parasitemia equal to or greater than 5%. On the other hand, for samples with parasitemia less than or equal to 4%, the detection of the parasite by PCR obtained negative results. According to the study by Freed & Cann, 2006 the biggest problem in malaria diagnosis in birds compared to humans is that birds have nucleated red blood cells and thrombocytes, while in humans, these cells lack nuclei. For this reason, avian malaria is complicated to detect by PCR because the parasite must be detected at a relatively lower DNA template concentration. The problems associated with the diagnosis of avian malaria by PCR, both in this pilot trial and in other investigations, suggest that to obtain better sensitivity results, greater attention should be paid to the correct storage of samples, the DNA purification protocol, the adequate design number of primers, and the standardization of the PCR cycle. In addition to the combination of different detection techniques, to improve the detection of avian malaria.

6. CONCLUSIONS AND RECOMMENDATIONS

6.1. Conclusions

The PCR test is a highly valuable tool for detecting avian malaria parasites. However, the sensitivity of this test is highly dependent on the PCR cycle and extraction parameters. This pilot study determined that the best extraction kit for detecting the avian malaria parasite was the GenElute Mammalian Genomic DNA Mini-Prep Kit. The standardization parameters of the PCR test were; for the denaturation stage, a cycle for ten minutes at 94°C, then for the annealing stage, it was a sequence of forty cycles, with forty minutes at 95°C, one minute at 56°C, and one minute at 72°C. Finally, for the extension stage, a ten-minute cycle at 72°C. An additional step of one cycle at 4°C for an indeterminate time. In addition, it was shown that the Plas-F (5'-GTA ACA GCT TTT ATG GGT TAC-3') and 4292Rw (5'-TGG AAC AAT ATG TAR AGG AGG AGT -3') primers used in the PCR test were capable of amplifying the genetic material of the parasite.

Despite the positive results, the avian malaria parasite was detectable only in individuals with parasitemia greater than 5%. In contrast, the analysis of blood smears presented a higher sensitivity in samples with parasitemia lower than 4%. Therefore, microscopic analysis of blood smears (see Appendix 8) had a sensitivity of 100%, compared to the 33% sensitivity of the PCR test for this population of individuals and with the primers mentioned above. These results are probably because the infections were in the early stages of infection. It is necessary to continue improving the parameters of the PCR test to increase its sensitivity in the early diagnosis of these parasitic infections. Therefore, to diagnose avian malaria adequately, it is necessary to combine several microscopic, serological, and molecular techniques.

6.2. Recommendations

- For the DNA sample extraction process, maintain the sterility of the entire process to avoid contamination of the samples.
- For future research, other PCR techniques are recommended, such as Real-Time PCR, which has shown greater sensitivity for detecting human and avian malaria parasites.
- To detect avian malaria with greater sensitivity, it is recommended to try other primers and use two or more tests together for a more reliable analysis of results.
- In future research, it is recommended to use the DNA extraction protocol and the optimization of the PCR in a greater number of samples to improve the sensitivity results of the test.

7. REFERENCES

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8. APPENDICES

Appendix 1

Reagent details provided by GenElute Mammalian Genomic DNA Miniprep Kit and DNeasy Blood & Tissue Kit used for standardization protocols and DNA extraction from blood from filter paper.

GenElute Mammalian Genomic DNA Miniprep Kit	
Reagents Provided	**G1N70/ 70 preps**
Resuspension solution	20 mL
Lysis Solution C	20 mL
Column Preparation Solution	60 mL
Wash Solution Concentrate	20 mL
Elution Solution (10m M Tris-HCl, 0.5m M EDTA, pH 9.0)	35 mL
Proteinase K	3 x 10 mg

DNeasy Blood & Tissue Kit	
Reagents Provided	**cat. nos. 6904**
Buffer AL	14 mL
Buffer AW1	12 mL
Buffer AW2	19 mL
Buffer AE	13 mL
Proteinase K	2x15mL

Appendix 2

Number of human blood samples used in the standardization procedure of the DNA extraction protocol from the filter paper. In addition, the quantity and purity results of the human DNA obtained are specified.

Sample name	ng/ul	A260/280	A260/A230	Kit name
1	3.3	1.45	0.18	DNeasy Blood & Tissues Kit de QIAGEN
2	2.3	1.08	0.13	
3	3.6	1.42	0.19	
4	2.3	1.59	0.14	
5	3.4	1.38	0.17	
6	2.1	2.28	0.12	
7	2.1	1.80	0.13	
8	3.9	1.78	0.19	
9	3.5	2.04	0.17	
10	4.9	1.82	0.22	
11	4.1	1.44	0.19	
12	2.4	1.50	0.10	
13	4.1	1.78	0.20	
14	1.6	1.67	0.09	
15	1.9	2.32	0.11	
16	1.1	2.11	0.06	
17	1.3	3.57	0.07	
18	1.2	4.70	0.02	
19	2.6	1.41	0.14	
20	1.9	2.23	0.10	
21	1.5	2.41	0.08	
22	0.9	1.85	0.05	
23	3.1	0.85	0.06	

24	1.6	0.99	0.08	DNeasy Blood & Tissues Kit de QIAGEN
25	0.6	1.52	0.03	
26	0.7	1.70	0.04	
27	1.7	1.09	0.01	
28	1.5	3.34	0.08	
29	4.3	1.99	0.11	
30	1.6	1.35	0.07	
31	2.6	2.03	0.10	
32	4.6	1.65	0.16	
33	20.4	1.38	0.39	
34	12.6	1.40	0.25	
35	19.2	1.33	0.46	
36	17.0	1.36	0.45	
37	10.3	1.33	0.33	
38	17.0	1.02	0.20	
39	16.4	1.36	0.36	
40	11.1	1.31	0.34	
41	9.5	1.58	0.31	
42	25.7	0.95	0.14	
43	0.3	0.88	0.01	
44	2.8	1.59	0.09	
45	0.5	4.05	0.02	
46	0.3	0.67	0.01	
47	0.8	1.96	0.03	
48	1.1	1.95	0.04	
49	52.0	1.68	0.79	GenElute Mammalian Genomic DNA Mini-Prep Kit, Catalog No. G1N70
50	58.6	1.80	1.01	
51	58.5	1.89	1.24	
52	58.8	1.88	1.12	

53	61.0	1.87	1.21	

Appendix 3

Results of the percentages of parasitemia obtained from the statistical analysis of the 15 individuals of the Puyucunapi Pilot Project Sampling Data project, exposed in the degree project Effects of Avian Malaria on Wild Birds in the Humid Forest of Pichincha Province in Ecuador (Abad et al., 2021). Which were used in this pilot test for the detection of avian malaria using PCR.

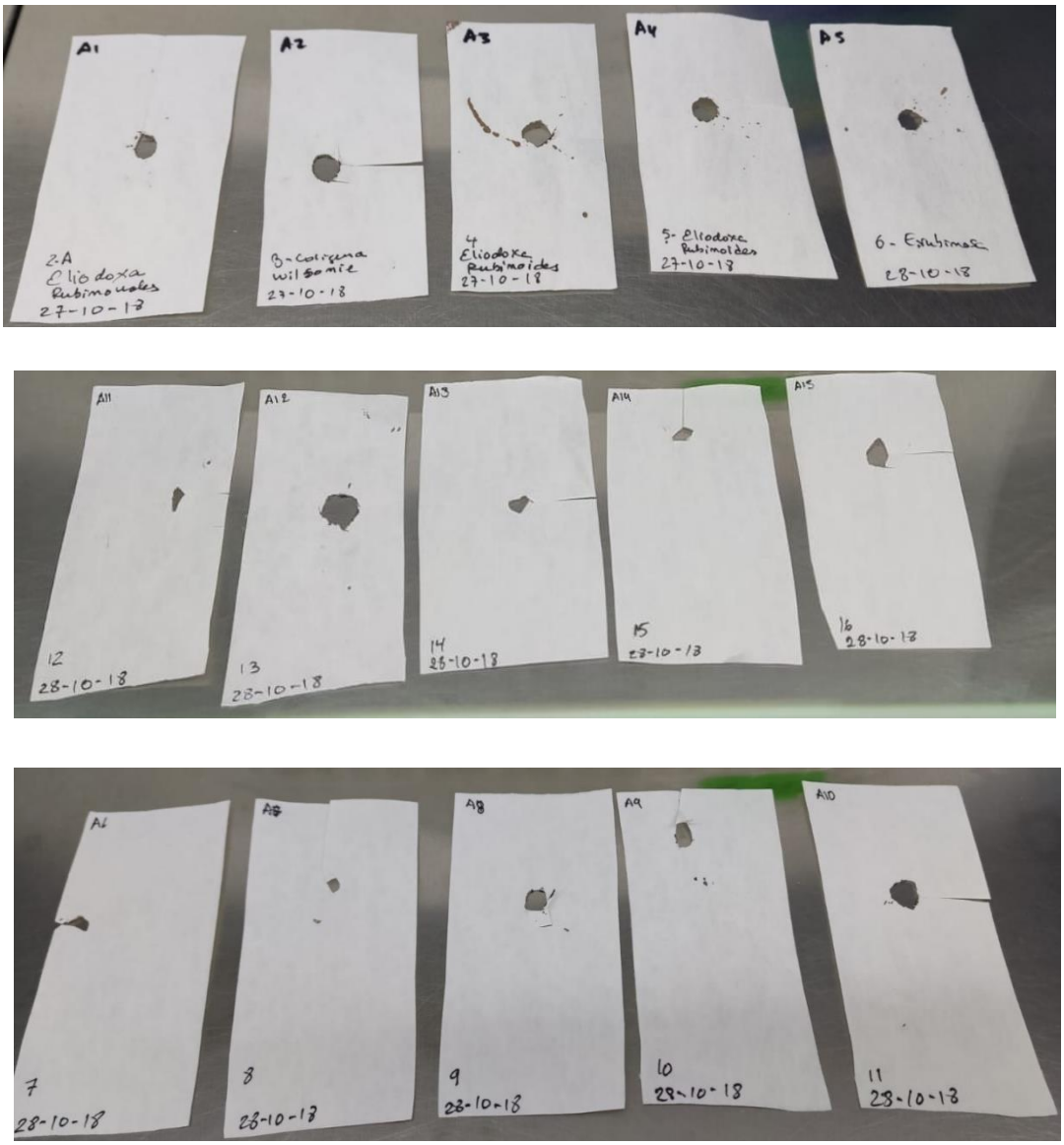
NANEGALITO (OCTOBER 27th AND 28th, 2018)					
Sample Name	**Common Name**	**Species**	**Code**	**Parasitemia (%)**	**References**
A1	**Fawn-breasted Brilliant**	***Heliodoxa rubinoides***	**S-27/10/2018-Helrub-2A**	**4%**	(Abad et al., 2021d)
A2	**Brown Inca**	**Coeligena wilsoni**	**S-27/10/2018-Coewil-3A**	**7%**	(Abad et al., 2021d)
A3	**Fawn-breasted Brilliant**	***Heliodoxa rubinoides***	**S-27/10/2018-Helrub-4A**	**3%**	(Abad et al., 2021d)
A4	**Fawn-breasted Brilliant**	***Heliodoxa rubinoides***	**-27/10/2018-Helrub-5A**	**4%**	(Abad et al., 2021d)
A5	**Fawn-breasted Brilliant**	***Heliodoxa rubinoides***	**S-28/10/2018-Helrub-6A**	**3%**	(Abad et al., 2021d)

A6	**Bran-colored Flycatcher**	***Myiophobus fasciatu***	**S-28/10/2018-Myifas-7B**	**4%**	(Abad et al., 2021d)
A7	**Violet-tailed Sylph**	***Aglaiocercus coelestis***	**S-28/10/2018-Aglcoe-8A**	**4%**	(Abad et al., 2021d)
A8	**Brown Inca**	***Coeligena wilsoni***	**S-28/10/2018-Coewil-9B**	**7%**	(Abad et al., 2021d)
A9	**Collared Inca**	***Coeligena torquata***	**S-28/10/2018-Coetor-10A**	**5%**	(Abad et al., 2021d)
A10	**Barred Becard**	***Pachyramphus versicolor***	**S-28/10/2018-Pacver-11A**	**8%**	(Abad et al., 2021d)
A11	**Fawn-breasted**	***Brilliant Heliodoxa rubinoides***	**S-28/10/2018-Helrub-12A**	**2%**	(Abad et al., 2021d)
A12	**Fawn-breasted Brilliant**	***Heliodoxa rubinoides***	**S-28/10/2018-Helrub-13B**	**3%**	(Abad et al., 2021d)
A13	**Fawn-breasted Brilliant**	***Heliodoxa rubinoides***	**S-28/10/2018-Helrub-14B**	**3%**	(Abad et al., 2021d)
A14	**Streak-necked Flycatcher**	***Mionectes striaticollis***	**S-28/10/2018-Miostr-15B**	**8%**	(Abad et al., 2021d)
A15	**Brown Inca**	***Coeligena wilsoni***	**S-28/10/2018-Coewil-16A**	**4%**	(Abad et al., 2021d)

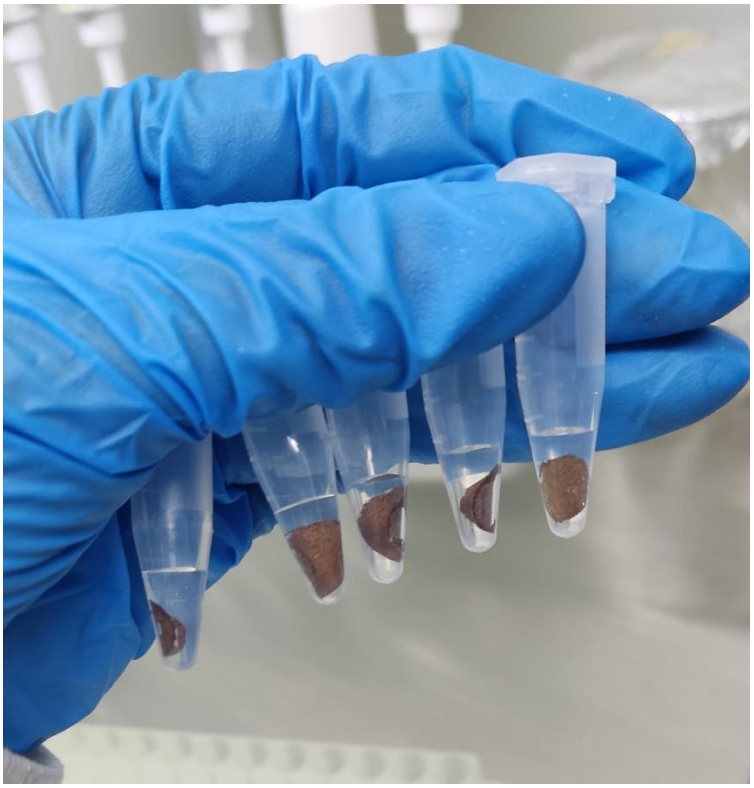
Appendix 4

Images of the parts of the DNA extraction process. A) Filter papers that stored the 15 blood samples from the hummingbirds, B) Sample after TE and thermoblock Thermo Scientific™ Digital Heating Shaking Drybath process C) Necessary equipment for the extraction.

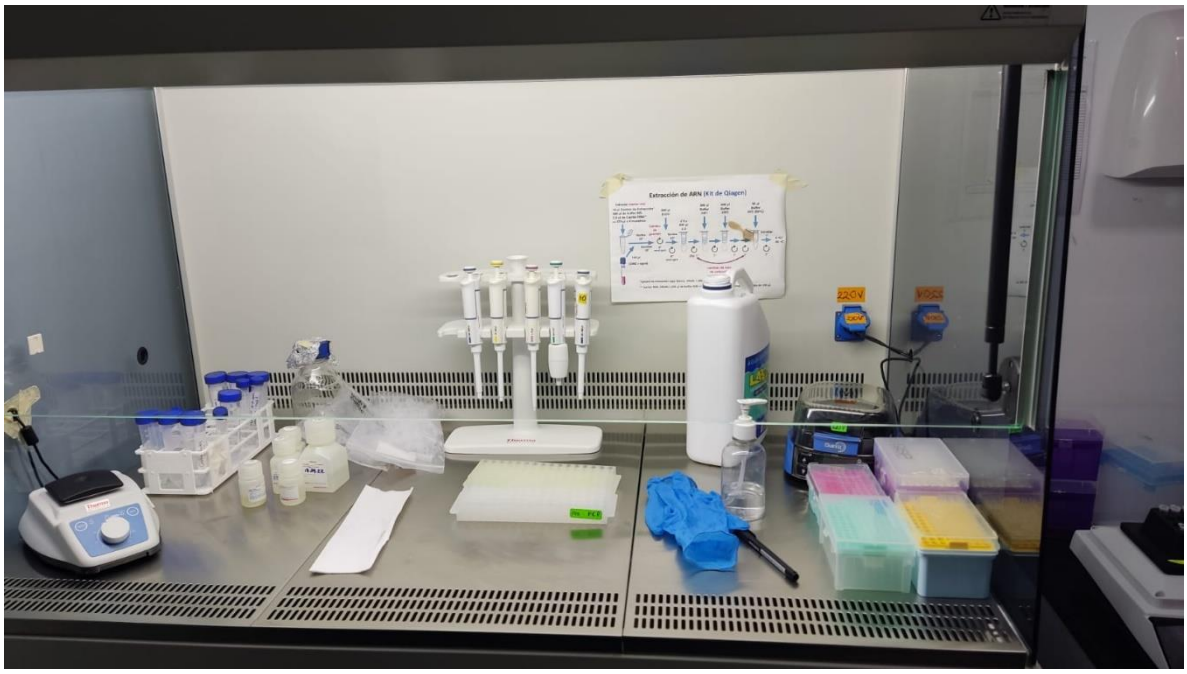
A)



B)

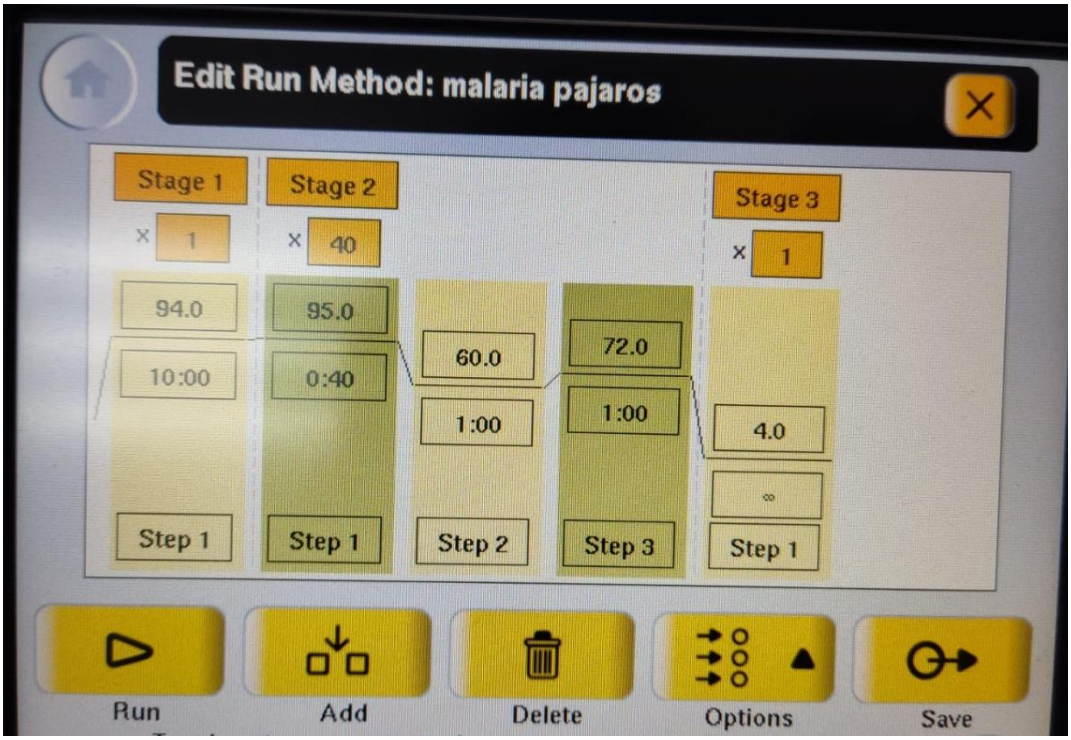


C)



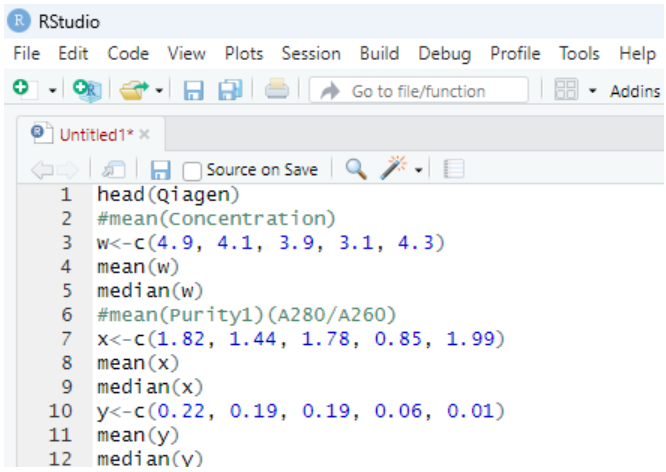
Appendix 5

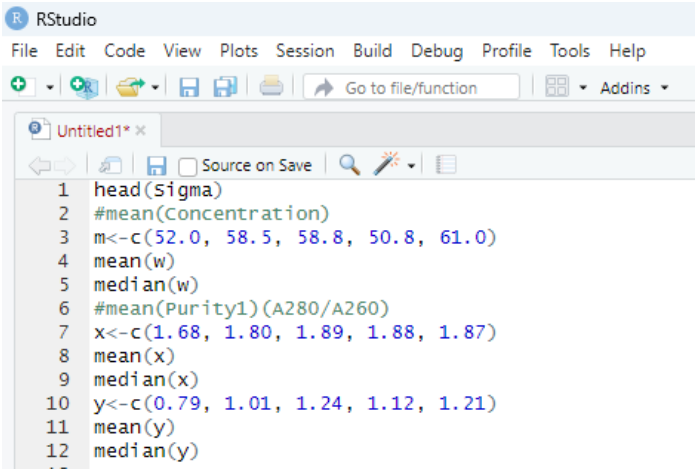
The programmed process in a Thermo Scientific thermocycler shows the PCR stages with the corresponding cycles, the temperature at which each stage was subjected and the time.



Appendix 6

Statistical calculation of the mean and median using Rstudio, comparing the concentration and purity data obtained, to determine which of the two kits GenElute Mammalian Genomic DNA Miniprep Kit and DNeasy Blood & Tissue Kit has the best performance.





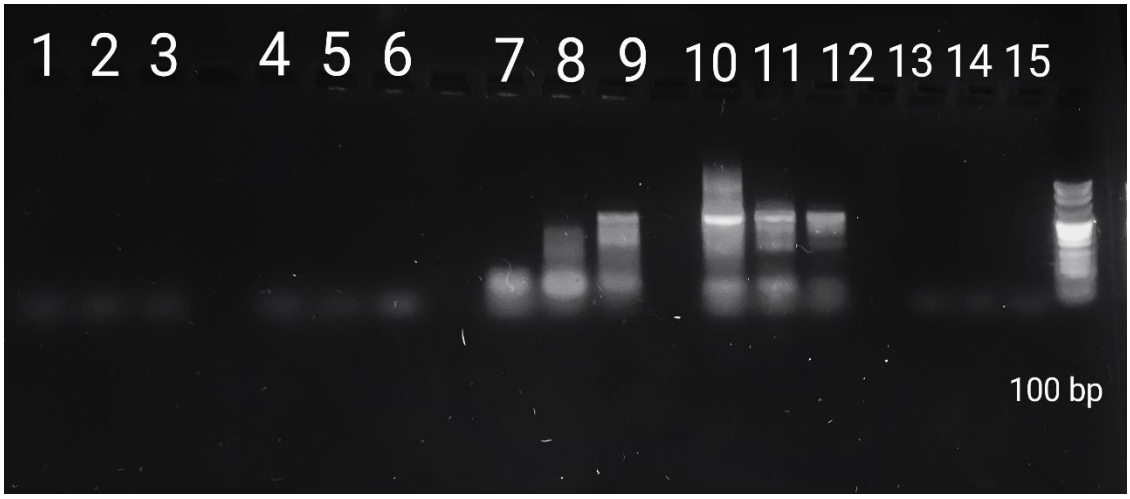

```
> head(Qiagen)
# A tibble: 5 × 4
  Number Concentration Purity1 Purity2
   <dbl>         <dbl>   <dbl>   <dbl>
1      1           4.9    1.82    0.22
2      2           4.1    1.44    0.19
3      3           3.9    1.78    0.19
4      4           3.1    0.85    0.06
5      5           4.3    1.99    0.01
> #mean(Concentration)
> w<-c(4.9, 4.1, 3.9, 3.1, 4.3)
> mean(w)
[1] 4.06
> median(w)
[1] 4.1
> #mean(Purity1)(A280/A260)
> x<-c(1.82, 1.44, 1.78, 0.85, 1.99)
> mean(x)
[1] 1.576
> median(x)
[1] 1.78
> y<-c(0.22, 0.19, 0.19, 0.06, 0.01)
> mean(y)
[1] 0.134
> median(y)
[1] 0.19
```

```
> head(Sigma)
# A tibble: 5 × 4
  Number Concentration Purity1 Purity2
   <dbl>         <dbl>   <dbl>   <dbl>
1      1            52    1.68    0.79
2      2          58.5    1.8     1.01
3      3          58.8    1.89    1.24
4      4          58.8    1.88    1.12
5      5            61    1.87    1.21
> #mean(Concentration)
> m<-c(52.0, 58.5, 58.8, 50.8, 61.0)
> mean(w)
[1] 4.06
> median(w)
[1] 4.1
> #mean(Purity1)(A280/A260)
> x<-c(1.68, 1.80, 1.89, 1.88, 1.87)
> mean(x)
[1] 1.824
> median(x)
[1] 1.87
> y<-c(0.79, 1.01, 1.24, 1.12, 1.21)
> mean(y)
[1] 1.074
> median(y)
[1] 1.12
>
```

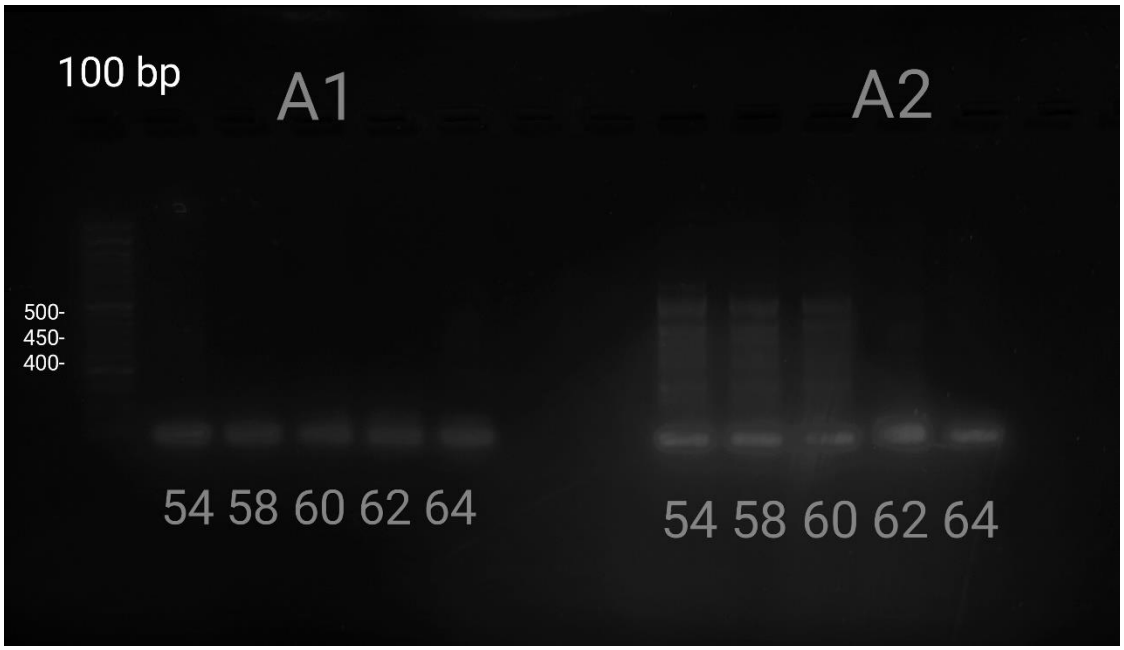
Appendix 7

Photographs of the UV visualization of the results of the standardization process. A) visualization of the results of the human DNA standardization, B) visualization of the results of the samples A1 and A2 with different annealing temperatures, and C) Visualization of the samples at two annealing temperatures.

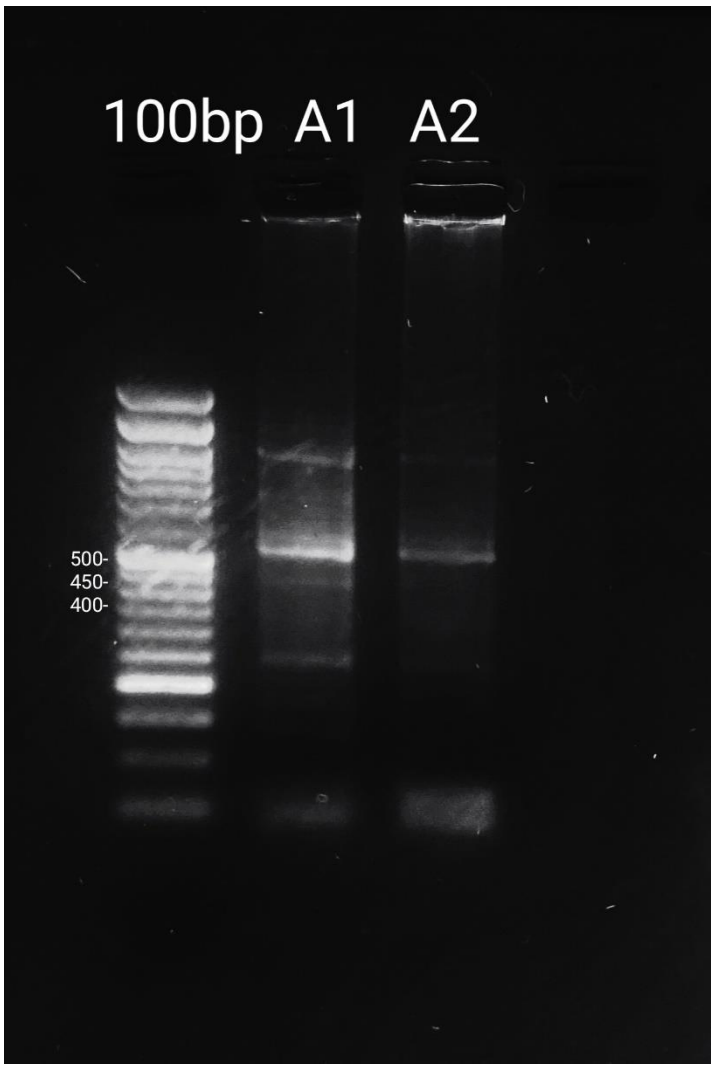
A)



B)



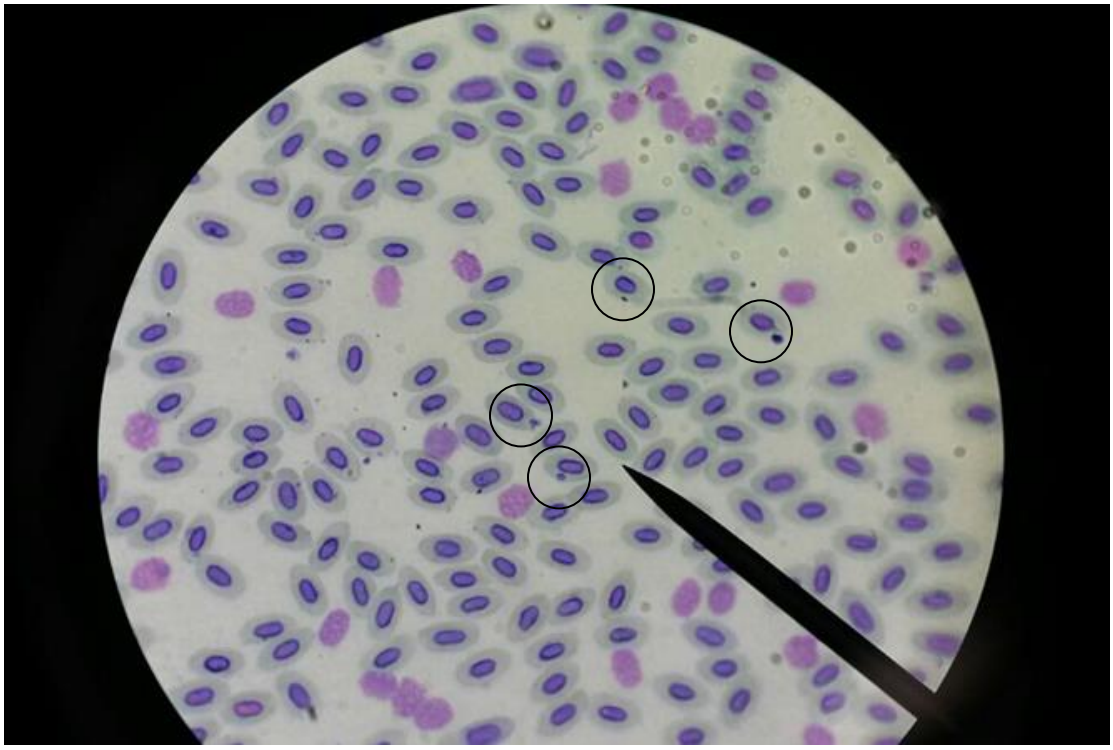
C)



Appendix 8

Images from the study of microscopic analysis of blood smears, from birds infected with the avian malaria parasite. A) visualization of merozoites in bird cells. B) visualization of schizonts in bird cells.

A)



B)