Molecular Detection and Identification of Parasites Involved in Human Disease

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DECLARATION

I declare that the work presented in this thesis is original, except where otherwise acknowledged and submitted to fulfill the requirements of a Master of Science in Biology at Lakehead University, Thunder Bay, Ontario, Canada.

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LIST OF ABBREVIATIONS

DNA	Deoxyribonucleic acid
A	Adenine
T	Thymine
G	Guanine
C	Cytosine
mtDNA	Mitochondrial DNA
kDNA	Kinetoplast DNA
aDNA	Ancient DNA
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
PCR	Polymerase chain reaction
bp	Base pair
T_m	Melting temperature
dNTP	Deoxynucleotide triphosphate
$MgCl_2$	Magnesium chloride
ddH_2O	Double distilled water
GuSCN	Guanidinium thiocyanate
pK	Proteinase K
DMSO	Dimethyl sulfoxide
BSA	Bovine serum albumin
UNG	Uracil-N-glycosylase
PAGE	Polyacrylamide gel electrophoresis
AGE	Agarose gel electrophoresis

EtBr	Ethidium bromide
DHFR-TS	Dihydrofolate reductase-thyidylate synthase
MSA	Major surface antigen
IMP-D	IMP dehydrogenase
AAP13LD	Amino acid permease 13 *Leishmania donovani*
ssrRNA	Small subunit rRNA
med RNA	Multicopy mini-exon derived DNA
MSP	Merozoite surface protein
RE	Reticuloendothelial
RPMI medium	Roswell Park Memorial Institute medium
NNN medium	Novy-Mac Neal-Nicolle medium
DAT	Direct agglutination test
IIF	Indirect immunofluorescence assay
IHA	Indirect hemagglutination assay
ELISA	Enzyme-linked immunosorbent assay
IgM	Immunoglobulin M
MPH	Microplate hybridization
RFLP	Restriction fragment length polymorphism
RAPD	Randomly amplified polymorphic DNA
AFLP	Amplified fragment length polymorphism
RT-PCR	Reverse transcriptase PCR
LB	Luria-Bertani
X-Gal	5-Bromo-4-chloro-3-indolyl-β-D-galactoside
IPTG	Isopropyl-β-D-thiogalactopyranoside

ABSTRACT

The detection and identification of disease-causing parasites via DNA and the polymerase chain reaction (PCR) offers a new approach for quick and efficient disease diagnosis. Three nucleic acid based technologies have been developed and optimized for the identification of six species of parasites that cause disease, and designed to amplify small fragments of DNA likely to remain in archaeological material. Two methods involve a species-specific identification by examining four gene fragments, one of which has been designed as a multiplex PCR to minimize the time required for diagnosis. The third method involves the use of one species-specific gene target per parasite in a multiplex PCR for the detection of multiple parasites in one reaction to expedite the testing process and confirmation of the disease in question in a clinical setting. Sensitivity and specificity have been proven through the use of a 1/1000 diluted DNA template and sequencing of products confirmed the presence of each species analyzed. With increased sensitivity, a less invasive sampling procedure would be required from the patient. Also, the earlier the presence of parasites in the tissues is detected, the earlier the diagnosis would occur. Tissues that contain very few parasites and had been classified as previously unusable for diagnosis or tissues from older medical cases can be analyzed for the presence of disease and the identification of causative species. The successful application of the singleplex PCR technique to *Plasmodium falciparum* and *Leishmania* degraded DNA samples leads to the feasibility of success for the multiplex PCR techniques involving degraded DNA analyses. Therefore, this methodology may be applied to samples of unknown disease content to either include or exclude the agents of disease. Future directions may extend these analyses to include a range of other agents of disease, thus allowing for the determination of disease antiquity through degraded DNA analyses.

CHAPTER 1: INTRODUCTION

The success of an organism or group of organisms depends on their ability to adapt and thrive in complex ecosystems. The parasitic way of life is highly successful because it evolved independently in nearly every phylum of animals, from protistan phyla to arthropods to chordates, as well as in many plant groups resulting in a greater abundance of parasitic organisms than nonparasitic organisms in the world (Roberts and Janovy Jr 2000). Parasites are defined as any life form – or any organic compound capable to multiply – that find their ecological niche in another living form (Araujo et al. 2003). They find in another organism their habitat and nourishment and during at least part of their lives depend upon another organism (Araujo et al. 2003).

Generally speaking, the study of the parasite-host relationship can be referred to as parasitology. A more common definition is a study of symbiosis, or literally, "living together", or in a wider sense, any two organisms living in close association, commonly one living in or on the body of the other, are symbiotic, as contrasted with free-living organisms (Roberts and Janovy Jr 2000). However, these symbiotic relationships can be characterized further into phoresis, mutualism, commensalisms and parasitism by specifying the nature of the interactions between the participants (Roberts and Janovy Jr 2000). These various forms of symbiosis are defined below.

Four categories of symbiotic relationships exist: phoresis, mutualism, commensalisms and parasitism. Phoresis exists when two symbionts are merely "travelling together", and there is no physiological or biochemical dependence on the parts of either participant (Roberts and Janovy Jr 2000). Mutualism describes a relationship, usually obligatory, in which both partners benefit from the association, and in commensalisms, one partner benefits from the association, but the host is neither helped nor harmed (Roberts and Janovy Jr 2000). Most attention is

focused on the parasitic relationship, which causes harm to the host. In parasitism, one of the participants, the parasite, either harms its host or in a sense lives at the expense of the host by causing mechanical injury, stimulating a damaging inflammatory or immune response, or simply by robbing the host of nutrition (Roberts and Janovy Jr 2000). Many researchers have invested interest and effort in and trying to understand the mechanism and evolutionary benefit behind such a phenomenon.

Parasite Evolution

The evolution of parasites can be directly linked to their ability to co-exist alongside and within a multitude of various species and ecological niches. A system is formed by the parasite, the host, and the environment where each one interacts and influences the other subsystem in such a way that any change in one subsystem affects the other two (Araujo et al. 2003). A parasite may be associated with a host because the two share a long evolutionary history (descent), or the host and parasite may be associated because the parasite has colonized the host in a manner analogous to the colonization of an island (Roberts and Janovy Jr 2000). With this, parasite life history traits (such as fecundity), life cycles themselves (loss or addition of stages), and virulence are all subject to evolutionary change (Roberts and Janovy Jr 2000). However, the question of why some parasites seem to be especially virulent while others are relatively benign has captured that attention of numerous researchers since a long and established paradigm states that parasites should evolve into less virulent forms, mainly because death of a host should have a negative effect on parasite survival (Roberts and Janovy Jr 2000). Nonetheless, some theories have suggested that parasites should evolve an optimal virulence that maximizes parasite numbers, "optimal" depending on numerous factors such as pathogenicity and transmission dynamics (Roberts and Janovy Jr 2000). However, parasitic infections and parasitic diseases are

two distinct situations since a parasite's presence is a necessary but not sufficient condition to launch a parasitic disease and the resultant parasitism does not necessarily result in injury or benefit to the host (Araujo et al. 2003). Thus, a parasitic disease can be described as an eventual outcome of a given parasite in a given host from a given population in a certain environment during a particular life co-evolution period of both protagonists (Araujo et al. 2003). The views concerning parasite virulence have generated interest on finding the evolutionary trends of many parasite-induced diseases and constructing a timeline of parasite evolution so as to extrapolate to trends of today.

Paleoparasitology

The analysis of cultural and biological specimens collected from the past can reveal many important facets of evolutionary history and begin to create a timeline of host-parasite events. Many significant remnants in the genome of parasites and hosts left during the co-evolution process of a host-parasite-environment system are transmitted and can reveal important features of the whole process (Araujo et al. 2003). Thus, the term paleoparasitology (also archaeoparasitology) has been applied to the development of a new field, with its unique relation to both parasitology and anthropology (especially parasite ecology and paleopathology), and its derivation of specialized analytical techniques (Reinhard 1990). It first appeared as a new branch of parasitology at the beginning of the 20th century, when a new technique to rehydrate desiccated tissues allowed the finding of *Schistosoma haematobium* eggs in infected kidneys of 3,200 year-old Egyptian mummies (Ruffer 1910; Loreille and Bouchet 2003) and thus was defined as the study of parasites in ancient material (Araujo et al. 1998). This approach allows inferences about disease patterns in the past, thus contributing to the knowledge of diseases in prehistoric populations (Ferreira et al. 2000). It offers the unique possibility of working with

ancient parasites and may shed light upon features concerning the antiquity of a given host-parasite complex as well as their origin and evolution (Araujo et al. 2003). Due to the development and application of new molecular techniques, which have allowed for the recovery of ancient DNA (aDNA) from archaeological material, the possibility for the study of human-parasite relationship through the finding of genetic traces of etiological agents of disease has been opened (Ferreira et al. 2000).

Polymerase Chain Reaction

The polymerase chain reaction (PCR) technique is considered by molecular analysts to have started an avalanche in technology and research. Originally described by Kary Mullis in 1985 (Mullis et al. 1986) this technique, although quite an extraordinary accomplishment contained one major drawback, the addition of fresh enzyme to every cycle of the reaction (Saiki et al. 1988). However, improvements were made through numerous experiments and it was found that a thermostable DNA polymerase, purified from the thermophilic bacterium, *Thermus aquaticus* (*Taq*) (Saiki et al. 1988) alleviated this problem. Following were substantial improvements of the overall performance, yield, sensitivity and length of targets that could be amplified (Saiki et al. 1988). These made possible the capacity to amplify specific segments of DNA and have transformed the way we think about approaching both fundamental and applied biological problems (Erlich 1989). PCR has provided an extremely sensitive and relatively straightforward means to amplify very small amounts of DNA or RNA – down to a single copy of a gene, to milligram amounts of the same sequences consisting of millions or billions of identical copies for detection, sequencing, cloning, diagnosis, etc. (Saiki et al. 1988). Before its introduction, DNA amplification could only be achieved by time-consuming *in vivo* cloning that

took several day, and now PCR can be set up and completed in a matter of hours (Saiki et al. 1988).

Methodology

PCR is an *in vitro* method for the enzymatic synthesis of DNA sequences, using two oligonucleotide primers that hybridize to opposite strands and flank the region of interest in the target DNA (Mullis et al. 1986; Erlich 1989). Reduced to its most basic terms, PCR merely involves combining a DNA sample with oligonucleotide primers, deoxynucleotide triphosphates (dNTPs), and the thermostable *Taq* DNA polymerase in a suitable buffer, then repetitively heating and cooling the mixture for several hours until the desired amount of amplification is achieved (Saiki et al. 1988; Erlich 1989). However, the reaction itself is based on three steps: denaturation, annealing and elongation phases, all of which are repeatedly in sequence, a process known as "cycling" (Saiki et al. 1988; Hummel 2003).

Initially, in the denaturation phase, the double-stranded DNA molecules denature into single strands through a high energy input that force open the hydrogen bonds between the complementary bases (Saiki et al. 1988; Hummel 2003). Following this, an annealing phase occurs, which enables the primers or oligonucleotides to hybridize to their recognition sites at temperatures dependent on the actual primer sequence (Saiki et al. 1988; Hummel 2003). The final elongation phase is where a heat-stable DNA polymerase begins to incorporate the complementary deoxynucleotide triphosphate bases starting from the 3′-end of the primers (Saiki et al. 1988; Hummel 2003) and results in the accumulation of a specific fragment whose termini are defined by the 5′-ends of the primers (Mullis et al. 1986; Erlich 1989). The repetitive nature of the technique ("cycling") thus enables newly synthesized primer extension products them to

serve as a template, resulting in an exponential increase, i.e. approximately doubling of the number of target DNA copies with each cycle (Mullis et al. 1986; Erlich 1989).

With these basic methodological steps involved in the PCR technique, it must be understood that there is no single set of conditions that will be optimal for all possible reactions (Erlich 1989). For example, the annealing and separation of the two strands depend on a number of factors, particularly temperature, salt concentration, pH, nucleotide composition and length of the sequence concerned (Saiki et al. 1988). However, considered to be of utmost importance is the DNA template itself. With improved molecular techniques arising, expanding the quantity and quality of genomic template analyzed, one fundamental concern involves understanding the mechanisms involved in the degradation process of DNA and how to overcome such an inhibiting factor.

Primer Design

The design and implementation of primers is one of the most important procedures for a specific and high quality PCR reaction. The primer sequences determine which particular sequence of a genome will be targeted in a PCR experiment and the specificity of the reaction is ensured when the primer sequences are a complete match with the intended hybridization site and are unique within the genome in focus (Saiki et al. 1988; Hummel 2003). In addition, the length of the primer and the annealing temperature of the PCR control the specificity (Dieffenbach et al. 1993).

The initial step in primer design begins with the length of the sequence required. Oligonucleotides between 18 and 30 bases (Erlich 1989; Dieffenbach et al. 1993) tend to be very sequence specific if the annealing temperature of the PCR is set within a few degrees of the

primer T_m (defined as the dissociation temperature of the primer/template duplex) (Dieffenbach et al. 1993). However, with a few simple guidelines, primers can be optimally designed.

1. Where possible, select primers with a random base distribution and with a GC content similar to that of the fragment being amplified (Erlich 1989). However, the length of the PCR product has an impact on the efficiency of amplification (Dieffenbach et al. 1993).
2. Avoid sequences with the potential for significant secondary structure, particularly at the 3′-end of the primer (Erlich 1989).
3. Check the primers against each other for complementarity (Erlich 1989). This is done to avoid the undesirable primer-dimer phenomena in which the PCR product obtained is the result of the amplification of the primers themselves (Dieffenbach et al. 1993).

If the guidelines have been followed, the specificity of the reaction will be greatly enhanced. However, altering PCR parameters for increased PCR optimization is also required. This requires an empirical approach to determine the correct concentration of reagents and optimal temperature for each PCR. Nonetheless, if the PCR is optimally designed, problems do still exist in the form of contaminating DNA. Therefore, researchers have imposed certain requirements for contamination control.

Multiplex PCR

Multiplex PCR is a variant of PCR in which two or more loci are simultaneously amplified in the same reaction. Since its first description in 1988, this method has been successfully applied in many areas of DNA testing, including analyses of deletions, mutations and polymorphisms, or quantitative assays and reverse transcription (Chamberlain et al. 1988; Henegariu et al. 1997). A short list of multiplex PCR applications now includes pathogen identification, gender screening, linkage analysis, forensic identification, template quantitation

and gender disease diagnosis (Edwards and Gibbs 1994). By simultaneously amplifying more than one locus in the same reaction, multiplex PCR is becoming a rapid and convenient screening assay in both the clinical and the research laboratory (Henegariu et al. 1997).

In multiplex assays, where closely related templates such as pathogen strains are distinguished by amplifying differing sequence, primers for a sequence common to all templates provide a positive control for amplification (Edwards and Gibbs 1994). The quality of the template may be determined more effectively in multiplex than in single locus PCR and the expense of reagents and preparation time is less in multiplex PCR than in systems where several tubes of singleplex PCRs are used (Edwards and Gibbs 1994). Consequently, a multiplex reaction is ideal for conserving costly polymerase and template in short supply (Edwards and Gibbs 1994). Therefore, for maximum efficiency of preparation time, the reactions can be prepared in bulk, randomly tested for quality, and stored frozen without enzyme or template until use (Edwards and Gibbs 1994).

Critical Components

There are certain unique components to take into account when designing a multiplex PCR. Multiplex PCR requires that primers lead to amplification of unique regions of DNA, both in individual pairs and in combinations of many primers, under a single set of reaction conditions (Markoulatos et al. 2003). The development of an efficient multiplex PCR usually requires strategic planning and multiple attempts to optimize the reaction conditions (Markoulatos et al. 2003). Thus, there are some important factors to consider.

Primer Concentration

The primer concentration is a significant constituent. When there is uneven amplification, with some of the products barely visible even after the reaction was optimized for

the cycling conditions, changing the proportions of various primers in the reaction is required, with an increase in the amount of primers for the "weak" loci and a decrease in the amount for the "strong" loci (Markoulatos et al. 2002). The final concentration of the primers (0.04-0.05μM) may vary considerably among the loci and is established empirically (Markoulatos et al. 2002).

dNTP Concentration

The amount of dNTPs added to a PCR is important for reliable unhindered amplification. Their role falls within the elongation phase of PCR, where the *Taq* DNA polymerase incorporates these nucleotides, thus extending the primers and producing two copies of the original target strands (Saiki et al. 1988; Markoulatas et al. 2003). Usually $MgCl_2$ concentration is kept constant, while the dNTP concentration is increased stepwise from 0.5-1.6mM (Markoulatos et al. 2002). However, the best results are between 200 and 400μM each dNTP values, above which the amplification is rapidly inhibited (Markoulatos et al. 2002).

$MgCl_2$ Concentration

The addition of $MgCl_2$ to a PCR contributes to the Mg^{2+} which must be optimized. Optimization of Mg^{2+} is critical since *Taq* DNA polymerase is a magnesium-dependent enzyme, and the template DNA primers and dNTPs bind Mg^{2+} (Markoulatos et al. 2002). Excessive Mg^{2+} concentration stabilizes the DNA double strand and prevents complete denaturation of DNA, which reduces yield and can also stabilize spurious annealing of primer to incorrect template sites (Markoulatos et al. 2002). On the other hand, inadequate Mg^{2+} concentration reduces the amount of product (Markoulatos et al. 2002).

PCR Buffer Concentration

The specific DNA polymerase buffer is also a critical reagent. Raising the buffer concentration improves the efficiency of the multiplex reaction (Markoulatos et al. 2002). Primer pairs with longer amplification products work better at lower salt concentrations, whereas primer pairs with short amplification products work better at higher salt concentrations (Markoulatos et al. 2002).

Use of Adjuvants

The most difficult multiplex PCR reactions can be significantly improved by using a PCR additive, such as dimethyl sulfoxide (DMSO), glycerol, formamide, betaine and bovine serum albumin (BSA), which relax DNA, thus making template denaturation easier (Markoulatos et al. 2002).

Contamination Sources and Control

Researchers utilizing the PCR technique, have to contend with the possibility of experimental contamination from extraneous DNA, such as bacterial, atmospheric or human. Many precautions must them be taken to avoid such contamination from sources such as these. Thus, a few sources of contamination and the requirements of control and prevention are presented.

One source of contamination is referred to as carry-over contamination. This occurs if amplification products are introduced to a pre-PCR analysis step (Handt et al. 1994; Hummel 2003), such as the air movement produced when opening PCR tubes or transferring liquids will create and disperse microscopic aerosol droplets, which can contain over a million copies of the template (Willerslev and Cooper 2005). A second source of contamination, referred to as cross-contamination, occurs as a result of sample-to-sample contamination (Hummel 2003), which can

be present in the specimens before samples are removed (Handt et al. 1994), and a third source, but not the last, is contamination via chemicals; the reagents used in the PCR (Hummel 2003). In order to minimize the risk, complete physical separation of pre- and post-PCR work spaces is necessary; technique devices should never be exchanged between these areas (Montiel et al. 2001). Regular controls without the addition of a template are suitable to monitor contaminations of the carry-over type (Handt et al. 1994; Hummel 2003), and most important, it is required that all PCR analysis steps, starting with sample preparation, DNA extraction and PCR set-up, following through to the end of the experiment, should be done with careful consideration (Hummel 2003).

The requirements of contamination control are basically the same at all stages of the PCR analysis (Hummel 2003). Strict separation for the handling of the samples, effective cleaning management for all equipment that is in direct contact with the samples, changing disposable gloves when handling different samples is required (Hummel 2003). Under no circumstances should touching of the inner surfaces of the reaction tubes occur, precise pipetting and finally, the use of positive displacement pipettes or aerosol-tight pipette tips are a necessity (Hummel 2003). If all precautions have been taken, the risk of contamination will be minimized. However, there are a few monitoring techniques implemented for PCR that may indicate the presence of contamination.

Contamination is a big problem when working with the PCR technique. Many researchers have thus used stringent controls to test the reaction for the possibility of contamination such as, the use of a blind control. This product undergoes all the extraction, purification and amplification procedures, except that it does not contain any sample material (Herrmann and Hummel 1994). This type of control will reveal even minor contaminations in

any of the reagents and chemicals used in the procedures (Herrmann and Hummel 1994). A second type of control is no-template control. This control serves only for the amplification reagents and conditions; it contains every amplification reagent except the DNA template (Herrmann and Hummel 1994). The inclusion of the aforementioned controls as well as other more research-specific requirements is of great importance to PCR research. Therefore, all reactions should implement them in the research as a form of authenticity of results.

DNA Degradation

A serious concern pertinent to the study of aDNA is the occurrence of postmortem damage in DNA extracted from archaeological material, since such damage may make impossible the application of many molecular biological techniques and/or cause erroneous sequence information to be obtained (Paabo 1989). The likely reason for this is damage to the DNA that occurs over time and eventually renders the DNA unable to serve as a template for PCR (Hoss et al. 1996). This damage may arise from environmental exposures such as low-level radiation or genotoxic chemicals (DNA damaging agents) or can be introduced during DNA extraction and/or purification processes (Mitchell et al. 2005). Therefore, overcoming the obstacles presented by postmortem DNA damage is a priority.

When an organism dies, the DNA molecules face an onslaught of destructive processes which put at risk its integrity. Firstly, bacteria, fungi and insects may be present at the site of organism decomposition and accelerate DNA damage by feeding on and degrading macromolecules (Paabo et al. 2004). Alternatively, or in conjunction with these organisms, cellular compartments within the decaying organism that normally sequester catabolic enzymes break down and the DNA is rapidly degraded by enzymes such as lysosomal nucleases (Paabo et al. 2004). Although, under fortunate circumstances, such as rapid desiccation, low temperatures

or high salt concentration, nucleases themselves become destroyed or inactivated before all nucleic acids are reduced to mononucleotides (Hofreiter et al. 2001).

Damage to DNA extracted from archaeological remains is exhibited by the nature of fragment size available for molecular analyses. On average, smaller fragmented DNA has a general size between 100 to 500 base pairs (bp) due to both enzymatic processes that occur shortly after death, and nonenzymatic hydrolytic cleavage (Paabo et al. 2004). Due to its hydrophilicity, DNA remains hydrated even in dry climates and hydrolytic damage will occur (Handt et al. 1994) such as cleavage of phosphodiester bonds in the phosphate-sugar backbone that generate single-stranded nicks and/or hydrolytic cleavage of the glycosidic bonds between nitrous bases and the sugar backbone resulting in abasic sites (Figure 1) (Paabo et al. 2004).

DNA sequence amplification is also limited by lesions induced by free radicals such as peroxide radicals ($\bullet O_2$), hydrogen peroxide (H_2O_2), and hydroxy radicals ($\bullet OH$), which are created by among other causes, background radiation (Paabo et al. 2004). Major sites of oxidative attack are the double bond of both pyrimidines and purines (Paabo et al. 2004), which may lead to high proportion of cytosine and thymine residues in extracts of ancient tissues that are oxidatively modified to hydantions (oxidative products of the pyrimidine bases cytosine and thymine), which block DNA polymerases and thus the PCR (Hofreiter et al. 2001).

DNA polymerases may also be hindered in function by cross-link damage in DNA (Paabo et al. 2004). Examples of such a phenomenon are referred to as Maillard products. Maillard products are formed by condensation reactions between sugars and primary amino-groups in proteins and nucleic acids, but can be broken by treatment with N-phenacylthiazolium bromide allowing for DNA sequence amplification from some ancient remains (Paabo et al. 2004; Vasan et al. 1996).

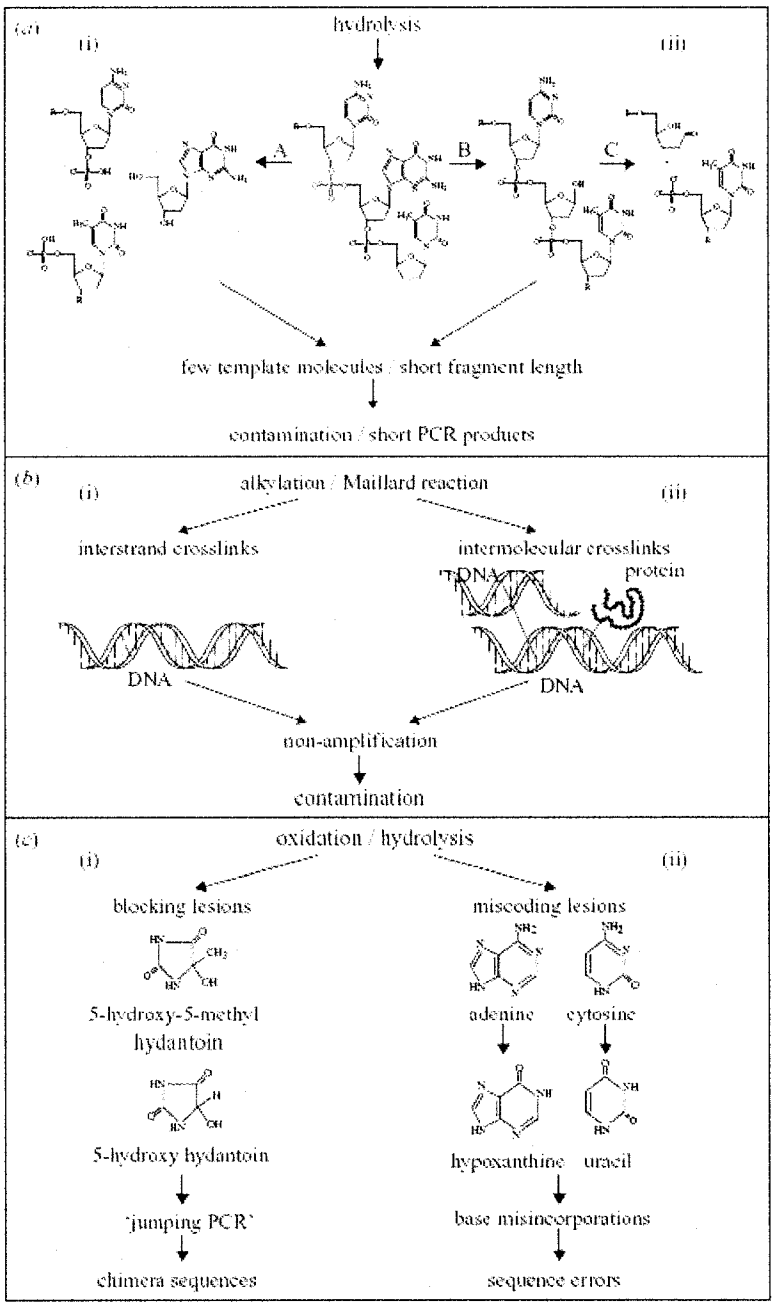


Figure 1. Post-mortem DNA modification in fossil remains, with the structures altered by damage shown in red (Willerslev and Cooper 2005). (*a*) Formation of strand breaks (single-stranded nicks) by hydrolytic damage. (i) Direct cleavage of the phosphodiester backbone (A). (ii) Depurination resulting in a baseless site (AP-site) (B) followed by breakage of the sugar backbone through β-elimination (C). Strand breaks are believed to be largely responsible for the short amplification length and the high rate of DNA loss in fossil remains. (*b*) Different types of crosslink formation. (i) Inter-strand crosslinks by alkylation. (ii) Intermolecular crosslinks by Maillard reaction. Crosslinks may prevent the amplification of endogenous template molecules, increasing the risk of contamination. (*c*) Oxidative and hydrolytic modification of bases resulting in (i) blocking lesions or (ii) miscoding lesions. Some oxidative damage results in lesions blocking the polymerase enzyme, and promoting chimeric sequences through 'jumping PCR'. Hydrolytic damage of bases may result in miscoding lesions, for exampled, deamination of cytosine and adenine to uracil and hypoxathine, respectively. These lesions will result in the incorporation of erroneous bases during amplification (Willerslev and Cooper 2005).

Modifications to DNA sequences by other known and unknown types of damage are common and problematic in aDNA analyses (Handt et al. 1994). Most common are hydrolytic loss of amino groups, from the bases adenine, cytosine, 5-methylcytosine, and guanine resulting in hypoxanthine, uracil, thymine, and xanthine respectively (Poinar 2003). The deamination products of cytosine (uracil) and of 5-methylcytosine (thymine) are of particular relevance for the amplification of ancient DNA since they cause incorrect bases (A instead of G, and C instead of T) to be inserted when new DNA strands are synthesized by a DNA polymerase (Handt et al. 1994). As time passes, the cumulative effects of damage to the DNA will be so extensive that no useful molecules remain (Hofreiter et al. 2001).

Authenticity of Degraded and Ancient DNA

Post-mortem DNA stability and survival is dependent on numerous circumstances that differ between all samples analyzed. However, in general, researchers have to contend with the fact that very little and often no DNA survives in ancient tissue (Hofreiter et al. 2001), thus in order to utilize PCR as a detection method for DNA in archaeological specimens, certain alterations of the protocol must be done. Usually PCR cycle number must be increased to ensure amplification of the degraded DNA, and because the DNA is degraded, primers must be designed to amplify DNA fragments with a maximum length of 300 base pairs (bp). Knowledge of the pervasive nature of contamination in aDNA technology has enhanced our ability to detect contamination, permitting a high degree of confidence when analyzing ancient, nonhuman organisms (Kolman et al. 1999). This usually low amount of authentic DNA in samples of up to several thousand years of age significantly increases the susceptibility of the PCR technique to contamination with modern DNA (Zink et al. 2002), due to its pervasiveness in the environment, both inside and outside of the laboratory (Hofreiter et al. 2001). Thus, the inclusion of a positive

PCR control of DNA extracted from modern specimens can inadvertently contaminate the archaeological specimen and provide template for the PCR even when DNA is stored separately from aDNA samples (Kolman et al. 1999). Therefore, strict precautions to avoid contamination with extraneous DNA in PCR have been imposed (Hofreiter et al. 2001), and there exists a general consensus on the clear necessity of strictly controlled conditions by all researchers working with aDNA (Zink et al. 2002). For example, the extraction and preparation of the PCR must be done in a laboratory that is rigorously separated from the work involving modern DNA (Hofreiter et al. 2001). Treatment of the laboratory equipment with bleach, UV irradiation of the entire facility, protective clothing and face shields are other routine precautions (Hofreiter et al. 2001). In addition, several criteria of authenticity are essential for believing that DNA sequences are ancient (Figure 2). Even when all precautions are followed perfectly, contamination may still occur, if for no other reasons than because the specimens themselves may be contaminated with modern DNA (Hofreiter et al. 2001).

Physically isolated work area
To avoid contamination, it is essential that all DNA work be carried out in an isolated lab dedicated to low copy number samples. A separate building, if possible where no genetic work is being carried out, would be the best possible situation. Post-DNA extraction, all PCR reactions can be set up in this laboratory space and then moved to a normal lab for subsequent typing and sequencing.

Extraction controls and PCR product
Mock extractions and PCRs without template DNA are elementary controls that should be carried out.

Decontamination of reagents and specimens
Bleach, acid, ultra-filtration, baking and UV irradiation of reagents and tools should be routine; particularly important in ancient human and microbial studies. The surface of samples should be removed.

Uracil-N-glycosylase (UNG) treatment
UNG removes deamination products of cytosine, and is particularly important in studies where results involve few substitution differences.

Inverse correlation between amplicon length and amplification efficiency
Generally, amplification of only short DNA pieces is possible.

Quantitation of numbers of template molecules
If the number of DNA molecules that initiate the PCR is less than ~1,000, at least three independent amplifications need to be analyzed, and the products need to be cloned and several clones should be sequenced.

Exclusion of nuclear insertions of mitochondrial DNA
Pieces of mitochondrial DNA (mtDNA) exist in the nuclear genome and represent a potential source of false results. Sometimes, they can be detected by the observation of several mtDNA sequences from an extract, or by finding the same sequence in another closely related species. If overlapping amplifications using different primers detect the same sequence over an area that is highly variable, it is unlikely that the sequence derives from a nuclear insertion.

Time-dependent pattern of damage and diversity
Provides strong support for antiquity in microbial studies.

Biochemical assay for macromolecular preservation
Amino-acid analysis should show that the state of preservation of the specimen is compatible with DNA preservation.

Amplification from a second extract
The reproduction of the results from a second, independent extract should show that the result is reliable.

Clone
Direct sequencing of PCR products should be verified by cloning amplified products and sequencing ten clones to determine the ratio of endogenous to exogenous sequences, and damaged induced errors.

Reproduction in a second laboratory
When a new or unexpected result is obtained, reproduction in a second laboratory, preferably from a sample that has been independently sent from the museum to the second laboratory, ensures that the laboratory-specific contamination is not the source of the sequence determined.

Associated remains
In studies of human remains where contamination is especially problematic, evidence that DNA targets survive in associated faunal material can provide critical supporting evidence.

Phylogenetic sense
Reproducible sequences should in the end be placed in a tree phylogenetically with other known haplotypes in order to ensure that as the final criteria they are indeed authentic. This approach is particularly important in the case of detecting mitochondrial pseudogenes in the nucleus.

Figure 2. Authenticity criteria to determine ancient DNA sequences (Hofreiter et al. 2001; Poinar 2003; Willerslev and Cooper 2005).

CHAPTER 2: PARASITES INVOLVED IN HUMAN DISEASE

Malaria

There are fours species of *Plasmodium* that cause human malaria. These protozoan parasites are concentrated in the sub-Saharan Africa, and have been estimated by the World Health Organization to cause 300 to 500 clinical cases per year, with more than two billion people at risk throughout the world, with *Plasmodium falciparum* being the most fatal version of the disease (Rich and Ayala 2000). Utilizing two species of mosquitoes as the host transport, *Anopheles gambiae* and *Anopheles funestus*, *P. falciparum* infection has greatly expanded due to stagnant pools of water for breeding and an increase in mosquito-human contact (McElroy and Townsend 1989).

Biology

When residing in mosquitoes, *Plasmodium* species are associated with the insect's gut and salivary glands. Transmission to humans occurs within an initial bite and transfer of saliva of an infected mosquito to a human host. This feeding allows for the introduction of the parasite in sporozoite form where they travel to the liver, develop into merozoites, and then invade and multiply the red blood cells via the trophozoite stage (Wirth 2002). The merozoites in s subset of infected red blood cells then develop into gametocytes (Wirth 2002). The cycle continues when another mosquito bites the infected host, taking up the gametocytes, which develop into male and female reproductive cells (gametes) (Wirth 2002). These fuse in the insect's gut to form a zygote, which in turn develops into the ookinete, crosses the wall of the gut and forms sporozoites-filled oocyst (Wirth 2002). The oocyst eventually bursts, releasing the sporozoites that move to the mosquito's salivary glands, and once this mosquito feeds on a host, the process begins again (Figure 3) (Wirth 2002).

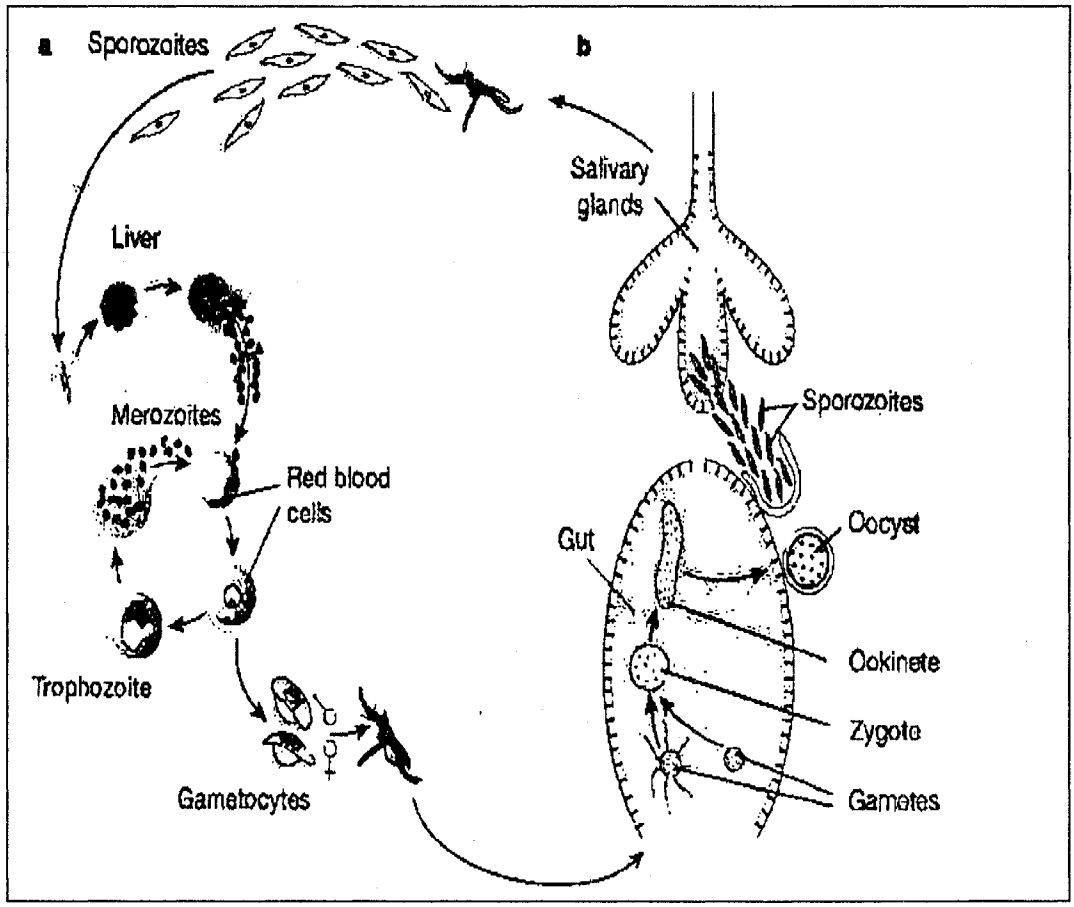


Figure 3. Life cycle of *Plasmodium falciparum* (Wirth 2002).

Pathogenesis

Most of the major clinical manifestations of malaria may be attributed to two general factors: (1) the host inflammatory response, which produces characteristic chills and fever, and (2) anemia, arising from enormous destruction of red blood cells (Roberts and Janovy Jr 2000). When the protozoa destroy the cells, usually at two or three-day intervals, the release of waste products and pigment brings on severe intermittent bouts of chills and fever (McElroy and Townsend 1989). The destruction of red blood cells during the erythrocytic cycle usually leads to anemia, which may be compounded by other immunopathological mechanisms (Taylor et al. 1997). The anemia caused by malaria is a serious hazard in pregnancy, and infection of the placenta by malaria parasites causes low birth weight in infants and in some cases miscarriage and stillbirth (McElroy and Townsend 1989). The most severe of the four species, *P. falciparum* causes acute symptoms in victims, especially among small children (McElroy and Townsend 1989).

Plasmodium falciparum is a cause of the more serious 'cerebral' malaria with continuous fever and jaundice, which can prove rapidly fatal (Taylor et al. 1997). Blackwater fever is an acute, massive lysis of erythrocytes, marked by high levels of free and breakdown products of hemoglobin in blood and urine, and by renal insufficiency (Roberts and Janovy Jr 2000). A combination of other severe manifestations leads to a condition known as algid malaria, which is the rapid development of shock and may be associated with septicemia, with toxemia and massive gastrointestinal hemorrhage (Roberts and Janovy Jr 2000).

Diagnoses

Diagnosis of this agent of disease may, to some extent, depend on the clinical manifestations of the disease itself, but the demonstration of the parasite in stained smears of

peripheral blood is most important (Roberts and Janovy Jr 2000). However, with microscopic identification, staining of the sample is critical for successful identification as well as timing of blood examination because, depending on the stage of the parasite's developmental cycle at which the blood sample is taken, various stages of the parasite will appear (Markell et al. 1992). An improved immunological method has also been employed with great success. It is referred to as the dipstick method, in which detection of a *P. falciparum* antigen in the blood of infected individuals provides a positive identification (Roberts and Janovy Jr 2000).

Schistosomiasis

Schistosomiasis is the general recognized term for a disease caused by species within the genus *Schistosoma*. *Schistosoma haematobium* is one member of the species producing the human disease urinary schistosomiasis (Markell et al. 1992). Originally, this disease apparently focused along the Nile Valley, where it is highly endemic (Markell et al. 1992). From there, this disease spread throughout Africa and Turkey (Markell et al. 1992).

Biology

This species, along with other members are dioecious with a male and female permanently paired throughout life (Ruppert and Barnes 1994) and adult worms inhabiting the veins that drain organs of their host's abdomen (Roberts and Janovy Jr 2000). Females usually reside in the gynecophoral canal of the males (Roberts and Janovy Jr 2000) but leave the males to deposit their eggs in small venules close to the lumen of the bladder (Markell et al. 1992). Then, the eggs must traverse the wall of the venule, some intervening tissue, and the bladder mucosa before they are in position to be expelled from the host (Roberts and Janovy Jr 2000) by means of an enzyme elaborated by the miracidium which diffuses through the egg shell and helps to digest the overlying tissue, or with some speculation, small spines on the eggs (Markell

et al. 1992). By the time the eggs are excreted with the urine of the host, they are completely embryonated and hatch when exposed to the lower osmolarity of fresh water (Roberts and Janovy Jr 2000) and the miracidium escapes. The miracidia are well supplied with sensory receptors and seek out particular species of freshwater snail; species of *Bulinus* and *Physopsis*, and possibly *Planorbarius* (Roberts and Janovy Jr 2000). If successful, the miracidium penetrates the snail, where it undergoes a cycle of development, giving rise to a large number of cercariae infective for humans (Markell et al. 1992).

The cercariae start to emerge from the snail host about four weeks after initial penetration by the miracidium (Roberts and Janovy Jr 2000). Once in contact with human skin, they attach and penetrate using enzymes and muscular boring movements (Ruppert and Barnes 1994). The now tailless "schistosomules" are carried to the bloodstream first to the lungs, then to the liver (Ruppert and Barnes 1994) where they mature in the sinusoids and migrate to reach the vesical, prostatic and uterine plexuses by way of the hemorrhoidal veins (Markell et al. 1992). The cycle then begins again when eggs are deposited in the walls of the bladder where they eventually break through into the lumen and escape with the urine (Figure 4).

Pathogenesis

Schistosomiasis is a debilitating disease and can be lethal (Ruppert and Barnes 1994). Pathogenesis is almost entirely due to the eggs and not to the adult worms (Roberts and Janovy Jr 2000). Egg penetration through the intestinal wall and bladder, aberrant lodging of eggs in various organs, and the developmental stages in the lung and liver can result in inflammation, necrosis or fibrosis, depending on the degree of infection (Ruppert and Barnes 1994). However, schistosomiasis is usually divided into three pathological phases: migratory, acute and chronic.

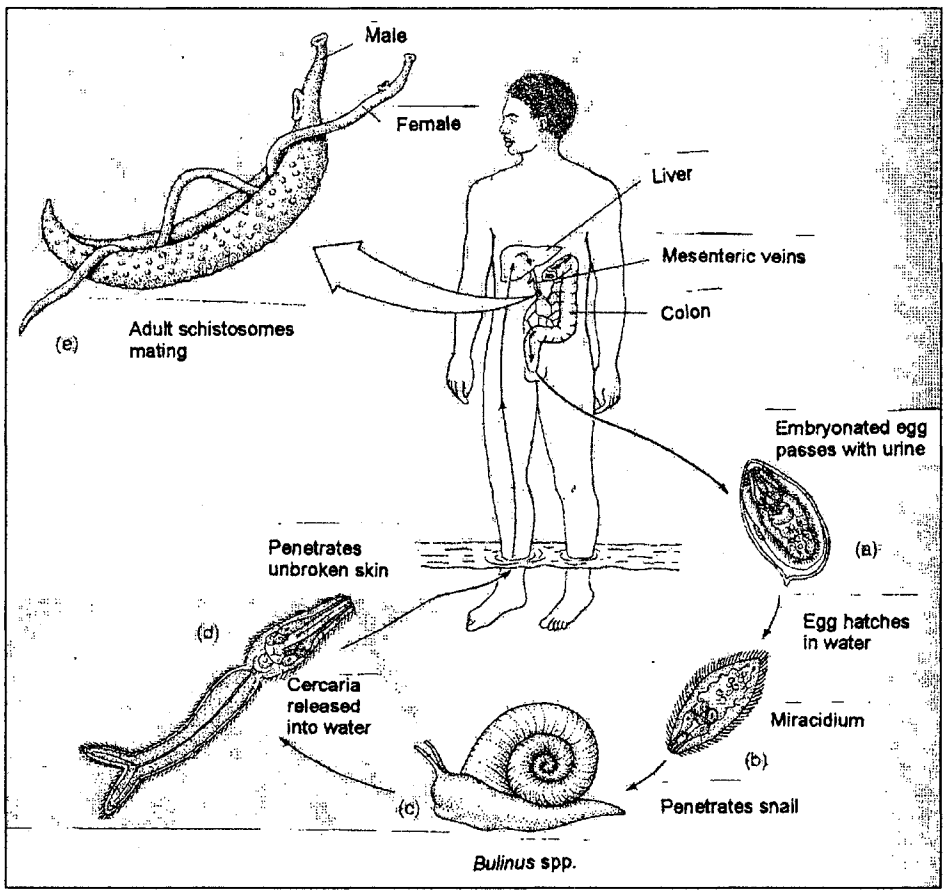


Figure 4. Life cycle of *Schistosoma haematobium* (modified) (Roberts and Janovy Jr 2000).

The migratory phase encompasses the time from penetration until maturity and egg production (Roberts and Janovy Jr 2000). Following cercariae penetration of the skin, transient toxic and allergic manifestations may occur, which may be very mild or even passed as unnoticed (Markell et al. 1992). Soon after, the developing schistosomes reach the liver and acute hepatitis may develop (Markell et al. 1992).

The acute phase, also known as katayama fever, occurs when schistosomes begin producing eggs about four to six weeks after initial infection (Roberts and Janovy Jr 2000). The host has considerable exposure to various schistosome antigens causing a change in the antigen-antibody ratio, which leads to the formation of large immune complexes marked by chill and fever, fatigue, headache, malaise, muscle aches, lymphadenopathy, and gastrointestinal discomfort (Roberts and Janovy Jr 2000). Granulomas surrounding the eggs may become fibrous or pseudotubercles, so called because of their resemblance to localized nodules of tissue reaction (tubercles) in tuberculosis (Roberts and Janovy Jr 2000). Many eggs however, may be carried by the hepatic portal circulation into the liver, lungs or other tissues where granuloma formation may be stimulated (Roberts and Janovy Jr 2000).

The chronic stage of the disease comes on gradually (Markell et al. 1992). Egg deposition in the smaller venules close to the lumen of the intestine or bladder may evoke the formation of minute abscesses, however, eggs that become dislodged and swept into circulation produce the most pathologic changes (Markell et al. 1992). Although this stage is commonly asymptomatic with *S. haematobium* infection, there may be pain on urination and blood in the urine (Roberts and Janovy Jr 2000).

Infection with *S. haematobium* is the considered the least serious of schistosomiasis (Roberts and Janovy Jr 2000). Adults of this species live in the venules of the urinary bladder

and the chief symptoms are associated with the urinary system (Roberts and Janovy Jr 2000). There is a gradual onset of hematuria with development of an ulcerated bladder as the disease progresses (Roberts and Janovy Jr 2000). These changes in the bladder wall are associated with a tissue-egg reaction causing the pseudotubercles, fibrous infiltration, thickening of the muscularis layer and ulceration (Roberts and Janovy Jr 2000). Hepatosplenic schistosomiasis is also common but tends to be subclinical or mild (Markell et al. 1992). Granulomas that form around the eggs may become lodged in the liver and become grossly enlarged (Markell et al. 1992). Portal hypertension may result from obstructive liver disease and lead to esophageal varices, which may bleed, and finally to massive ascites (Markell et al. 1992).

Diagnoses

Identification of this parasite comes in the form of a positive demonstration of the eggs of this species in the urine (Roberts and Janovy Jr 2000). However, due to minimal numbers of eggs produced by the females of this genus, as compared to other helminths that infect humans (Roberts and Janovy Jr 2000), centrifugation or sedimentation of urine must occur for the recovery of the characteristics eggs (Markell et al. 1992). A more invasive approach for diagnostic purposes may be either a biopsy from the bladder wall or vagina of an infected individual (Markell et al. 1992). If conditions permit, serological tests based on the detection of antibodies in the patient's blood may also be employed (Roberts and Janovy Jr 2000).

Leishmaniasis

The species of *Leishmania* that infect humans are widely distributed. It is thought that the transport of slaves to the Western world from Africa through the Middle East and Asia spread *Leishmania* into previously uncontaminated areas, where they now apparently are evolving rapidly into new strains (Roberts and Janovy Jr 2000).

Three species of *Leishmania* were recognized, corresponding to the clinical entities of cutaneous, mucocutaneous and visceral leishmaniasis (Markell et al. 1992). However, *Leishmania donovani*, the species that causes visceral leishmaniasis, locally known in India as Dum-Dum fever and widely known as Kala-Azar (Roberts and Janovy Jr 2000), is of interest.

Biology

Leishmania donovani lives within the cells of the reticuloendothelial (RE) system of the viscera, including the spleen, liver, mesenteric lymph nodes, intestine and bone marrow (Roberts and Janovy Jr 2000). However, this species is not confined to the cells of the subcutaneous tissues and mucous membranes, but may be found throughout the body (Markell et al. 1992).

The intermediate host and vector of *L. donovani* are sandflies of the genus *Phlebotomus* (Roberts and Janovy Jr 2000). When these insects ingest the blood of an infected animal, they also ingest amastigote forms of the parasite where they lodge in the midgut of the insect and begin to multiply (Roberts and Janovy Jr 2000). The parasites attach to the walls of the fly's gut and replicate where they transform into promastigotes and move forward to the esophagus and pharynx and accumulate (Roberts and Janovy Jr 2000). The feeding sandfly pumps the esophageal contents in and out to clear the obstruction, thereby inoculating promastigotes into the skin of the victim (Roberts and Janovy Jr 2000). Once in the mammalian host, the parasite is immediately engulfed by a macrophage, in which it divides by binary fission, eventually killing the host cell, it then escapes and the process repeats severely damaging the RE system (Figure 5) (Roberts and Janovy Jr 2000).

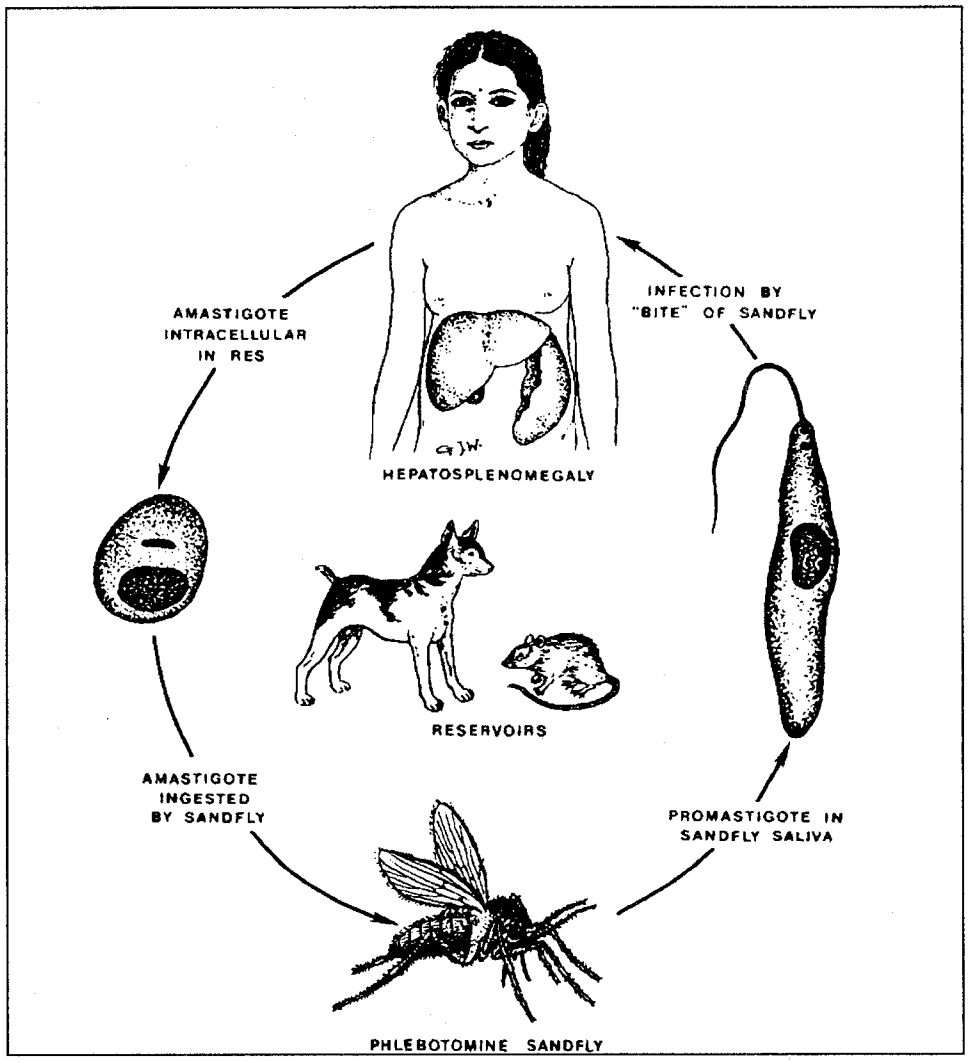


Figure 5. Life cycle of *Leishmania donovani* (Markell et al. 1992).

Pathogenesis

Leishmania donovani does not in most areas cause skin lesions, although in some cases dermal lesions (presumably at the site of infecting bite) if present may be erythematous or depigmented macules distributed over the entire body or in patches (Markell et al. 1992). A butterfly distribution over the nose is not uncommon with later lesions becoming nodular, and at this stage may be mistaken for leprosy nodules (Markell et al. 1992).

It is a progressive disease, usually beginning slowly with low-grade fever and malaise, followed by progressive wasting and anemia, protrusion of the abdomen from enlarged liver and spleen, and finally death (if untreated) in two or three years (Roberts and Janovy Jr 2000). Accompanying this are edema, especially of the face, and bleeding of the mucous membranes (Roberts and Janovy Jr 2000).

However, the species that cause visceral leishmaniasis may be viewed as a disease of the RE system (Roberts and Janovy Jr 2000) parasitizing the cells of this system throughout the body (Markell et al. 1992). Proliferation of the phagocytic cells, which are so important in defending the host against invasion, are themselves the habitat of the parasites (Roberts and Janovy Jr 2000). Thus, compensatory production of macrophages and other phagocytes can lead to an increase in parasite numbers, particularly in the spleen and liver, resulting in massive hypertrophy of these organs (Markell et al. 1992). Thus, splenomegaly with stasis of blood in the sinusoids may result in increased destruction of both red and white blood cells (Markell et al. 1992). Also, glomerular involvement, with deposition of subendothelial and mesangial immune complexes resembling those found in the kidney in human cases of hepatosplenic schistosomiasis have been found (Markell et al. 1992).

Diagnoses

Finding amastigotes in tissues or secretions, such as a spleen or liver puncture, blood or nasal smears, bone marrow, should be examined and cultures from these should be attempted (Roberts and Janovy Jr 2000). However, such procedures may be risky, such as a spleen puncture, or not as productive as one would like, such as a liver puncture (Markell et al. 1992). Culturing of the parasite is an alternative method. It was found that Schneider's Drosophila medium, microscopy using Giemsa-stained smears, RPMI medium 1640, and NNN medium were found to range from most to least sensitive, respectively, for detection (Markell et al. 1992). Also, fluorescent antibody tests, enzyme-linked immunosorbent assay (ELISA) (Harith et al. 1987), and direct agglutination test (DAT) have been developed (Pappas et al. 1984). However, one of the most simplistic means of identification could be the elimination of the diseases that have symptoms similar to Kala-Azar (Roberts and Janovy Jr 2000).

Trypanosomiasis

Trypanosomiasis is the recognized term for a disease caused by species within the genus *Trypanosoma* generally referred to as trypanosomes. Trypanosomes are agents of numerous diseases of humans in subtropical and tropical regions of the world (Ruppert and Barnes 1994). They are divided into two broad groups, or "sections", based on characteristics of their development in the insect hosts (Roberts and Janovy Jr 2000). If a species develops in the anterior portions of the digestive tract, it is said to undergo anterior station development and is relegated to the section Salivaria, such as in the case of *Trypanosoma brucei*, but if a species develops in the hindgut of its invertebrate host, it is said to undergo posterior station development and is placed in the section Stercoraria, such as in the case of *Trypanosoma cruzi* (Roberts and Janovy Jr 2000). In general, part of the life cycle of these parasites is passed within

or attached to gut cells of blood-sucking insects, and another part of the cycle is spent in the blood or in white blood cells and lymphoid cells of the vertebrate host, although other tissues may be invaded (Ruppert and Barnes 1994). Intracellular stages are aflagellate, but during the life cycle there are motile, extracellular flagellate stages in the bloodstream or in the invertebrate host (Ruppert and Barnes 1994).

Trypanosoma brucei

African sleeping sickness is the name given to the disease caused by two subspecies of *Trypanosoma brucei*: *T. b. gambiense*, which is the cause of Gambian or the West African form of the disease, and *T. b. rhodensiense,* which causes Rhodesian or East African sleeping sickness (Markell et al. 1992). However, due to the similarity between the life cycles of each subspecies, their biology will be described in terms of the *T. brucei* complex. This parasite occurs as trypomastigotes in the bloodstream, lymphatics, and cerebrospinal fluid, although, it has been reported to occur as nonflagellate cryptic forms in the choroids plexus, and possibly other organs (Markell et al. 1992).

Biology

The insect vectors, tsetse fly, that participate in the life cycle of *T. brucei* fall within the genus *Glossina* (Roberts and Janovy Jr 2000). When the parasite is ingested by the fly, along with a blood meal, *T. brucei* locates in the posterior section of the midgut of the insect where it multiplies in the trypomastigote form for about 10 days (Roberts and Janovy Jr 2000). Migration of the parasite into the foregut occurs between the 12^{th} and 20^{th} day of development, followed by further migration into the esophagus, pharynx, and hypopharynx where they enter the salivary glands and transform into epimastigotes (Roberts and Janovy Jr 2000). Several asexual generations produces metacyclic trypomastigotes, the only stage in the vector that is infective to

the vertebrate host, and when feeding, the tsetse fly may inoculate the host with several thousand flagellates (Figure 6) (Roberts and Janovy Jr 2000).

Once in the vertebrate host, the trypanosomes multiply as trypomastigotes in the blood and lymph (Roberts and Janovy Jr 2000). In chronic situations, many invade the central nervous system, multiply, and enter intercellular spaces within the brain (Roberts and Janovy Jr 2000).

Pathogenesis

Initially in human infections, a small sore (chancre) often develops at the site of metacyclic trypanosome inoculation but soon may disappear after one or two weeks (Roberts and Janovy Jr 2000). These protozoa then gain entrance to the blood and lymph channels, where they reproduce rapidly, producing a parasitemia and invading nearly all organs of the body (Roberts and Janovy Jr 2000). It is at this stage that the similarities between the two subspecies begin to lessen.

Trypanosoma brucei rhodensiense rarely invades the nervous system, but usually causes a more rapid course toward death when compared to *T. b. gambiense* (Roberts and Janovy Jr 2000). Once this parasite reaches the lymph channels, the lymph nodes in the neck, groin and legs become swollen and congested, with a characteristic swelling of the nodes at the base of the skull referred to as Winterbottom's sign (Roberts and Janovy Jr 2000). Weight loss is common with involvement of the heart, resulting in death within a few months (Roberts and Janovy Jr 2000). However, there is no sign of somnambulism or other protracted nervous disorders as with *T. b. gambiense* due to rapid death of the host (Roberts and Janovy Jr 2000).

Early in infection, *T. b. gambiense* is found in the choroids plexus where a blockage of capillaries may occur causing localized edema and obstruction of the cerebral spinal fluid (Markell et al. 1992). In the acute stage of infection, evidence suggests that it may be due to

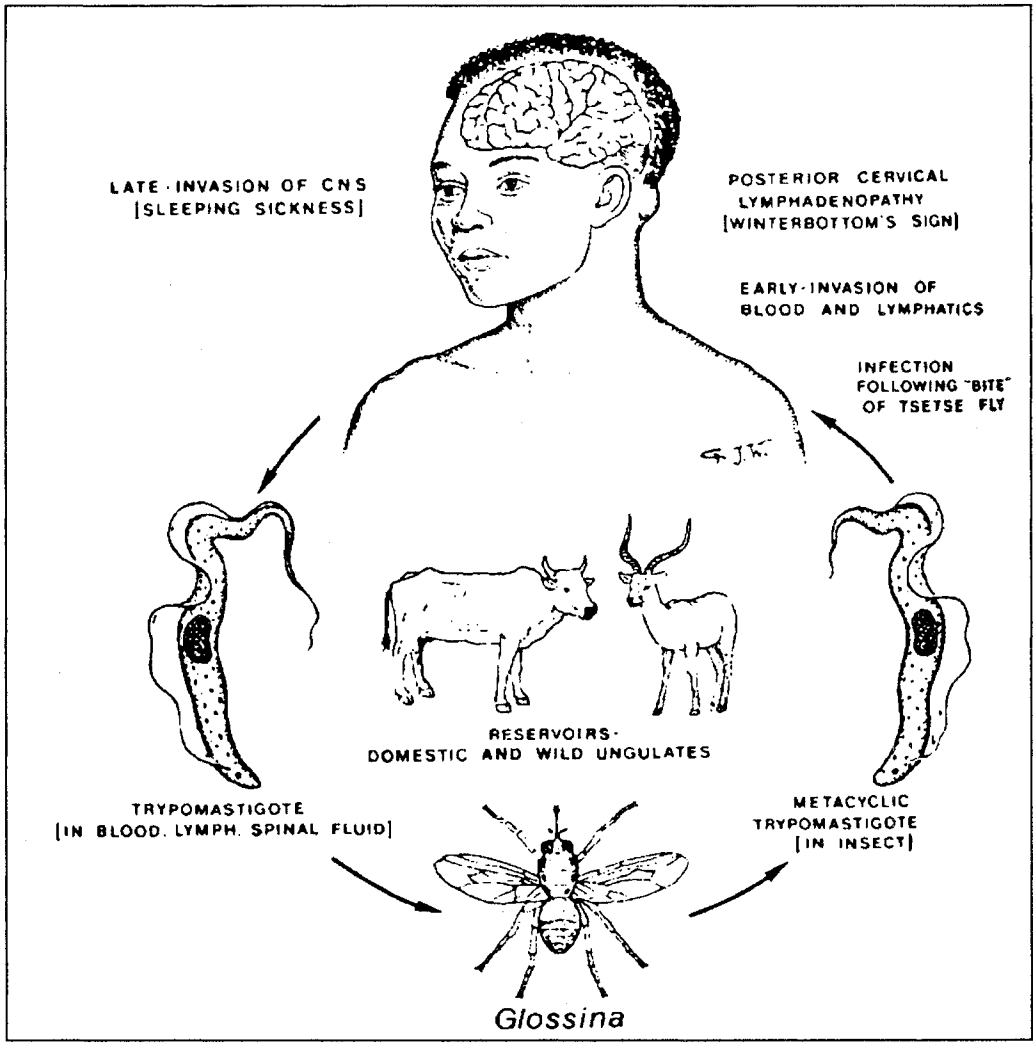


Figure 6. Life cycle of *Trypanosoma brucei* complex (Markell et al. 1992).

antigenic "performance" of trypanosomes and the host's immune reaction (Roberts and Janovy Jr 2000). At this stage, trypanosome antigens adsorb to the surface of some host cells, such as red blood cells, causing lysis and accounting for the resultant anemia (Roberts and Janovy Jr 2000). In the chronic stage of infection, trypanosomes invade the central nervous system (Roberts and Janovy Jr 2000). Mental and physical instabilities follow, which may lead to coma and death resulting from malnutrition, pneumonia, heart failure, or other parasitic infection (Roberts and Janovy Jr 2000).

Diagnoses

Demonstration of parasites in the blood, bone marrow or cerebrospinal fluid established diagnosis (Roberts and Janovy Jr 2000). Centrifugation of the spinal fluid increases the possibility of trypanosome detection, however, it was found that double centrifugation was at least twice as sensitive as single centrifugation and increased the early detection of late-stage cases (Markell et al. 1992). Serological tests for *T. brucei* identification include immunofluorescence (IIF), indirect hemagglutination (IHA), ELISA and complement fixation techniques: serum and spinal fluid immunoglobulin M (IgM) measurements (Markell et al. 1992). Differential diagnosis between the subspecies may be made on geographical grounds, but there are some areas, such as Uganda, where the two infections coexist, therefore much reliance in placed on the patient's clinical history and physical findings (Markell et al. 1992).

Trypanosoma cruzi

The etiological agent of Chagas' disease or American trypanosomiasis is *Trypanosoma cruzi*. This protozoan parasite is distributed throughout some southern parts of the United States through Mexico and Central America, and in South America as far south as Argentina (Markell et al. 1992). This organism differs from other trypanosomes infecting humans in that it has an

intracellular amastigote stage in cardiac muscle and other tissues, as well as trypanosome forms in the circulating blood (Markell et al. 1992).

Biology

Trypanosoma cruzi develops successfully in a large number of insects, but it is considered that reduviid bugs are the only vectors of importance and only those species that invade houses and habitually defecate during the process of feeding or immediately thereafter are major vectors of the human disease (Markell et al. 1992). Their feces may contain metacyclic trypanosomes, which gain entry into the body of the vertebrate host through the bite, scratched skin, or mucous membranes that are rubbed with fingers contaminated with the insect's feces (Roberts and Janovy Jr 2000). Trypomastigotes do not reproduce until they have entered a cell, such as reticuloendothelial cells of the spleen, liver, and lymphatics and cells in cardiac, smooth, and skeletal muscles, and have transformed, by binary fission, into amastigotes killing and lysing the cell (Roberts and Janovy Jr 2000). Some intermediate forms (promastigotes and epimastigotes) complete the metamorphosis into trypomastigotes and find their way into the blood (Roberts and Janovy Jr 2000).

Trypomastigotes ingested by triatomine bugs pass through to the posterior portion of the insect's midgut, where they become short epimastigotes, multiply by longitudinal fission, and eight to 10 days later, metacyclic trpomastigotes appear in the insect's rectum(Roberts and Janovy Jr 2000). These pass with the feces, and once inoculated into a mucous membrane or wound in the skin, the cycle begins again (Figure 7) (Roberts and Janovy Jr 2000).

Pathogenesis

Upon its entry into the vertebrate host, *T. cruzi* produces an acute local inflammatory reaction (Markell et al. 1992). Within one to two weeks of infection, the metacyclic

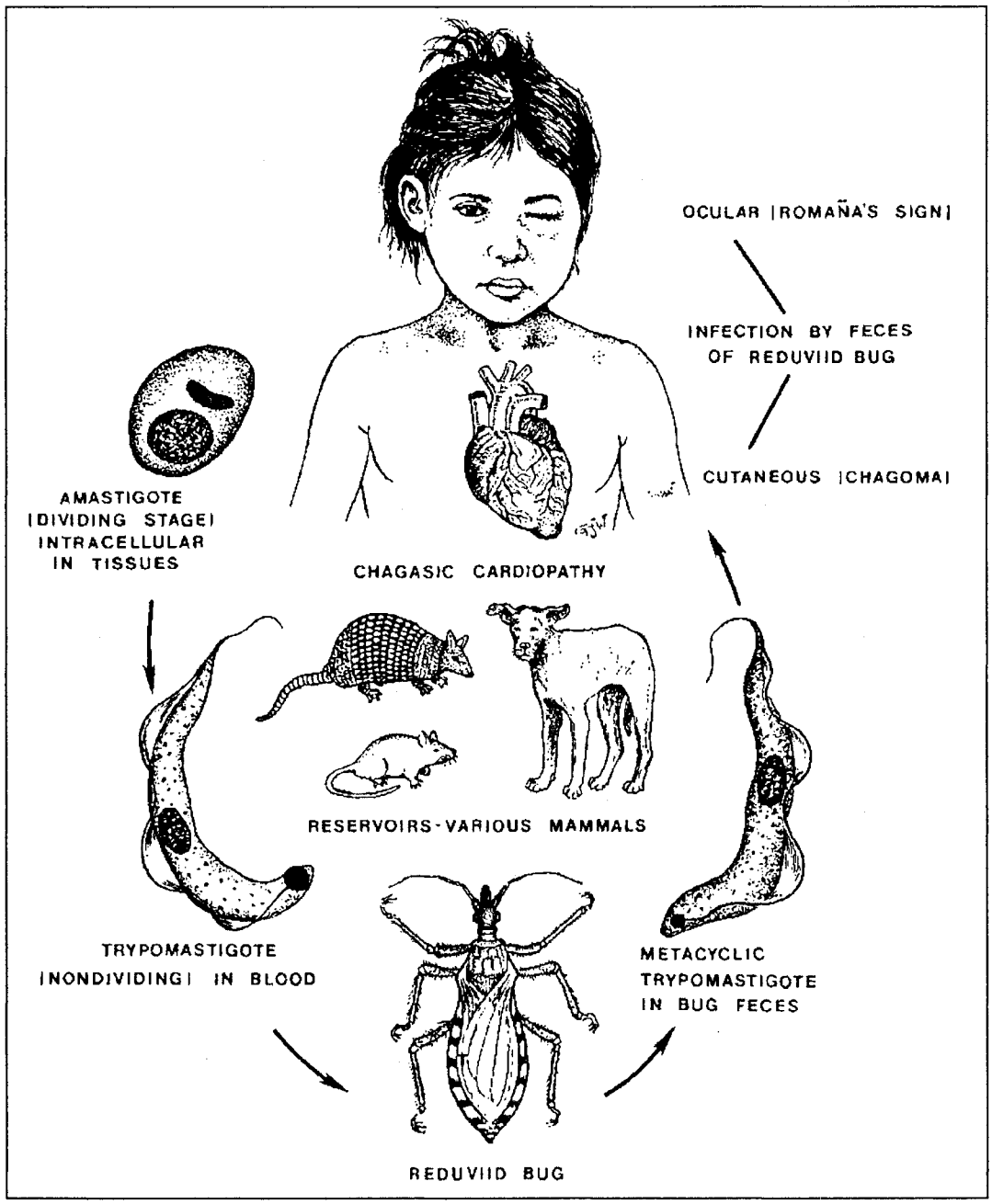


Figure 7. Life cycle of *Trypanosoma cruzi* (Markell et al. 1992).

trypanosomes spread to the regional lymph nodes and begin to multiply in the cells that phagocytose them, generating an accumulation intracellular amastigotes, which produces a so-called pseudocyst (Roberts and Janovy Jr 2000). Trypomastigotes that burst out of the pseudocyst cause an acute, local inflammatory response, with degeneration and necrosis (cell or tissue death) of nerve cells in the vicinity (Roberts and Janovy Jr 2000).

An acute phase of the disease is initiated by inoculation into the wound of the trypanosomes from the bug's feces resulting in a local inflammatory response and producing a small red nodule known as a chagoma (Roberts and Janovy Jr 2000). Lipogranulomas form after the lymphatic spread of the parasites to the regional nodes (Markell et al. 1992). Pseudocysts may be found throughout the body, as the disease progresses, invading the heart muscle and resulting in an 80% loss of the cardiac ganglion cells (Roberts and Janovy Jr 2000).

The chronic phase of the disease revolves mostly around the heart and surrounding tissues. Massive cardiomegaly may develop, with diffuse inflammation of the myocardium as well as fibrosis and infiltration of lymphocycts, macrophages, and plasma cells, and the heart weight increases (Markell et al. 1992). Damage to the autonomic nervous system of the heart parallels that to Auerbach's plexus in the walls of the digestive tract (Markell et al. 1992). Hypertrophy of the muscle layers and diminution in number of the ganglion cells are seen in affected portions of the digestive tract, most frequently in the esophagus and colon (Markell et al. 1992).

Diagnoses

Positive identification of this parasite may rely on the demonstration of trypanosomes in blood, cerebrospinal fluid, fixed tissues or lymph, with trypomastigotes most abundant in peripheral blood during periods of fever via smear of biopsy (Roberts and Janovy Jr 2000). In

young children, this technique is easily accomplished, however, in older children and adults, it is more difficult thus requiring concentration techniques or culture (Markell et al. 1992). Blood can be inoculated into a suitable host animal (guinea pig, mouse) where heart smears from these animals can be examined for the presence and identification of the parasite (Roberts and Janovy Jr 2000). Xenodiagnosis may also be employed. This technique involves the use of laboratory-reared triatomines, which are allowed to feed on a patient and after a suitable period of time (10 to 30 days) they are examined for intestinal flagellates (Roberts and Janovy Jr 2000). A more specific test, a complement fixation test referred to as the Machado-Guerreiro test, relies on antigen specificity or radioimmunoassay (RIA) developed for immunodiagnosis (Markell et al. 1992).

Ascariasis

Ascariasis is a disease caused by two species of *Ascaris*: *A. lumbricoides* and *A. suum*, however, it is the species that causes the human form of the disease that is of interest. *Ascaris lumbricoides*, and the species in general, are characterized by their great size; females ranging from 20cm to 49cm long and 3mm to 6mm wide, and males 15cm to 31cm long and 2mm to 4mm wide; having three prominent lips, each with a dentigerous ridge, and no interlabia or alae (Roberts and Janovy Jr 2000). Both sexes are creamy white, sometimes with a pinkish cast, and the cuticle has fine circular striations (Markell et al. 1992).

Mature female worms can produce up to 200 000 eggs daily, each recognizable once fertilized (Markell et al. 1992). These fertilized eggs are oval to round, 45μm to 75μm long by 35μm to50μm wide, with thick, lumpy outer shell (mammallated, uterine or proteinaceous layer) (Roberts and Janovy Jr 2000).

The possibility of infection with *A. lumbricoides* is common. This nematode is quite abundant and has a cosmopolitan distribution (Roberts and Janovy Jr 2000). Infection persists throughout the temperate and tropical areas of the globe, and under conditions of poor sanitation virtually 100% of the population harbour the parasite (Markell et al. 1992).

Biology

Infection with Ascariasis begins when unsegmented eggs are passed; under favourable conditions they require a period of about two or three weeks outside the host to develop to the infective stage (Markell et al. 1992). When the fully embryonated eggs are swallowed with either contaminated food or water, they hatch in the duodenum, penetrate the mucosa and submucosa and enter the lymphatics or venules (Roberts and Janovy Jr 2000). After passing through the right heart, they enter the pulmonary circulation and break out of capillaries into air spaces (Roberts and Janovy Jr 2000). There they grow and molt, and after about 20 days migrate through the respiratory passages to reach the esophagus and eventually once again the small intestine (Markell et al. 1992). Two or three months after ingestion of the eggs, the mature worms commence egg laying in the intestine (Figure 8) (Markell et al. 1992).

Pathogenesis

Damage caused by ascarids is initiated when juveniles break out of lung capillaries into the respiratory system, where they cause a small hemorrhage at each site (Roberts and Janovy Jr 2000). However, with heavy infections, small pools of blood may accumulate, which may initiate edema with resultant clogging of air spaces by white blood cells and dead epithelium known as Ascaris pneumonitis (Roberts and Janovy Jr 2000).

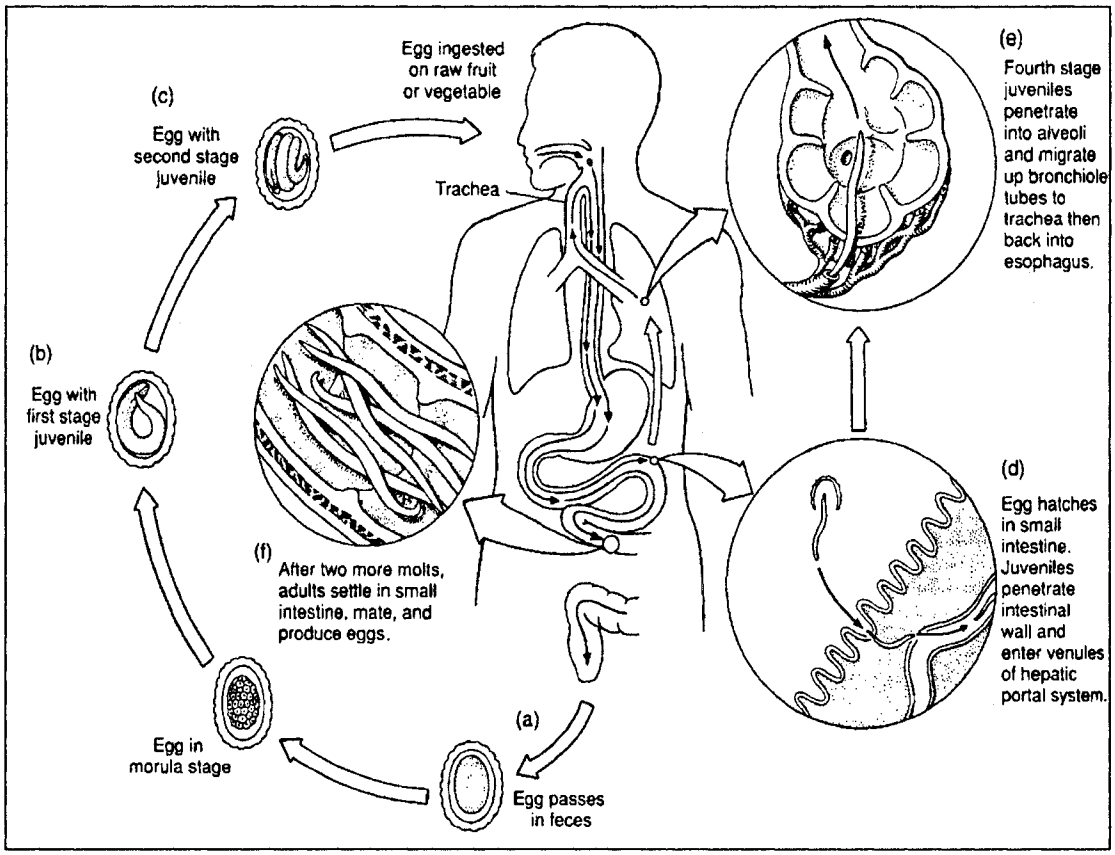


Figure 8. Life cycle of *Ascaris lumbricoides* (Roberts and Janovy Jr 2000).

It is not uncommon for female worms to leave the intestine. This occurs from a lack of male presence and/or overcrowding, commonly known as "wandering worms" (Roberts and Janovy Jr 2000). If worms wander downstream, they make their way to the appendix, which can be clogged or penetrated, or to the anus (Roberts and Janovy Jr 2000). Upstream wandering leads to the pancreatic and bile ducts, possibly occluding them with grave results (Roberts and Janovy Jr 2000). Worms reaching the stomach are aggravated by the acidity and writhe about, causing nausea (Roberts and Janovy Jr 2000). Suffocation or lung damage is possible if the worms reach the esophagus and crawl into the trachea; may crawl into the eustachean tubes and middle ears causing damage; or may simple exit through the nose or mouth, causing predictable consternation (Roberts and Janovy Jr 2000).

Diagnoses

A highly used method to diagnose this parasite involves identifying the characteristic, mammillated eggs in stool, or by the worm itself (Roberts and Janovy Jr 2000). There are usually an abundance of eggs, therefore, one or two direct fecal smears are usually sufficient to demonstrate infection (Roberts and Janovy Jr 2000). Providing there is an experienced technician with an abundance of experience identifying juveniles of this species, demonstration of the juveniles in the sputum via a smear is a definite diagnostic tool (Roberts and Janovy Jr 2000). Other methods, slightly more tiresome on the patient, involves the diagnosis of the parasite on radiography, either as worm-shaped radiolucent areas in a barium-filled intestine, sometimes with their own intestinal tracts outlined as well by the barium they have ingested, or in cholangiograms (Markell et al. 1992).

Although the methods used in these simplistic, conventional diagnostic procedures seem to allow for an accurate identification of the parasite, there are a few disadvantages with the

methods. Firstly, the more invasive the test, the less likely patients would be willing to go through such a procedure. For all of the parasites outlined, microscopic identification of the parasite was the most efficient method and the most commonly used. However, only a minimal number of slides may be read by one microscopist per day, which makes this method rather unsuitable for application in large epidemiological field situations (Jelinek et al. 1996), and an expert is usually required for each species identification. Finally, with the more advance serological tests, several problems do still exist. Many times, these tests only become positive some time after infection, they only become negative some time after cure, many tests cannot differentiate between closely related species or between current and cured cases of the disease, and many cross react with other species of the same genera, such as in helmintic infections (Roberts and Janovy Jr 2000). That is why new techniques are required, which allow for specific, efficient, and immediate diagnosis of disease. One such technique that falls within all these parameters is the polymerase chain reaction.

CHAPTER 3: PARASITE DIAGNOSTICS VIA PCR

Parasites and PCR

The PCR technique has been an innovative tool in molecular analyses of DNA. This technique has been able to revolutionalize molecular research through its ability to progress alongside all other techniques, although decades old. It has stood firm on its own and in combination with other techniques to still be considered a "new" molecular technique. Thus, through innovative research methodology and expanding knowledge of biological properties, PCR has opened up new horizons to how research employs critical analyses of nucleic acids. With this and alongside other ground-breaking techniques, it has given rise and provided us with substantial research inquiries into the realm of parasite analyses via nucleic acid research.

An abundance of research involving PCR has been performed on DNA analyses of various parasites. Kinetoplast DNA (kDNA) has been used extensively in research and identification of *L. donovani*, such as the identification of species from mixed infections (Ibrahim et al. 1994) or testing for the presence of the parasite in unknown or suspected samples showing signs of disease (Smyth et al. 1992; Bhattacharyya et al. 1996; Salotra et al. 2001) and finding a correlation between seroreactivity of the multicopy mini-exon derived RNA (med RNA).

Other parasites have also been examined. DNA studies suggested that *S. haematobium* or a hybrid of *S. haematobium* and *Schistosoma mattheei* parasite infected dog in Zambia (Chiti et al. 1999), and *DraI* repeat could be used for monitoring free-living cercariae and infected snails only in bodies free from cross-hybidizing species of *Schistosoma* (Hamburger et al. 2001). Trypanosome PCR analyses have also provided a means to identify species, whether it be a determination of optimal oligonucleotides for *T. cruzi* (Virreira et al. 2003) through the determination of allelic frequencies in geographical areas and nature of multilocus genotypes in

two geographically separate *T. brucei* stocks (Mbati et al. 1999), or to determine how effective drug treatment against *T. brucei* is (MacLeod et al. 2001). However, PCR analyses involving a combination of methods have expanded the utility of PCR.

A combination of two PCR techniques has been termed nested PCR. It involves a PCR followed by a subsequent PCR with primers of the second flanking a region inside or outside the first PCR primers, and the initial PCR product used as the template. Many investigators have used nested PCR in parasite analyses. With *Plasmodium* species geographical distributions overlapping, nested PCR has been used to identify the distribution of mixed species (Mehlotra et al. 2000) or to establish the genetic structure of *P. falciparum* in areas with a different degree of endemicity (Montoya et al. 2003). Also, linkage disequillibrium was analyzed to determine the frequency of recombination events in merozoite surface protein-1 (MSP-1) (Sakihama et al. 2001). In addition to this, a technique referred to as heminested PCR was used to genetically type *T. cruzi* lineages I and II (Freitas et al. 2005).

Combining immunological methods and PCR has proven effective in research. PCR-ELISA has demonstrated its reliability for diagnosing visceral leishmaniasis when compared to direct examination and culture (Costa et al. 1996). However, when investigating *T. brucei* parasitemia, PCR-ELISA, although increasingly more effective than microscopy, only produced an efficiency of 85% (Masake et al. 2002). The addition of microplate hybridization (MPH) to PCR based ELISA (PCR-MPH) was used to target the DNA sequence of the 8S small subunit ribosomal RNA (rRNA) gene and hybridize with species-specific probes for *P. falciparum* (Laoboonchai et al. 2001). Thus, combining PCR with techniques that are solely efficient has proven a reliable means of parasite analysis.

Genetic characterization of parasites has revolved around randomly amplified polymorphic DNA (RAPD). RAPD was used to describe the phylogenetic groups of *T. cruzi* (I and II) (Zalloum et al. 2005). It was used to examine the relationship between genetic diversity of *S. haematobium* and the clinical outcome of samples (Brouwer et al. 2003), to examine the genetic diversity occurring in a population of schistosomes (Shiff et al. 2000), and to cross-sectionally study the genetic population structure of the *Bulinus globosus-Schistosoma haematobium* system (Davies et al. 1999). Also, relatedness was analyzed between *T. brucei*, *Trypanosoma evansi* and *Trypanosoma equiperdum* (Lun et al. 2004).

Fragment length polymorphisms in combination with PCR have provided many techniques for DNA analyses. Differentiation between species, subspecies, groups or hosts are a few applications of this technique. Restriction fragment length polymorphism (RFLP) combined with PCR has been used to differentiate between *P. vivax* and *P. falciparum* (Patsoula et al. 2003), between *S. haematobium* and *Schistosoma bovis* (Barber et al. 2000) and to show *A. lumbricoides* infections in dogs (Traub et al. 2002) and pigs (Zhu et al. 1999). Combined with amplified fragment length polymorphism (AFLP) analysis, a technique for whole genome fingerprinting, it was able to provide information that humans had acquired *Ascaris* from pigs and may therefore be considered a zoonotic disease (Nejsum et al. 2005).

A PCR based method for the quantitation of parasite infections involves the use of real-time PCR. This method has proven its accuracy and sensitivity on many occasions. This methodology was used to amplify a 195-bp repeat in *T. cruzi* DNA with accurate identification and quantitation of tissue parasite burden in animals (Cummings and Tarleton 2003). Primers designed for targeting a 177-bp repeat satellite DNA of *T. brucei* were designed and applied

using real-time PCR to human blood and found to be efficient, rapid and sensitive (Becker et al. 2004).

A more innovative approach in parasite identification involves the amplification process of converting RNA to DNA. This method, known as reverse transcriptase PCR (RT-PCR), has been used in numerous studies. It has proven to be a sensitive and specific method for *P. falciparum* detection (Abdullah et al. 1996). RT-PCR analyzed the migratory pattern of three schistosomes through human skin via cytokine pattern analysis (Poinar 2003). Following suppression subtractive hybridization analysis, RT-PCR further validated the identification of differentially expressed genes in different *T. cruzi* strains, but no specific correlation between gene expression and the classification of groups could be found (Dost et al. 2004).

As seen, there are many PCR-based techniques for parasite identification. The parasites investigated and presented are of great interest and importance to parasitological studies. They offer insights into genus and species biology, ecology distribution, which coincides with society's life and well being. Therefore, further molecular analyses involving each species will be investigated and discussed.

Parasites and Multiplex PCR

Multiplex PCR has been used in a variety of applications involving parasites. Many investigations focus on the differentiation of species when working with multiplex PCR because many species have morphologic and symptomatic similarities that classic diagnostic procedures would not be feasible, or singleplex PCRs too time consuming. The use of multiplex PCR has greatly enhanced the specificity of molecular analyses as well as providing a more sensitive and less time consuming technique.

This analysis has been able to enhance the identification of species by increasing the number of targets analyzed in a single reaction. By applying this methodology and using minisatellite repeats from *Schistosoma mansoni* mitochondria DNA (mtDNA) for multiple targets, this parasite was found to be the cause of infection in one intermediate host, *Biomphalaria glabrata* (Jannotti-Passos et al. 1997). Multiplex PCR for the identification of various strains of a single species has been used to identify *P. falciparum* (Sidhu and Madhubala 2000) or to confirm that dual drug-resistant clones carried both episomal drug-resistant marker plasmids from *T. cruzi* lineages I and II, revealing a genetic exchange (Stothard et al. 1999). Other researchers have taken this approach further and used multiple species targets.

Differentiation between genus and species has increased the specificity of detection. Using targets designed specifically for the genus, and others designed to be species-specific would allow for production of an amplification product of at least one target if the genus is present. This technique has been applied to a variety of clinical samples in order to detect *Leishmania braziliensis* complex (Belli et al. 1998). The use of a multiple competitive real time PCR was used to detect either *Plasmodium* or *P. falciparum* with a diagnosis in two hours (Fabre et al. 2004). However, more common is the differentiation and identification between species of the same genus.

The spliced leader (mini-exon) gene repeats was targeted in a multiplex PCR for differentiation of the three New World *Leishmania* complexes (*L. braziliensis*, *L. mexicana*, *L. donovani*) (Harris et al. 1998). Due to the morphological similarities and overlapping geographical and host distributions *T. rangeli* and *T. cruzi* I and II, differentiation via multiplex PCR is common (Souto et al. 1999; Fernandes et al. 2001). Utilizing a multiplex real time PCR, species-specific probes were created for the causative agents of human malaria: *P. falciparum*, *P.*

vivax, *P. malariae* and *P. ovale* (Rougemont et al. 2004). Differential diagnosis between *P. falciparum* and *P. vivax* from blood has been studied on many occasions (Zaman et al. 2001; Kho et al. 2003; Patsoula et al. 2003), as well as between all four human malaria species (Rubio et al. 1999; Patsoula et al. 2003), whether in single or mixed infections. Even the differentiation between intermediate hosts has begun, such as in the creation of distinct profile for *B. glabrata*, *B. tenagophila* and *B. straminea*, intermediate hosts of *S. mansoni* (Jannotti-Passos et al. 1997). Therefore, due to the rapid nature of this detection system, able to increase the sensitivity and specificity as well as the use of multiple target analysis in one reaction, multiplex PCR has begun to modernize the world of molecular analyses.

Parasites and aDNA Analyses

The absence of evidence of a pathogen in any given human remains is not evidence of its absence (Herrmann and Hummel 1998). The vast majority of infectious diseases in ancient populations cannot be diagnosed directly because they are undetectable: most of them are not bone-seeking disease, and even bone-seeking infectious disease cause characteristic tissue alterations only after a more or less long-term chronic infection (Herrmann and Hummel 1998). Due to the nature of parasitic diseases, we usually look for evidence of the disease in the past which almost exclusively comes from two kinds of source material: (i) historical material: written sources, tools for medical treatment, pharmacological materials indicating the presence of diseases (sometimes even specific diseases); and (ii) biological materials that are the substrates for diseases: remains of humans or other organisms bearing signs of diseases, sometimes enabling specific diagnosis (Herrmann and Hummel 1998). Once all preliminary factors have been investigated, following through with aDNA analysis is the next step.

PCR-based amplification of specific DNA fragments are used to amplify ancient DNA (aDNA) residues in human remains to provide evidence for a presumed infectious disease (Zink et al. 2002). Success with this technique came in the 1990s on a 1000-year-old South American mummy (Salo et al. 1994), and an Egyptian mummy from the New Kingdom (1550-1080 BC) (Nerlich et al. 1997), in which *Mycobacterium tuberculosis* DNA was detected. Several other microbes have been detected in the following years widening the diagnostic ability, such as *Mycobacterium leprae* (Rafi et al. 1994), *Yersinia pestis* (Drancourt et al. 1998), *Plasmodium falciparum* (Taylor et al. 1997), *Trypanosoma cruzi* (Guhl et al. 1999), *Treponema pallidum* (Kolman et al. 1999), *Escherichia coli* (Fricker et al. 1997), *Ascaris* spp. (Loreille et al. 2001) and *Corynebacterium* spp (Zink et al. 2001), which were mostly based on single findings or on small series of selected samples (Zink et al. 2002).

Plasmodium falciparum was identified as the causative agent of disease when a semi-nested PCR developed for identifying *Plasmodium* species nucleic acid was applied to two human rib bones from separate individuals who had died almost 60 years previously with anemia thought to be due to malaria (Taylor et al. 1997). A hemi-nested PCR, employing primers designed to anneal to part of the 18S ribosomal RNA (rRNA) genes of the four *Plasmodium* species, which infect humans, was used to amplify successfully malarial DNA from a rib bone from one individual who had died in 1937 with anemia believed to be caused by malaria, and *P. falciparum* was confirmed as the pathogen by sequencing the PCR product and obtaining a 98% sequence identity (Sallares and Gomzi 2001).

A PCR-based identification system amplifying a fragment of kinetoplast DNA from *T. cruzi* was used to identify the species from spontaneously desiccated mummified tissue from the coastal areas of the Atacama desert of northern Chile (Guhl et al. 1999) and from coastal and low

valley sites in northern Chile and southern Peru (Aufderheide et al. 2004). Aufderheide and colleagues (2004) further analyzed the amplified PCR products by reacting (hybridizing) them with a species-specific probe to discover a 41% positive identification. PCR amplification of the conserved region of the minicircle molecule found four of six mummified abdominal/chest cavity tissue samples positive for *T. cruzi* (Ferreira et al. 2000). The amplified products corresponding to genetic fragments of the parasite were then tested by hybridization experiments, with positive results for *T. cruzi* specific molecular probe (Ferreira et al. 2000).

To date, only one study has been published regarding the identification of *Ascaris* from archaeological remains. Coprolites mixed with soil organic matter were recovered from a 15th century latrine from the Middle-Age site of 'Place d'Armes' in Namur (Belgium) (Loreille et al. 2001). The parasitic eggs discovered in the remains were morphologically identified as *Ascaris* sp. (Loreille et al. 2001), however, PCR amplification would further confirm this identification. Thus, PCR was used to amplify and sequence fragments of the cytochrome b and 18S rRNA genes in the 600-year-old *Ascaris* eggs (Loreille et al. 2001). It was found that the 18S rRNA sequence was identical to that of *A. suum*, but the cytochrome b sequence exhibited two and three substitutions when compared to *A. lumbricoides* and *A. suum* respectively (Loreille et al. 2001). Therefore, it was difficult to identify the species present because there was no archaeological evidence to favour one of these identifications (Loreille et al. 2001).

When working with parasites and disease, it must be remembered that it is known that many infections are asymptomatic, and even when death does occur, it is frequently caused not by the agent alone, but by the synergistic interactions with other diseases (Sallares and Gomzi 2001). Thus, it would not be surprising if it turned out to be impossible in practice to detect many cases of disease in the past using even the most sophisticated biomolecular techniques

which could be devised (Sallares and Gomzi 2001). Thus the preservation of the specimen and the parasites must be taken into consideration when working with PCR and ancient material. Parasites utilize their human host for growth and sometimes reproduction, but if that host dies, the parasite dies along with it. There have been few evolutionary defence mechanisms evolved by the parasite to avoid death once host death has occurred, such as in bacteria that initiate the formation of a cyst when the environment is not suitable for survival. Therefore, the majority of parasites will succumb to DNA degradation similar to that of its human host. Thus PCR methods must take into consideration the degradation of the parasitic DNA, therefore a minimum of three criteria for malaria detection methods have been imposed: high sensitivity, rapidity, and simplicity (Tirasophon et al. 1994).

CHAPTER 4: MOLECULAR DETECTION AND IDENTIFICATION OF PARASITES INVOLVED IN HUMAN DISEASE: METHODOLOGICAL OPTIMIZATION

The field of molecular research involving the use of nucleic acids and the PCR technique has expanded throughout the decades. An increase of information available to researchers has provided a basis of study involving many aspects of parasitic disease diagnoses, parasite identification and an understanding of the factors involved when these parasites interact with humans today. Therefore, the following study explores the basis of molecular analyses of the six parasites: (1) *Plasmodium falciparum*, (2) *Schistosoma haematobium*, (3) *Leishmania donovani*, (4) *Trypanosoma brucei*, (5) *Trypanosoma cruzi* and (6) *Ascaris lumbricoides*, via PCR and nucleic acid research.

Objectives

1. To design and develop methods of detection and identification that are highly specific and highly sensitive for each parasite involved in disease by utilizing the PCR technique and nucleic acid based technology in three steps:

   STEP I: parasite identification via PCR

   STEP II: parasite identification via multiplex PCR

   STEP III: multiple parasite identification via multiplex PCR.

2. Application of the technique to degraded DNA samples to study the presence of disease and the causative species:

   Degraded DNA Analysis I: parasite identification in medical archived samples

   Degraded DNA Analysis II: parasite identification in archaeological samples.

Institute of Study

This study was conducted at the Paleo-DNA Laboratory on campus at Lakehead University in Thunder Bay, Ontario, Canada.

Materials and Methods

Primer Design

Genes within the genome of each species were chosen as targets to design sets of species-specific oligonucleotide primers on the basis of literature research and stability and function within the genome using the sequence of each species as templates (Table 1). Initial design began with locating a suitable sequence of bases within an 18 to 30 base-length and coupling with a second sequence to produce an amplicon size of no greater than 300 bp. A standard nucleotide-nucleotide BLAST (blastn) (Altschul et al. 1997) search was performed on each individual primer to verify the species-specific origin of the sequence of the sequence in question, followed by the determination of disassociation temperature using QIAGEN's Oligos Toolkit (http://oligos.quiagen.com/oligos/toolkit.php). Amplicon analysis continued with a computer simulated PCR program (Amplify version 1.2 for Mac software) to determine the primability and stability of match, and to visualize the sensitivity and specificity of match of each amplicon.

General Laboratory Procedures

Certain precautions were taken to guard against contamination of the sample with extraneous DNA: (i) double gloves were worn (frequently changed when moving stations); (ii) PCR preparation and analysis was performed in three separate areas: one area for PCR reagent set-up (hood), one area for PCR itself, and one area for PCR analysis; (iii) sterile disposable laboratory instruments were utilized whenever possible, as well as positive displacement pipettes and barrier-tipped pipette tips, with each area containing its own set of equipment that were not interchanged; (iv) all areas prior to and following use were thoroughly cleaned using 10% sodium hypochlorite, ddH_2O, 70% ethanol, and hoods were UV irradiated for at least 30

Table 1. Sequences of primers used for each species.

Parasite: *Plasmodium falciparum*							
Primer	Sequence 5′ to 3′	Genome Position	bp	T_m (°C)	Source	NCBI Accession	Nucleotide Position
DHFR-TS3	AAT GAT GAC AAA GAT ACA TG	DHFR-TS	280	52.2	(Arai et al. 1994)	J04643	757-776
DHFR-TS2	TTC GAT CAC TTT GTT TAT TTC CAT T	DHFR-TS		56.4	(Arai et al. 1994)		1036-1012
KI-14-P2	CGT GTA CCA TAC ATC CTA CCA AC	pBRK1-14 clone	206	63.5	(Jelinek et al. 1996)	AF003109	538-560
KI-14-P1	GCT ACA TAT GCT AGT TGC CAG AC	pBRK1-14 clone		63.5	(Jelinek et al. 1996)		742-720
MSA-1B	GAG TTC TTT AAT AGT GAA CAA G	MSA-1	100	55.9	(Laserson et al. 1994)	M55001	301-282
MSA-1D	GGT GCT GCA AAT CCT TCA GAT GAT T	MSA-1		62.9	THIS STUDY		202–226
MSA-2C	GTT CCA CCC ACT CAA GAT GC	MSA-2	131	62.4	THIS STUDY	L19052	472–491
MSA-2B	CCT GTA CCT TTA TTC TCT GG	MSA-2		58.3	(Laserson et al. 1994)		602–583
Parasite: *Schistosoma haematobium*							
Primer	Sequence 5′ to 3′	Genome Position	Bp	Tm (°C)	Source	NCBI Accession	Nucleotide Position
SH2F	ACG GAA AAC AAA GCG CAC CAC	Ribosomal IGS	85	63.3	THIS STUDY	AJ223838	1083–1103
SH2R	CTT TAC ACA TGG TCT TAC TCG CC	Ribosomal IGS		63.5	THIS STUDY		1167–1145
SH3F	TAG TTT CTA ACC GAG ATG TCA AG	*nad6* (modified)	121	59.9	(Kane et al. 2003)	AJ271051	2191-2213
SH3R	CCT AAA CCT GAC CCA TAC TTA TC	Partial mito. genome		61.7	THIS STUDY		2311-2289
SH5F	GAT TTT TTG GTC ATC CAG AGG TG	Cytochrome C oxidase	161	61.7	THIS STUDY	AY157209	608-630
SH5R	GGC TCA AAC TAC ACT TCC TAA G	Cytochrome C oxidase		61.5	THIS STUDY		768-747
SH6F	GCA CTT AGA GAG GCA CAA CTC	Egg shell protein	128	63.3	THIS STUDY	M27659	150-170
SH6R	GAG CGA CGA TCT CAT TTT TCC	Egg shell protein		61.4	THIS STUDY		278-258
Parasite: *Leishmania donovani*							
Primer	Sequence 5′ to 3′	Genome Position	Bp	Tm (°C)	Source	NCBI Accession	Nucleotide Position
LD1F	TGC GTG CGT GTG TGT GAC ATC	IMP-D	116	65.3	THIS STUDY	M55667	382-402
LD1R	TAC GTC AGT CCA TCG CCC CG	IMP-D		66.5	THIS STUDY		497-478
LD2F	AGA CAA GCT GGG TCG TAA CTC	AAP13LD	129	63.3	THIS STUDY	AY453650	201-221
LD2R	AAG ACG ACG AGG TAG CCA ATA G	AAP13LD		63.4	THIS STUDY		329-308
LD3F	GGA GTA GCC TCA GGA CTT TAG	Kinetoplast minicircle	182	63.7	(Salotra et al. 2001)	Y11401	541-561
LD3R	TAG GTA CAC TCT ATC AGT AGC AC	Kinetoplast minicircle		61.7	THIS STUDY		722-700
LD4F	GCT GGA AGT GAA GGT TGT CTG C	Adenylate kinase	218	65.2	THIS STUDY	AF156853	189-210
LD4R	GAT AGA TGC GTC CCG ACT TGG	Adenylate kinase		65.3	THIS STUDY		406-386
Parasite: *Trypanosoma brucei*							
Primer	Sequence 5′ to 3′	Genome Position	Bp	Tm (°C)	Source	NCBI Accession	Nucleotide Position
TB3F	TCG GGC AGG ATT TTC GTA TGG	Aspartate aminotransferase	157	62.5	THIS STUDY	AF326990	29-49
TB3R	CTT GCA ATT CCT GTG ACT GGG	Aspartate aminotransferase		62.5	THIS STUDY		185-165
TB7F	TCA GGT TGA GAA GAT TGG CGA AG	P-type ATPase gene	124	62.7	THIS STUDY	M73769 M37894	1698-1720
TB7R	TTT CCA TTT CCC CTC GCA AAG TG	P-type ATPase		62.7	THIS		1821-1799

		gene			STUDY		
21F	TTT AGC GGC GGT CAC TTT TCA C	HSP70 gene	108	63.4	THIS STUDY	M32140	1991-2012
21R	CCT CGT CAT GTC TAT TAT CCT CTT G	HSP70 gene		62.9	THIS STUDY		2098-2074
22F	CTA AAC GTC TCG GCG ACT TCA AC	Basic copy gene of surface glycoprotein	196	65.2	THIS STUDY	V01384 J01232	1081-1103
22R	GAG CGA GCT TGA GGT CCT TTA G	Basic copy gene of surface glycoprotein		65.2	THIS STUDY		1276-1255

Parasite: *Trypanosoma cruzi*

Primer	Sequence 5′ to 3′	Genome Position	Bp	Tm (°C)	Source	NCBI Accession	Nucleotide Position
TC1F	AAC GAG GTG CTT ATG AAA CCG TG	HSP70 gene	143	62.7	THIS STUDY	M73627	109-131
TC1R	AGA GGA GCC ATC ACC ATG ATT TC	HSP70 gene		62.7	THIS STUDY		251-229
TC3F	GGA AGT AAC TTG AAG ATT GGG ATT G	GTP binding protein gene	154	61.3	THIS STUDY	AY178827	86-110
TC3R	ATC GAT CGT CAG GAA TGT TAA TGT C	GTP binding protein gene		61.3	THIS STUDY		239-215
TC5F	TTC ACT GAA AAA CTC ACA GCC CTT G	Structural maintenance of chromo. Protein 1 mRNA	124	62.9	THIS STUDY	AY528744	781-805
TC5R	CTT TGC GGA TCT TTT TAA GTT CCT C	Structural maintenance of chromo. Protein 1 mRNA		61.3	THIS STUDY		904-880
TC14F	CTT CTT CCA ACA TGA AGA GGG TG	MCAR-1 gene	172	62.7	THIS STUDY	AJ704363	992-1014
TC14R	ATC TTC TCA TTC CAT ACC CTC GG	MCAR-1 gene		62.7	THIS STUDY		1163-1141

Parasite: *Ascaris lumbricoides*

Primer	Sequence 5′ to 3′	Genome Position	Bp	Tm (°C)	Source	NCBI Accession	Nucleotide Position
ASFB	GGT TAT ATG CTT ATC TCA AAG GC	18S ssrRNA gene	246	59.2	THIS STUDY	U94366	1-23
ASRB	CAG CTA TAG TTA TTC AGA GTC ACC	18S ssrRNA gene		61.1	(Loreille et al. 2001)		247-224
ASFE	GCT GCG TAC GGC ACT GCG AAA	Trans-spliced mRNA encoded protein gene 12	120	66.4	(Anderson and Jaenike 1997)	M33757	1091-1111
ASRE	GAA TAA CGG GAA ACA CTG ACC G	Trans-spliced mRNA encoded protein gene 12		62.6	THIS STUDY		1210-1189
AL2F	GTT TTC AGA GGT GCA CAA GTT C	rDNA w 18S rRNA gene 5' end	228	60.8	THIS STUDY	X05836	1813-1834
AL2R	CTA GAA TTA CCA CAG TTA TCC ACG	rDNA w 18S rRNA gene 5' end		61.1	THIS STUDY		2040-2017
AL3F	GAT GCT AGC GAC GGG AAA TGA TTG	Spliced leader & 5S rRNA	124	64.5	THIS STUDY	M27961	451-474
AL3R	TAG AAG CAC TGA GCT GAA ACA CGG	Spliced leader & 5S rRNA		64.5	THIS STUDY		574-551

minutes; (v) extraction and reagent blanks (negative controls) were simultaneously run alongside with the detection PCR.

Samples

Species specific samples were required for each parasite either in the form of DNA or a preserved specimen (Biosafety Level 1 or 2). DNA samples specific for *P. falciparum* and *T. cruzi* were commercially obtained from the American Type Culture Collection (ATCC). DNA samples for *S. haematobium* was donated by Dr. Fred Lewis and DNA samples for *L. donovani* and *T. brucei* were donated by Dr. Marc Ouellette. Due to the nature of the samples received, no extraction or purification steps were required for DNA isolation. A preserved worm specimen, morphologically identified as *A. lumbricoides*, was donated by Dr. Don Martin. This specimen required extraction and purification of the tissue for DNA isolation.

***Ascaris lumbricoides* Extraction/Purification**

Sample preparation began with a sectioning of the worm tissue into small fragments, approximately 1.0 centimetre in length. DNA extraction and purification of the tissue sections commenced with a proteinase K (pK) extraction followed by the phenol:chlorofom separation and ethanol precipitation purification methods.

PCR Optimization and Detection

To facilitate PCR optimization, a positive control of DNA from each species was analyzed via PCR involving a range of annealing temperatures in order to generate an optimal annealing temperature for primer and reaction specificity. Further analysis included the use of a diluted DNA template in order to test primer sensitivity (up to 10 000-fold) and specificity. Once parameters had been finalized, a 25.0μL reaction volume for PCR containing 1x PCR buffer, 1mM magnesium chloride ($MgCl_2$), 0.5mM of each dNTP, 0.5 units of *Taq* Polymerase,

optimized concentrations of each forward and reverse primer and 10.0μL of DNA template was run to receive the final amplified target of interest. The reactions were carried with the following program: initial denaturation of two minutes at 94°C followed by 45 cycles of denaturation, primer annealing and primer extension at 94°C for one minute, 60°C for one minute and 72°C for two minutes, respectively. Following amplification, PCR products were analyzed by 6% polyacrylamide gel electrophoresis (PAGE) using a 100bp molecular marker (2.0μL) (Fermentas) for size approximation, loaded with 5.0μL of PCR product and 3.0μL of 6X loading dye [2.5% xylene cyanol (12.0μL), 2.5% bromophenol blue (12.0μL), 35% Ficoll (432.0μL) and 544.0μL of ddH_2O and a 15 to 20 minute staining period in ethidium bromide (EtBr) solution (1/20 dilution using 5.0μL of EtBr stock solution by STRATAGENE ®) and photographed.

Sequencing

Sequencing of the PCR products confirmed the identification of the species analyzed. It occurred in a reaction of varying cycles (dependent on the intensity of the amplified target) of denaturation, annealing and extension at 96°C for 30 seconds, 50°C for 15 seconds and 72°C for four minutes respectively, employing the ABI Prism® 310 or 3100 Genetic Analysis instrument. A gene fragment comparison utilizing the sequence product and the sequenced gene fragment and the corresponding published gene was carried out by means of BioEdit Sequence Alignment Editor.

Cloning

An alternative technique to confirm the species-specific results of DNA amplification was used for the PCR products with incompatible primers for direct sequencing. Cloning of the PCR products was employed as a suitable option. DNA amplification of the products that required cloning utilized the same cycling parameters as outlined above, however a final

extension at 72°C for 10 minutes was added to ensure that all PCR products are full length and 3' adenylated. PCR products were cloned using T4 DNA ligase to insert the DNA fragment of interest into a pure linear pCR®4-TOPO® vector. The ligated vector was introduced into competent *Escherichia coli* TOP10 cells and grown overnight on Laria-Bertani (LB) Agar containing 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) and isopropyl-β-D-thiogalactopryranoside (IPTG) to identify successful pCR®4-TOPO® insertion. The resulting glossy, white colonies were plucked and cultured overnight in LB broth. The isolation and purification of the resulting DNA sequence was accomplished with the QIAprep plasmid purification system and amplified via PCR with sequence specific primers. Sequencing of PCR products occurred in a reaction of 40 cycles of 94°C for 30 seconds, 50°C for 15 seconds and 72°C for four minutes employing the ABI Prism® 310 or 3100 Genetic Analysis instrument.

STEP I: PCR-BASED DETECTION AND IDENTIFICATION OF PARASITES INVOLVED IN HUMAN DISEASE

Aim

The aim of this section of study is to design and optimize a PCR-based detection and identification system for six parasites involved in human disease.

Parasites Studied

1. *Plasmodium falciparum*
2. *Schistosoma haematobium*
3. *Leishmania donovani*
4. *Trypanosoma brucei*
5. *Trypanosoma cruzi*
6. *Ascaris lumbricoides*

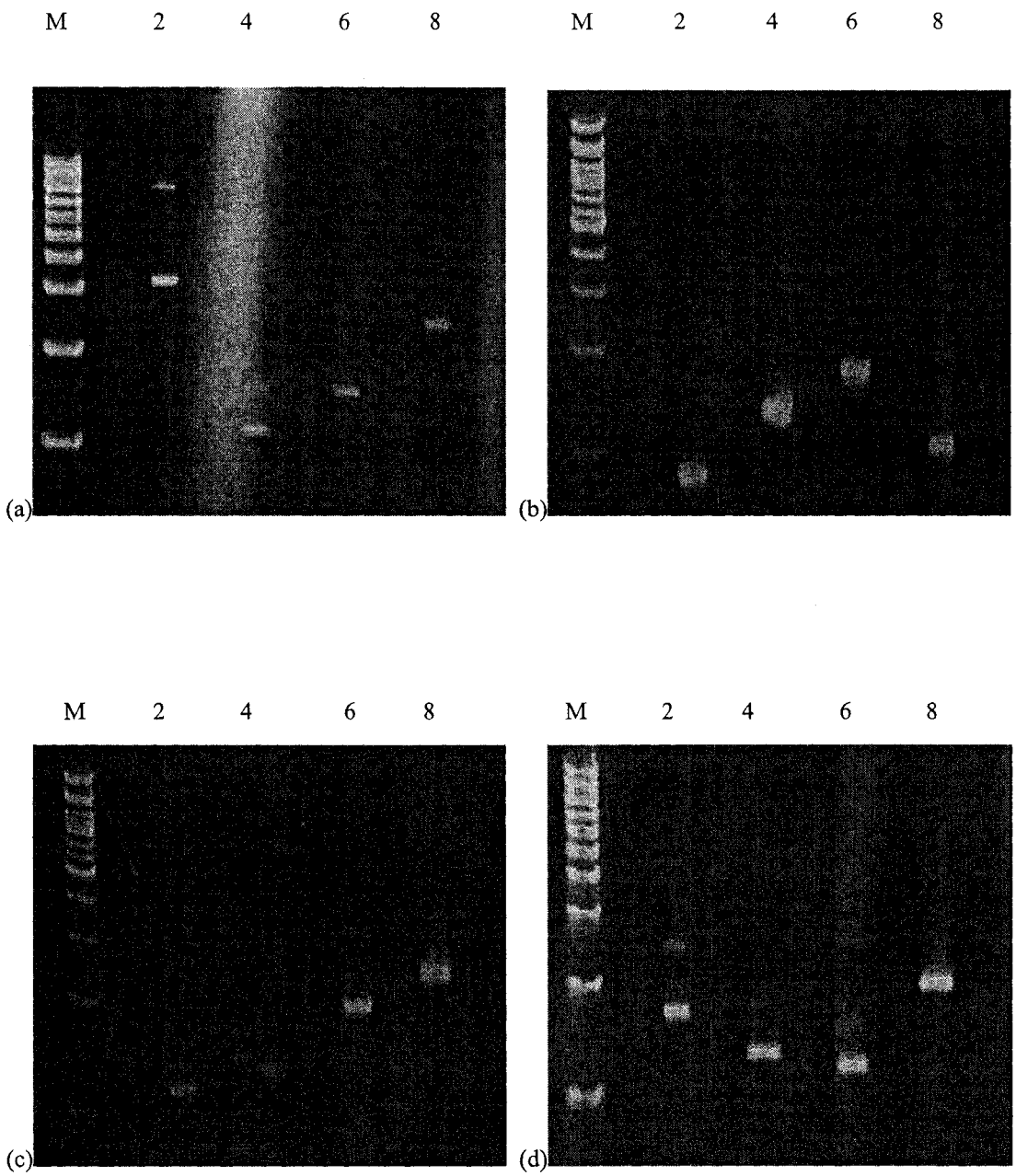
PCR Optimization

PCR parameters for each primer set were previously determined (page 60). Throughout this process, a standard primer concentration of 0.20μM for each primer in the singleplex PCR was used. A 1000-fold sensitivity of the DNA template was applied to the reaction.

Results

All positive controls were subjected to PCR using each primer set along with PCR negatives (no DNA) to test the quality of the PCR reagents and examine for contamination. The resulting PCR products were then electrophoresed on 6% polyacrylamide gel and visualized (Figure 9).

Each amplified DNA product corresponded to the expected amplicon size previously determined for each primer set and all PCR negatives were devoid of DNA. Sequencing of the



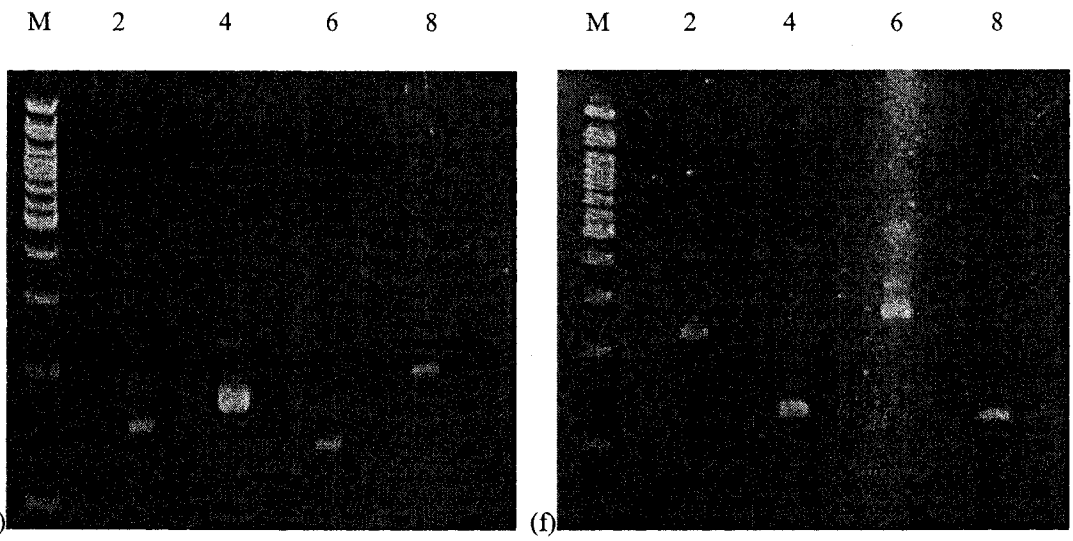


Figure 9. 6% PAGE images of individual parasite singleplex PCR results. Image schematics as follows: M represents 100bp molecular marker; (a) *Plasmodium falciparum*: lanes 2, 4, 6, 8 represent reaction involving primer sets DHFT-TS3/DHFR-TS3, MSA-1D/MSA-1B, MSA-2C/MSA-2B and K1-14-P2/K1-14-P1 respectively; lanes 3,5, 7, 9 represent corresponding PCR negatives; (b) *Schistosoma haematobium*: lanes 2, 4, 6, 8 represent reaction involving primer sets SH2F/SH2R, SH3F/SH3R, SH5F/SH5R and SH6F/SH6R respectively; lanes 3, 5, 7, 9 represent corresponding PCR negatives; (c) *Leishmania donovani*: lanes 2, 4, 6, 8 represent reaction involving primer sets LD1F/LD1R, LD2F/LD2R, LD3F/LD3R and LD4F/LD4R respectively; lanes 3, 5, 7, 9 represent corresponding PCR negatives; (d) *Trypanosoma brucei*: lanes 2, 4, 6, 8 represent reaction involving primer sets TB3F/TB3R, TB7F/TB7R, 21F/21R and 22F/22R respectively; lanes 3, 5, 7, 9 represent corresponding PCR negatives; (e) *Trypanosoma cruzi*: lanes 2, 4, 6, 8 represent reaction involving primer sets TC1F/TC1R, TC3F/TC3R, TC5F/TC5R and TC14F/TC14R respectively; lanes 3, 5, 7, 9 represent corresponding PCR negatives; (f) *Ascaris lumbricoides*: lanes 2, 4, 6, 8 represent reaction involving primer sets AL2F/AL2R, AL3F/AL3R, ASFB/ASRB and ASFE/ASRE respectively; lanes 3, 5, 7, 9 represent corresponding PCR negatives.

PCR products followed, employing the parameters previously outlined. A gene fragment comparison utilizing the sequenced gene fragment and the corresponding published gene was carried out by means of BioEdit Sequence Alignment Editor (Figures 10 to 15). Comparing the two sequences, certain polymorphisms were detected, either as base insertions, base deletions or base transitions. These have been outlined in Table 2 along with the percent match of the experimentally attained sequence to the published gene fragment. Since the electropherogram output reads each base according to the intensity of the signal produced, some base transition polymorphisms had a second less intense base reading at the same position and these bases have also been outlined. With the inclusion of these anomalies in the sequence output, the percent match between the experimentally attained sequence and the published sequence is increased. Overall, the resultant sequences in each analysis confirmed the identification of each species within the amplified PCR products. Only one of the sequences did not produce a species-specific identification. This occurred with the SH2F sequence, which identified *S. haematobium* as well as *S. intercalatum*. Since these two species have very similar gene sequences, the experimentally attained sequence was not able to distinguish between the two species. However, the primers designed for the analysis are species-specific.

```
DHFR-TS GENE TAACAGAATT TTACAAAAAT GTAGACAAAT ATAAAATTAA TTATGAAAAT GATGATGATG
DHFR-TS3     -----GAATT TTACAAAAAT GTAGACAAAT ATCAAATTAA TTATGAAAAT GATGATGATG

DHFR-TS GENE ATGAAGAAGA AGATGATTTT GTTTATTTTA ATTTTAATAA AGAAAAAGAA GAGAAAAATA
DHFR-TS3     ATGAAGAAGA AGATGATTTT GTTTATTTTA ATTTTAATAA AGAAAAAGAA GAGAAAAATA

DHFR-TS GENE AAAATTCTAT ACATCCAAAT GATTTTCAAA TATATAATAG CTTGAAATAT AAATATCATC
DHFR-TS3     AAAATTCTAT ACATCCAAAT GATTTTCAAA TATATAATAG CTTGAAATAT AAATATCATC

DHFR-TS GENE CTGAATACCA ATATTTAAAT ATTATTTATG ATATTATGAT GAATGGAAAT AAACAAAGTG
DHFR-TS3     CTGAATACCA ATATTTAAAT ATTATTTATG ATATTATGAT GAATGGAAAT AAACAAAGTG

DHFR-TS GENE ATCGAACG.. .......... .......... .......... .......... ..........
DHFR-TS3     ATCGAA--.. .......... .......... .......... .......... ..........


pBRK1-14     CGTGTACCAT ACATCCTACC AACATCGAAA AATACCAAAT ACATCACATC TATTCTTTCA
*K1-14-P1    ---GTACCAT ACATCCTACC AACATCGAAA AATACCAAAT ACATCACATC TATTCTTTCA

pBRK1-14     ATAATTTTAT ATATTGGAGT TTTTTCTTCA TTTTTTTTTT TCTTCGATAA CGTAAAATCA
*K1-14-P1    ATAATTTTAT ATATTGGAGT TTTTTCTTCA TTTTTTTTTT TCTTCGATAA CGTAAAATCA

pBRK1-14     AATAAATAAT TAACATAACT AAAGCTATAA CCACTATTGC AACGATAGA. ..........
*K1-14-P1    AATAAATAAT TAACATAACT AAAGCTATAA CCACTATTGC AACGATAGA. ..........


MSA-1 GENE   CAGATGCTAA ATCTTACGCT GATTTAAAAC ATAGAGTTCA AAATTACTTG TTCACTATTA
MSA-1D       CAGATGCTAA ATCTTACGCT GATTTAAAAC ATAGAGTTCA AAATTACTTG TTCACTATTA

MSA-1 GENE   AAG....... .......... .......... .......... .......... ..........
MSA-1D       AAG....... .......... .......... .......... .......... ..........


MSA-2 GENE   ACAAGCTGAA AATTCTGCTC CAACAGCCGA ACAAACTGAA TCCCCCGAAT TACAATCTGC
MSA-2C       ACAAGCTGAA AATTCTGCTC CAACAGCCGA ACAAACTGAA TCCCCCGAAT TACAATCTGC

MSA-2 GENE   ACCAGAGAAT AAAGGTACAG GA........ .......... .......... ..........
MSA-2C       ACCAGAGAAT AAAGGTACAG GA........ .......... .......... ..........
```

Figure 10. Sequence alignment of the four genes analyzed in *Plasmodium falciparum*. Alignment illustrates the published gene fragment sequence followed by the experimentally attained sequence of the corresponding primer. * represents the reverse complement of the sequence attained. Sequence polymorphisms highlighted in red.

```
SH2 GENE    CGAAGTTAGG GGTAGTCTCC CTATATAACA TGGCGAGTAA GACCATGTGT AAAG......
SH2         CGAAGTTAGG GGTAGTCTCC CTATATAACA TGGCGAGTAA GACCATGTGT AAAG......

SH3 GENE    ATTTAGTGGT AGTATGATTT TAATTAATAA AATTAATTAA ATGTATATAG GATGCCAGAT
SH3F        ATTTAGTGGT AGTATGATTT TAATTAATAA AATTAATTAA ATGTATATAG GATGCCAGAT

SH3 GENE    AAGTATGGGT CAGGTTTAGG .......... .......... .......... ..........
SH3F        AAGTATGGGT CAGGTTTAGG .......... .......... .......... ..........

SH5 GENE    CCTGGATTTG GAATAGTTAG TCATATATGT ATGAGGATAA GTAATAATGA TTCATCGTTT
SH5F        CCTGGATTTG GAATAGTTAG TCATATATGT ATGAGGATAA GTAATAATGA TTCATCATTT

SH5 GENE    GGGTATTATG GATTGATTTG TGCTATGGCT TCGATAGTTT GCTTAGGAAG TGTAGTTTGA
SH5F        GGGTATTATG GATTGATTTG TGCTATGGCT TCGATAGTTT GCTTAGGAAG TGTAGTTTGA

SH5 GENE    GCC..... .......... .......... .......... .......... ..........
SH5F        GCC..... .......... .......... .......... .......... ..........

SH6 GENE    TGCACTTAGA GAGGCACAAC TCTTCCAATC CATAATCAAA AACAATATAT AAATGACAAA
SH6F        ---------- ---------- ---------- -----TCAAA AACAAGATAT AAATGACAAA
*SH6R       TGCACTTAGA GAGGCACAAC TCTTCCAATC CATAATCAAA AACAATATAT AAATGACAAA

SH6 GENE    TCACACTAGT TTACATATCA TCACACCGAA TACAACAACA CAGAC..... ..........
SH6F        TCACACTAGG TTACATATCA TCACACCGAA TACAACAACA CAGAC..... ..........
*SH6R       TCACACTAGT TTACATATCA CCACACCGAA TACAACAACA CAGAC..... ..........
```

Figure 11. Sequence alignment of the four genes analyzed in *Schistosoma haematobium*. Alignment illustrates the published gene fragment sequence followed by the experimentally attained sequence of the corresponding primer. * represents the reverse complement of the sequence attained. Sequence polymorphisms highlighted in red.

Figure 12. Sequence alignment of the four genes analyzed in *Leishmania donovani.* Alignment illustrates the published gene fragment sequence followed by the experimentally attained sequence of the corresponding primer. Sequence polymorphisms highlighted in red.

```
TB3 GENE      GGAAGGTGAA TTTGTCTATT GGTGTGTACC GCGACGACGC CGACCAGCCT TTTGTGCTGG
TB3F          GGAAGGTGAA TTTGTCTATT GGTGTGTACC GCGACGACGC CGACCAGCCT TTTGTGCTGG

TB3 GENE      AATGTGTGAA ACAAGCCACA TTAGGCACCA ACATGGACTA CGCCCCAGTC ACAGGAATTG
TB3F          AATGTGTGAA ACAAGCCACA TTAGGCACCA ACATGGACTA CGCCCCAGTC ACAGGAATTG

TB3 GENE      CAAG...... .......... .......... .......... .......... ..........
TB3F          CAAG...... .......... .......... .......... .......... ..........


TB7 GENE      CCTTTTGGTA ATGAGTGAGA AATTCGCAAA TATAAAAGGT GACTCTGCGG TAAACGCCTT
TB7F          CCTTTTGGTA ATGAGTGAGA AATTCGCAAA TATAAAAGGT GACTCTGCGG TAAACGCCTT

TB7 GENE      CCGTACACTT TGCGAGGGGA AATGGAAAAA .......... .......... ..........
TB7F          CCGTACACTT TGCGAGGGGA AATGGAAAAA .......... .......... ..........


21 GENE       TAGAAAAATA GGCAACGGAA GAACAAAGTA AGGCGAA-CG ACGGGAGAAC GACGAAAAAA
*21R          TAGAAAAATA GGCAACGGAA GAACAAAGTA AGGCGAAGCG ACGGGAGAAC GACGAAAAAA

21 GENE       ACAAGAGGAT AATAGACATG ACGAGGAA.. .......... .......... ..........
*21R          ACAAGAGGAT AATAGACATG ACGAGGAA.. .......... .......... ..........


22 GENE       CAAGACAACC GAATGCAACA CAGAGTCACC GGAAGACACA AAAGAGCCAG ATCAAACAAC
22F           CAAGACAACC GAATGCAACA CAGAGTCACC GGAAGACACA AAACAGCCAG ATCAAACAAC

22 GENE       TCTGTCCAAA AA......... .......... .......... .......... ..........
22F           TCTGTCCAAA AA......... .......... .......... .......... ..........
```

Figure 13. Sequence alignment of the four genes analyzed in *Trypanosoma brucei*. Alignment illustrates the published gene fragment sequence followed by the experimentally attained sequence of the corresponding primer. * represents the reverse complement of the sequence attained. Sequence polymorphisms highlighted in red.

```
TC1 GENE    ACCTCTTACA TGTGTCATCT TCCGTGCTTA CCTTTTTTTT CTATCTCTAC CGTAGGAAAT
TC1F        ACCTCTTACA TGTGTCATCT TCCGTGCTTA TCTTGTTTTT CTATCTCTAC CGTAGGAAAT

TC1 GENE    CATGGTGATG GCTC..... ..........  .......... .......... ..........
TC1F        CATGGTGATG GCTC..... ..........  .......... .......... ..........

TC3 GENE    AGTCCACATT TTTCAACGTT CTATCTAAAA AAGGTGTTCC CGCTGAAAAT CGGCCATTTT
TC3F        AGTCCACATT TTTCAACGTT CTATCTAAAA AAGGTGTTCC CGCTGAAAAT CGGCCATTTT

TC3 GENE    GCACCATTGA TCCCAACACC GCGGACATTA ACATTCCTGA CGATCGAT.. ..........
TC3F        GCACCATTGA TCCCAACACC GCGGACATTA ACATTCCTGA CGATCGAT.. ..........

TC5 GENE    GACGTACTTG GAGGAACTTA AAAAGATCCG CAAAGA.... ..........  ..........
TC5F        GACGTACTTG GAGGAACTTA AAAAGATCCG CAAAGA.... ..........  ..........

TC14 GENE   CTTTGCTATC CGCTGCATGT CATGCTGCGC TATGAAATTG AGCGCGATCT GATGGACGGG
TC14F       CTTTGCTATC CGCTGCATGT CATGCTGCGC TATGAAATTG AGCGCGATCT GATGGACGGG

TC14 GENE   AATATTGAAG CGGAGGAAGT ACCGAGGGTA TGGAATGAGA AGAT...... ..........
TC14F       AATATTGAAG CGGAGGAAGT ACCGAGGGTA TGGAATGAGA AGAT...... ..........
```

Figure 14. Sequence alignment of the four genes analyzed in *Trypanosoma cruzi*. Alignment illustrates the published gene fragment sequence followed by the experimentally attained sequence of the corresponding primer. Sequence polymorphisms highlighted in red.

```
AL2 GENE    AAGTGTGCCG GTTATTAACT GTGCATCTCC CCGATTGATT CTGTCGGCGG TTATATGCTT
AL2F        AAGTGTGCCG GTTATTAACT GTGCATCTCC CCGATTGATT CTGTCGGCGG TTATATGCTT

AL2 GENE    ATCTCAAAGG CTAAGC-ATG CATGTCTAAG TTCAAATGGC CTATAAAGGT GAAACCGCGA
AL2F        ATCTCAAAGG CTAAGCCATG CATGTCTAAG TTCAAATGGC CTATAAAGGT GAAACCGCGA

AL2 GENE    ACGGCTCATT ACAACAGCTA TTATATACTT GATCTTGATA TCCTACGTGG ATAACTGTGG
AL2F        ACGGCTCATT ACAACAGCTA TTATATACTT GATCTTGATA TCCTACGTGG ATAACTGTGG

AL2 GENE    TAATTCTAG. .......... .......... .......... .......... ..........
AL2F        TAATTCTAG. .......... .......... .......... .......... ..........


AL3 GENE    ACTCGTTTTT GCACTTACCG ATTGTGGGGA TGATCTCGAT GGTTTAATTA CCCAAGTTTG
AL3F        ACTCGTTTTT GCACTTACCG ATTGTGGGGA TGATCTCGAT GGTTTAATTA CCCAAGTTTG

AL3 GENE    AGGTAATTCC GTGTTTCAGC TCAGTGCTTC TA........ .......... ..........
AL3F        AGGTAATTCC GTGTTTCAGC TCAGTGCTTC TA........ .......... ..........


ASB GENE    AATGGCCTAA AAAGGTGAAA CCGCGAACGG CTCATTACAA CAGCTATTAT ATACTTGATC
ASFB        AATGGCCTAT AAAGGTGAAA CCGCGAACGG CTCATTACAA CAGCTATTAT ATACTTGATC

ASB GENE    TTGAAATCCT ACGTGGATAA CTGTGGTAAT TCTAGAGCTA ATACATGCAC CAAAGCTCCG
ASFB        TTGATATCCT ACGTGGATAA CTGTGGTAAT TCTAGAGCTA ATACATGCAC CAAAGCTCCG

ASB GENE    AATTTTTGAC GAGCGCATCT ATTAGATTAA AACCAATCGG GTTTCGGCCC GTAAATTGGT
ASFB        ATTTTCTGAC GAGCGCATCT ATTAGATTAA AACCAATCGG GTTTCGGCCC GTCAATTGGT

ASB GENE    GACTCTGAAT AACTATAGCT GA........ .......... .......... ..........
ASFB        GACTCTGAAT AACTATAGCT GA........ .......... .......... ..........


ASE GENE    CTCATCAATG GGAGTGATGA GGCCGGAGCT GATCATGAAA TCCGTTATTC CTGTCATCAT
ASFE        CTCATCAATG GGAGTGATGA GGCCGGAGCT GATCATGAAA TCCGTTATTC CTGTCATCAT

ASE GENE    GGCCGGTCAG TGTTTCCCGT TATTC..... .......... .......... ..........
ASFE        GGCCGGTCAG TGTTTCCCGT TATTC..... .......... .......... ..........
```

Figure 15. Sequence alignment of the four genes analyzed in *Ascaris lumbricoides*. Alignment illustrates the published gene fragment sequence followed by the experimentally attained sequence of the corresponding primer. Sequence polymorphisms highlighted in red.

Table 2. Resultant polymorphisms of the published gene fragment sequence compared to the experimentally attained gene fragment.

Parasite: *Plasmodium falciparum*				
Primer	Sequence % Match to Gene	Sequence Polymorphism (published to experimental)	Sequence Possibility	Base Location in Gene
DHFR-TS3	99	A to C transition	-	823
K1-14-P1	100	-	-	-
MSA-1D	100	-	-	-
MSA-2C	100	-	-	-
Parasite: *Schistosoma haematobium*				
Primer	Sequence % Match to Gene	Sequence Polymorphism (published to experimental)	Sequence Possibility	Base Location in Gene
SH2F	100	-	-	-
		-	-	-
SH3F	100	-	-	-
SH5F	99	G to A transition	G	702
SH6F	97	T to G transition	-	194, 218
SH6R	99	T to C transition	T	229
Parasite: *Leishmania donovani*				
Primer	Sequence % Match to Gene	Sequence Polymorphism (published to experimental)	Sequence Possibility	Base Location in Gene
LD1F	98	T to C transition	-	472
LD2F	100	-	-	-
LD3F	98	A to G transition	-	671
		C to T transition	-	695
LD4F	97	Unknown base	-	247
		G to A transition	-	343
		A to T transition	-	348
Parasite: *Trypanosoma brucei*				
Primer	Sequence % Match to Gene	Sequence Polymorphism (published to experimental)	Sequence Possibility	Base Location in Gene
TB3F	100	-	-	-
TB7F	100	-	-	-
21R	98	G insertion	-	2050.1
22F	98	G to C transition	G	1219
Parasite: *Trypanosoma cruzi*				
Primer	Sequence % Match to Gene	Sequence Polymorphism (published to experimental)	Sequence Possibility	Base Location in Gene
TC1F	97	C to T transition	-	204
		T to G transition	-	208
TC3F	100	-	-	-
TC5F	100	-	-	-
TC14F	100	-	-	-
Parasite: *Ascaris lumbricoides*				
Primer	Sequence % Match to Gene	Sequence Polymorphism (published to experimental)	Sequence Possibility	Base Location in Gene
AL2F	99	C insertion	-	1928.1
AL3F	100	G to C transition	-	493
ASFB	98	A to T transition	-	56, 111
ASFE	100			

STEP II: MULTIPLEX PCR-BASED DETECTION AND IDENTIFICATION OF PARASITES INVOLVED IN HUMAN DISEASE

Aim

The aim of this section of study is to design and optimize a multiplex PCR-based detection and identification system for five parasites involved in human disease.

Parasites Studied

1. *Plasmodium falciparum*
2. *Schistosoma haematobium*
3. *Leishmania donovani*
4. *Trypanosoma brucei*
5. *Trypanosoma cruzi*

PCR Optimization

Since primer optimization and PCR optimization of individual species primer sets was carried out, the PCR optimization for this section of study involved multiple trials of PCR runs to facilitate a reaction for each species involving the use of multiple amplicons in one reaction. A 25.0μL reaction volume for PCR containing 1x PCR buffer, 1mM magnesium chloride ($MgCl_2$), 0.5mM of each dNTP, 0.5 units of *Taq* Polymerase and 10.0μL of 1000-fold sensitive DNA template was run to receive the final amplified target of interest. Primer concentration was altered in order to produce an optimal reaction with each amplicon producing a similar intensity (Table 3). The reaction parameters followed the original program as stated above (page 60). Following amplification, PCR products were analyzed by electrophoresis on 6% polyacrylamide gel and photographed.

Table 3. Primers and concentrations used in the multiplex PCR.

Plasmodium falciparum		*Schistosoma haematobium*		*Leishmania donovani*	
Primer	Concentration (μM)	Primer	Concentration (μM)	Primer	Concentration (μM)
DHFR-TS3	0.12	SH2F	0.18	LD1F	0.20
DHFR-TS2	0.12	SH2R	0.18	LD1R	0.20
KI-14-P2	0.12	SH3F	0.14	LD2F	0.20
K1-14-P1	0.12	SH3R	0.14	LD2R	0.20
MSA-1	0.20	SH5F	0.14	LD3F	0.20
MSA-1	0.20	SH5R	0.14	LD3R	0.20
MSA-2	0.20	SH6F	0.20	LD4F	0.16
MSA-2	0.20	SH6R	0.20	LD4R	0.16
Trypanosoma brucei			*Trypanosoma cruzi*		
Primer	Concentration (μM)		Primer	Concentration (μM)	
TB3F	0.16		TC1F	0.20	
TB3R	0.16		TC1R	0.20	
TB7F	0.12		TC3F	0.12	
TB7R	0.12		TC3R	0.12	
21F	0.16		TC5F	0.20	
21R	0.16		TC5R	0.20	
22F	0.16		TC14F	0.20	
22R	0.16		TC14R	0.20	

Results

All positive controls were subjected to multiplex PCR using each primer set along with PCR negatives (no DNA) to test the quality of the PCR reagents and examine for contamination. The resultant PCR products were then electrophoresed on 6% polyacrylamide gel for visualization (Figure 16).

Each amplified DNA product corresponded to the expected amplicon size previously determined for each primer set and all PCR negatives were devoid of DNA. Sequencing of the PCR products and gene fragment comparison was previously determined (Step I).

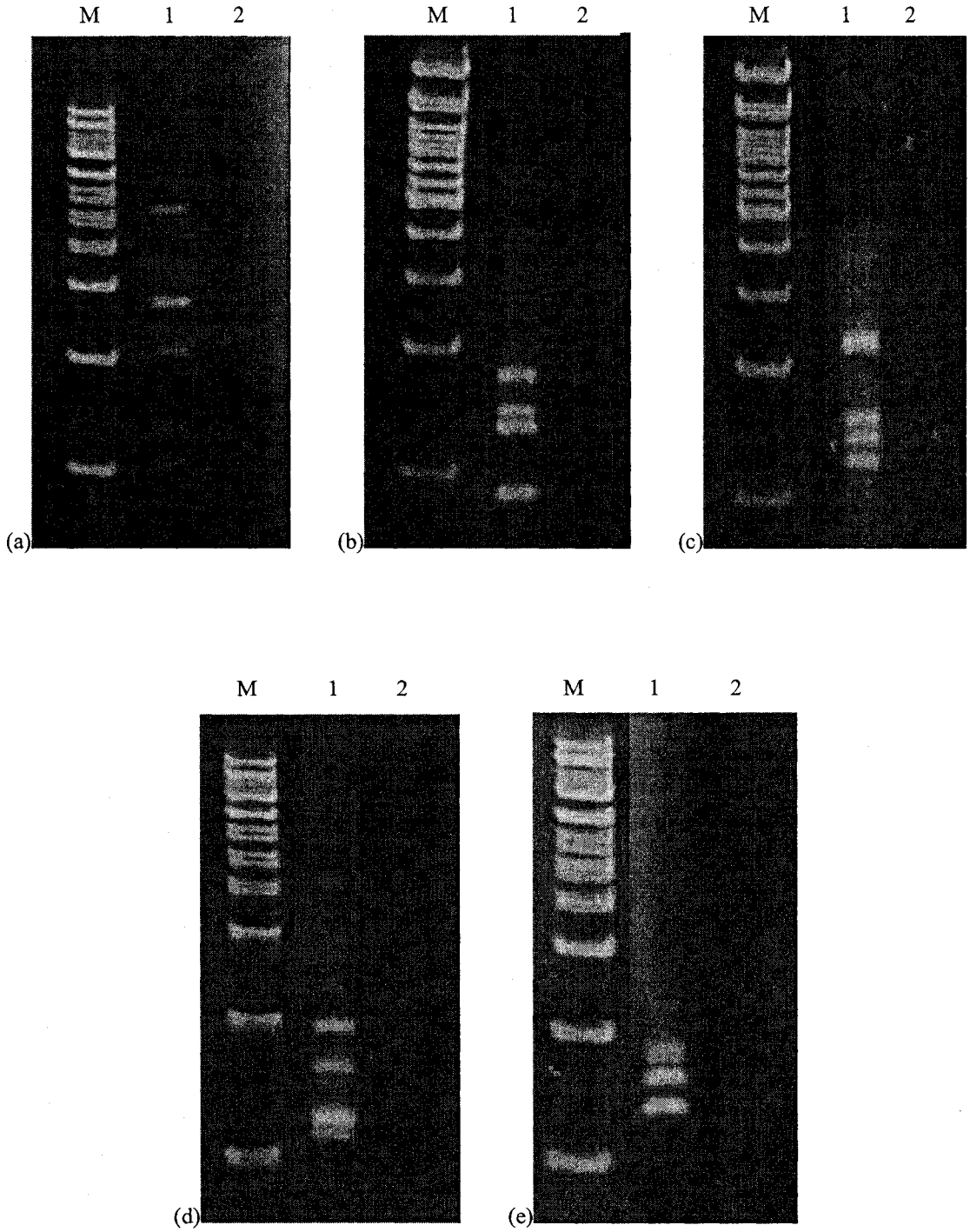


Figure 16. 6% PAGE images of individual parasite multiplex PCR results. Image schematics as follows: M represents 100bp molecular marker; lane 1 represents DNA amplification products via multiplex PCR; lane 2 represents PCR negative. (a) *Plasmodium falc*iparum (b) *Schistosoma haematobium* (c) *Leishmania donovani* (d) *Trypanosoma brucei* (e) *Trypanosoma cruzi.*

STEP III: MULTIPLE PARASITE MULTIPLEX PCR-BASED DETECTION AND IDENTIFICATION OF PARASITES INVOLVED IN HUMAN DISEASE

Aim

The aim of this section of study is to design and optimize a multiple parasite multiplex PCR-based detection and identification system for six parasites involved in human disease.

Parasites Studied

1. *Plasmodium falciparum*
2. *Schistosoma haematobium*
3. *Leishmania donovani*
4. *Trypanosoma brucei*
5. *Trypanosoma cruzi*
6. *Ascaris lumbricoides*

PCR Optimization

PCR optimization for this section of study involved multiple trials of PCR runs to facilitate a reaction involving the use of one primer set per parasite (each of differing amplicon size) in one reaction. However, due to the interactions of primers within the reaction, an additive was included into the reaction to avoid any interactions between primers and relax the DNA template. It was found that a 25.0μL reaction volume for PCR containing 1x PCR buffer, 1mM magnesium chloride ($MgCl_2$), 0.5mM of each dNTP, 0.6% of DMSO, 0.5 units of *Taq* Polymerase and 10.0μL of 1 000-fold sensitive DNA template received the final amplified target of interest for each species. However, primer concentration was altered in order to produce an optimal reaction with each amplicon producing a similar intensity (Table 4). The reaction parameters followed the original program as stated above (page 60). Following amplification,

Table 4. Primers and concentrations used in the multiple parasite multiplex PCR.

Parasite	Primer	Concentration (μM)	bp
Plasmodium falciparum	DHFR-TS3	0.20	280
	DHFR-TS2	0.20	
Schistosoma haematobium	SH6F	0.16	128
	SH6R	0.16	
Leishmania donovani	LD4F	0.08	218
	LD4R	0.08	
Trypanosoma brucei	21F	0.08	108
	21R	0.08	
Trypanosoma cruzi	TC3F	0.08	154
	TC3R	0.08	
Ascaris lumbricoides	ASFB	0.08	246
	ASRB	0.08	

PCR products were analyzed by electrophoresis on 6% polyacrylamide gel applying the same parameters as in Step I.

Results

One species-specific primer set was chosen and included in a multiple parasite multiplex PCR. All positive controls were subjected to this multiplex PCR along with a PCR negative (no DNA) to test the quality of the PCR reagents and examine for contamination. The resultant PCR products were then electrophoresed on 6% polyacrylamide gel for visualization (Figure 17).

Each amplified DNA product corresponded to the expected amplicon size previously determined for each primer set and the PCR negative was devoid of DNA. Sequencing of the PCR products and gene fragment comparison was previously determined (Step I).

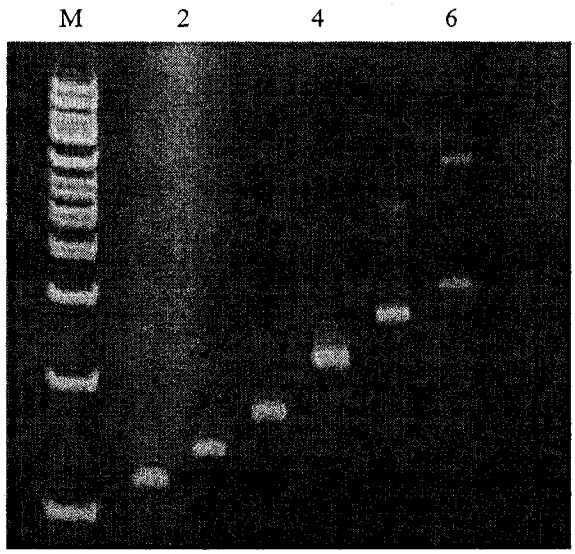


Figure 17. 6% PAGE image of the multiple parasite multiplex PCR results. Image schematics as follows: M represents 100bp molecular marker; lane 1 represents amplification of *Trypanosoma brucei* DNA using primer set 21F/21R; lane 2 represents amplification of *Schistosoma haematobium* DNA using primer set SH6F/SH6R; lane 3 represents amplification of *Trypanosoma cruzi* DNA using primer set TC3F/TC3R; lane 4 represents amplification of *Leishmania donovani* DNA using primer set LD4F/LD4R; lane 5 represents amplification of *Ascaris lumbricoides* DNA using primer set ASFB/ASRB; lane 6 represents amplification of *Plasmodium falciparum* DNA using primer set DHFR-TS3/SHFR-TS2; lane 7 represents PCR negative.

DISCUSSION

Modern human infectious diseases are the product of interactions between the biology of the infectious agent(s), the environment and human behaviour/biology (Aufderheide et al. 2004). If these potential variables do not change, the expression of the disease within a population can be expected to remain constant (Aufderheide et al. 2004). Significant alteration in one or more of these variables, however, can cause the disease to present itself in a very different fashion (Aufderheide et al. 2004). Thus, the development of effective prevention and control measures of many infections depends upon knowledge of how the etiological agents survive and are transmitted in different environments (Morgan and Thompson 1998). However, the lack or inaccurate data currently available may in part be due to variation in diagnostic protocols between diagnostic laboratories (Morgan and Thompson 1998). Furthermore, studies need to be coupled with newer, more sensitive diagnostic techniques that can provide an accurate picture of prevalence in asymptomatic and symptomatic individuals (Morgan and Thompson 1998). PCR has provided the basis for the development of a new generation of diagnostics through its sensitivity, ease of use and the ability to analyze large numbers of samples simultaneously (Morgan and Thompson 1998).

Six parasites involved in the diseases of malaria, schistosomiasis, leishmaniasis, trypanosomiasis and ascariasis have been studied. Most of these diseases are tropical diseases; however the advent of air transportation has led to the possibility for these diseases to occur in geographical areas uncharacteristic of the environment for the proliferation of the diseases. This has brought about a number of problems in the medical profession, where clinicians are unfamiliar with the signs and symptoms of each condition. The diagnosis has relied heavily on numerous tests to confirm the presence or absence of a specific infectious agent. Therefore,

through the presented research, three sensitive and species-specific detection and identification systems have been designed and developed for each parasite involved in disease by utilizing the PCR technique and nucleic acid based technology.

The initial investigation involved the design and optimization of a PCR for each parasite analyzing four different species-specific gene fragments through the design and optimization of oligonucleotide primers unique to each gene investigated (Step I). With this design and development, the genus-specific detection and identification of each parasite via PCR has been proven by DNA amplification and sequencing of each gene fragment. Secondly, each amplicon studied was applied to a multiplex PCR specific for each parasite (Step II). However, due to the nature of the oligonucleotide primers designed for the detection and identification of *A. lumbricoides*, a multiplex PCR was not feasible due to extensive primer interaction. Nevertheless, a multiplex PCR detection and identification system was developed for the remaining parasites investigated with DNA product sequencing of each amplicon confirmed in Step I. Thirdly, one primer set was chosen to incorporate into a multiple parasite multiplex PCR for all six species (Step III). For each species analyzed, we utilized a unique gene fragment with a discrete amplicon size in order to visualize by electrophoresis which species would be present. In this way, a single test is able to screen for a range of parasitic diseases to expedite the testing process and confirmation of the disease in question in a clinical setting. The sensitivity of these studies was an important issue for a number of reasons. Firstly, the greater the sensitivity, the less invasive the sampling procedure would have to be from the patient. Secondly, in the process of infection, the earlier the presence of parasites in the tissues is detected, the earlier a diagnosis of disease would occur. Thirdly, with increased sensitivity, tissues that contain very few

parasites and had been classified as previously unusable for diagnosis can be analyzed for the presence of parasites and thus diagnosed.

Due to the potential risk of contaminating the reaction, each analysis followed a strict protocol to ensure minimizing the possibility of cross-contamination and introductory contamination from an outside source. The reagent preparation and sample preparation were performed in their own separate rooms. All surfaces and instrumentation are cleaned prior to and following every use. Each and every step in the analysis has its own dedicated equipment and separate hoods. The analysts wear a lab coat, double sleeves and double gloves, and hair net and face mask.

Resulting DNA products for each analysis produced thick, high intensity bands due to the abundance of DNA generated in the reaction and the fact that a modern DNA template was used in each analysis. However, the modern DNA template used in each reaction was of a 1 000-fold sensitivity. Since each product visualized by electrophoresis was of high intensity, it can be stated that the reaction designed and optimized is highly sensitive. Also, the lack of contamination, represented by the absence of amplified DNA in the PCR negative (no DNA template added) can thus eliminate the possibility of reagent contamination in the analyses, and none of the sequences generated were identified as contamination from any of the laboratory personnel or outside source.

The results of this study thus confirm the identification of each parasite as expected with the use of modern positive DNA samples in the analyses. Since a few primers were designed based on previously published data, the results also verify those previous findings. However, during the amplification reaction, some of the primer sets produced a second amplified DNA product larger than the targeted amplicon size. Due to the parameters of the PCR, optimization

for intended degraded DNA analysis, and the fact that modern DNA was used as the template, this product could be considered product dimer due to its size as indicated by electrophoresis; double the size of the intended product. Nonetheless, this did not interfere with the sequencing of each DNA product and may not likely occur if each system was applied using degraded DNA due to the fragmented nature of the DNA and diminished quantity of available DNA.

Each system designed and optimized for the detection of identification of parasites involved in human disease can be utilized solely or in conjunction with one another. In the individual singleplex PCR, four gene fragments are used for identification of species. The time of this reaction would be reduced if the multiplex PCR for each species were utilized first. A multiplex PCR has an advantage over the singleplex PCR in that all four gene targets would be simultaneously detected in one reaction. All the amplicons chosen for each multiplex PCR vary in size, some greater than others, however, once the PCR products have been electrophoresed, the DNA products are easily distinguished. Then again, if the sample to be analyzed was unknown as to which parasite was the cause of the disease represented, use of the multiple parasite multiplex PCR first would allow for a single identification to occur. Following this identification, proceeding with the species-specific multiplex PCR would verify the initial identification of the parasite by the simultaneous amplification of four species-specific gene fragments. Each gene fragment could then be analyzed solely for the identification of the parasite via DNA sequencing and gene fragment comparison and alignment with a published gene.

Each primer set for the PCR system developed was designed to amplify small fragments of species-specific DNA likely to remain in archived and archaeological material. Thus, the parameters utilized in each reaction follow this objective. Therefore, the initial aim of this study

was to develop a system of parasite detection applicable for use with modern, archived and archaeological material. By analyzing archaeological samples for the detection of parasites involved in human disease, we may be able to reconstruct historical data and apply this to further our understanding of parasite-host evolution, as well as establishing a foundation of awareness for the antiquity of disease.

CHAPTER 5: MOLECULAR DETECTION AND IDENTIFICATION OF PARASITES INVOLVED IN HUMAN DISEASE: DEGRADED DNA ANALYSES

DISEASES IN ANTIQUITY

The parasite-human host relationship has evolved as a battle between two organisms, with neither of the two a successor. During our relatively short history on Earth, humans have acquired an amazing number of parasites, about 300 species of helminth worms and over 70 species of protozoa (Ashford and Crewe 1998; Cox 2002). One reason for this is that the evolution of humans and parasites has run hand in hand (Cox 2002). It has been estimated that sometime, about 150 000 years ago, *Homo sapiens* emerged in eastern Africa (Tishkoff et al. 2001) and spread throughout the world, possibly in several waves (Templeton 2002), until 15 000 years ago at the end of the Ice Age humans had migrated to and inhabited virtually the whole of the face of the Earth, bringing some parasites with them and collecting others on the way (Cox 2002). The role that disease has played in human dispersal and demography has often been discussed by historians and geographers and to some extent anthropologists (Taylor et al. 1997). The development of settlements and cities facilitated the transmission of infections between humans, and the opening up of trade routes resulting in the wider dissemination of parasitic infections (Cox 2002). Also, the slave trade, which flourished for three and a half centuries from about AD 1500, brought about new parasites to the New World from the Old World (Desportes et al. 1985; Cox 2002). The discovery of such information has allowed many researchers to construct an evolutionary time line and examine the movement of parasites and the diseases they cause throughout history. Thus, we are beginning to learn a lot about the past history of parasitic infections from studies of archaeological artefacts, such as the presence of helminth eggs or protozoan cysts in coprolites, naturally or artificially preserved bodies (Cox 2002) or written records, which brings about a foundation for the investigation of the antiquity of disease.

Early written records of parasitic infections offer insights into understanding the antiquity of disease. The first written records of what are almost certainly parasitic infections came from a period of Egyptian medicine from 3000 to 400 BC (Cox 2002). Other descriptions of disease were found throughout the world as in the writings of physicians in Greece from 800 to 300 BC, China from 3000 to 300 BC, India from 2500 to 200 BC, Rome from 700 BC to 400 AD and the Arab Empire in the latter part of the first millennium (Cox 2002). However, these described conditions may have or may not have been caused by parasites (Cox 2002). New, innovative techniques of piecing together the information concerning the antiquity of disease are required. Therefore, the molecular detection of infections in ancient remains represents an emerging field of research (Zink et al. 2002).

Molecular detection of the agents of disease via PCR has greatly advanced over the years. Besides the benefit of a more precise diagnosis in ancient material, it has advanced from isolated reports on diagnosis of infectious diseases to much more profound studies on disease frequencies in ancient populations and evolutionary aspects of host-pathogen interactions (Zink et al. 2002). As a result, theories are emerging as to the origin and transmission of disease (Zink et al. 2002) thus contributing to our understanding of the antiquity of disease.

We know that many of the important parasites encountered today not only existed, but were widespread, and our early ancestors must have been aware of the presence of the largest and most common worms and of some of the diseases caused by parasites (Cox 2002). There exists a lot of historical and archaeological evidence of infectious diseases and many have been detected in ancient specimens by the successful amplification of the corresponding parasitic or pathogenic DNA (Zink et al. 2002). However, there is still a lack of extended studies to gain more information about the frequency, transmission and probable evolution of these diseases

(Zink et al. 2002). The ongoing research in the field of ancient DNA analysis has the potential to contribute significantly to a better understanding of host-pathogen or more specifically host-parasite interaction, transmission and spread of infectious diseases in historic and modern times (Zink et al. 2002). Without a doubt, infectious diseases have changed through pre-historic times to present day including changes in virulence and pathogenecity that were associated with the progressive development of civilization (Araujo et al. 2003). The subsequent history of human parasitology revolves around a number of factors: early descriptions of a particular disease and the identification of the parasite causing the disease, the elaboration of the life cycle and the establishment of the causal relationship between the parasite and the disease (Cox 2002). Presented here are two studies involving degraded DNA analyses that will contribute to the understanding of the antiquity of diseases.

STEP IV: APPLICATION OF A PCR-BASED DETECTION AND IDENTIFICATION SYSTEM OF PARASITES INVOLVED IN DISEASE TO DEGRADED SAMPLES

Aim

The aim of this section of study is to apply the PCR-based detection and identification system designed in Step I to degraded samples indicative of disease.

Parasites Studied

1. *Plasmodium falciparum*
2. *Leishmania donovani*

DEGRADED DNA ANALYSIS I: THE DETECTION AND IDENTIFICATION OF PLASMODIUM FALCIPARUM IN CLINICAL ARCHIVAL SAMPLES

Introduction

Human malaria, caused by four *Plasmodium* species: *P. vivax*, *P. ovale*, *P. malariae* and *P. falciparum*, has been estimated by the World Health Organization to cause 300-500 clinical cases per year, with more than two billion people at risk (Rich and Ayala 2000). Classical diagnoses of malaria employed microscopic examination of stained blood smears (Tirasophon et al. 1994), with an estimated 60 malaria slides read by one microscopist per day (Jelinek et al. 1996). However, due to the unsuitability of this application in large epidemiological field trials (Jelinek et al. 1996), new and different epidemiological tools that can diagnose malaria on a population level, distinguish between present and past infections, and distinguish between different strains of parasite are needed (Laserson et al. 1994).

The PCR technique for *in vitro* DNA amplification has provided information pertaining to malaria epidemics and seasonal transmission occurrences, proven highly effective in the identification of malarial "mixed" infections, as well as having the capacity to discriminate between recrudescences of previous infections and completely new infections, following drug treatment (Sallares and Gomzi 2001). Analyses of archaeological remains via PCR have made available important information concerning disease antiquity. Taylor and colleagues (1997) successfully amplified and sequenced *P. falciparum* DNA from a rib bone of an anemic individual who had died in 1937. Thus, PCR offers the possibility to compare modern data on epidemiology with the situation prevalent in previous times providing direct insights into the evolution of pathogens and the interaction with their hosts, which are crucial to understand the transmission and spread of particular infectious diseases (Zink et al. 2002).

Aim

The aim of this study is to apply the designed and optimized PCR-based detection and identification system for *P. falciparum* (Step I) on archived clinical samples.

Materials and Methods

General Laboratory Procedures

To guard against contamination of the sample with extraneous DNA, several precautions were taken: (i) throughout the entire extraction procedure, a face mask and double gloves were worn (frequently changed when moving stations); (ii) the sampling and extractions were carried out within a room devoid of any modern malaria DNA; (iii) PCR preparation and analysis was performed in three separate areas: one area for PCR reagent set-up (hood), one area for PCR itself, and one area for PCR analysis; (iv) sterile disposable laboratory instruments were utilized whenever possible, as well as positive displacement pipettes and barrier-tipped pipette tips, with each area containing its own set of equipment that were not interchanged; (v) all areas prior to and following use were thoroughly cleaned using 10% sodium hyperchlorite, ddH_2O, 70% ethanol, and hoods were UV irradiated for at least 30 minutes; (vi) extraction and reagent blanks (negative controls) were simultaneously run alongside with the detection PCR; (vii) no positive, modern control was run with the PCR.

Primer and PCR Optimization Design

Four sets of species-specific primers were designed optimized previously (Step I). Each set of primers targets a unique gene within the *P. falciparum* region, and has been designed with a suitable amplicon size target as research indicates for degraded DNA analyses. PCR optimization had also been previously executed, and parameters are followed as such.

Samples

Five archived clinical samples of dried seven-year-old blood stored on FTA® GeneCards were obtained from individuals from Ghana, Africa. Each sample was analyzed for the presence of *P. falciparum* DNA due to a suspected malarial infection. However, it was unknown as to which, if any, were positive for the disease. Thus, the study was done blind.

Extraction/Purification

Each sample was extracted using the Guanidinium Thiocyanate Extraction procedure (Boom *et al.*, 1990) along with a corresponding extract negative control. Purification was executed using two trials of Performa® DTR Gel Filtration Cartridges for optimal purification.

PCR and Detection

A 25.0μL reaction volume was used for the PCR containing 1X PCR reaction buffer, 2mM $MgCl_2$, 0.2mM dNTPs, 0.2μM of each primer, 0.5 units *Taq* polymerase, 4.0μL of sample extract and 15.9μL of ddH_2O. The PCR began with an initial two-minute denaturation at 94°C, followed by 45 cycles of denaturation, annealing, and extension at 94°C for one minute, 60°C for one minute and 72°C for two minutes, respectively (no final extension). PCR products (6.0μL) were added to 3.0μL of 6X loading dye (produced in laboratory): 12.0μL of 2.5% xylene cyanol, 12.0μL 2.5% bromophenol blue, 432.0μL of 35% Ficoll and 544.0μL of ddH_20) and electrophoresed in a 6.0% (w/v) polyacrylamide gel in 1 X TBE at 120 volts for 50 minutes. Amplified DNA was visualized by ethidium bromide staining exposed to ultraviolet light and photographed. PCR products were purified and a sequencing reaction of 40 cycles of 96°C for 30 seconds, 50°C for 15 seconds and 72°C for four minutes was executed. The ABI Prism® 3100 sequencer and analyzer sequenced the products.

Results

All extracts were subjected to PCR using each primer set along with extract and PCR negatives (no DNA) to test the quality of the PCR reagents and examine for contamination. The resultant PCR products were then electrophoresed on 6% polyacrylamide gel for visualization (Figure 18).

Amplified DNA was detected in samples 2, 3, and 4 for three primer sets (Figure 18a, 18c, and 18d) and one primer set amplified DNA from samples 2 and 4 (Figure 18b). Each amplified DNA product corresponded to the expected amplicon size previously determined for each primer set and all extract and PCR negatives were devoid of DNA. The repetitive nature of amplified DNA products in and between the samples provides evidence for the presence of *P. falciparum* DNA within due to multiple gene target analyses via PCR. However, the identification of this agent of disease is conditional upon sequencing.

Sequencing of the PCR products was easily attained due to the specific nature of the amplified DNA. A gene fragment comparison utilizing the sequenced gene fragment and the corresponding published gene was carried out by means of BioEdit Sequence Alignment Editor (Figure 19). The resultant sequences thus confirmed the presence of *P. falciparum* DNA within the three samples however DNA sequencing utilizing primer set K1-14-P primer set was unsuccessful (Table 5).

Discussion

The antiquity of malaria has proven difficult to attain due to the many questions concerning its history and evolution. Conflicting theories have arisen based on newer studies involving molecular evolution. Many researchers have proposed that the spread of malaria in Africa coincided with the advent of agriculture (Sallares and Gomzi 2001). However, studies in

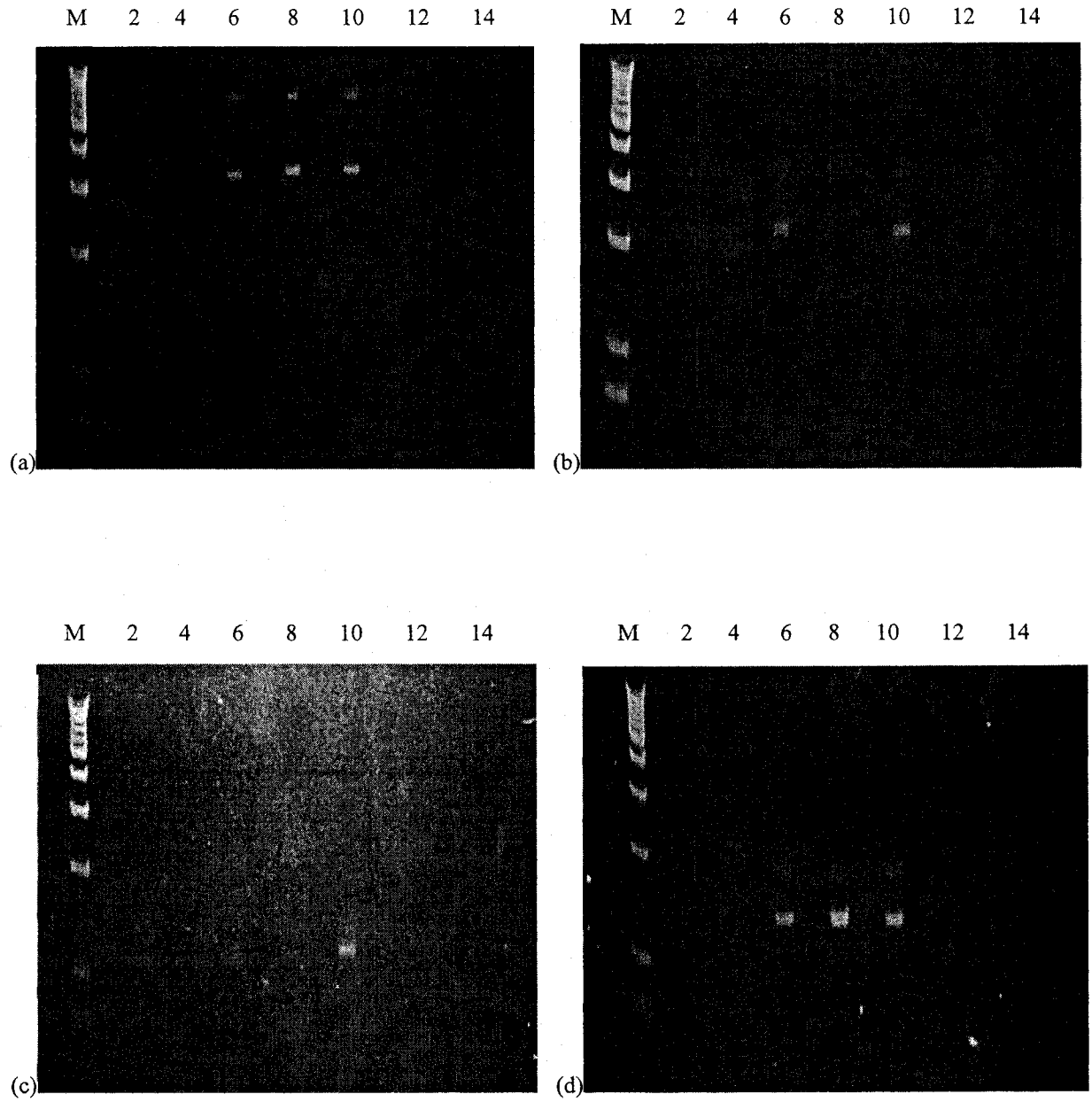


Figure 18. 6% PAGE images of the singleplex PCR results for the five archival samples analyzed. Image schematics as follows: M represents the 100bp molecular marker; lanes 4, 6, 8, 10 and 12 represent samples 1 to 5 respectively; lanes 5, 7, 9, 11 and 13 represent sample 1 to 5 extract negatives respectively; lane 14 represents PCR negative. (a) reaction involving primer set DHFR-TS3/DHFR-TS2 (b) reaction involving primer set K1-14-P2/K1-14-P1 (c) reaction involving primer set MSA-1D/MSA-1B (d) reaction involving primer set MSA-2C/MSA-2B.

```
     DHFR-TS GENE   AACAGAATTT TACAAAAATG TAGACAAATA TAAAATTAAT TATGAAA--- ATGATGATGA
     SAMPLE 2       ----GAATTT TACAWWWATG TAGACRAATA TCARRTTGAT TATGAAATGC ATGMTGATGA
     SAMPLE 3       ----GAATTT TACAAAAATG TAGACRAATA TCARRTTGAT TATGAAATGC ATGMTGATGA
     SAMPLE 4       ----GAATTT TACAWWWATG TAGACRAATA TCARRTTGAT TATGAAATGC ATGMTGATGA

     DHFR-TS GENE   TGATGAAGAA GAAGATGATT TTGTTTATTT TAATTTTAAT AAAGAAAAAG AAGAGAAAAA
     SAMPLE 2       TGATGAAGAA GAAGATGATT TTGTTTATTT TAATTTTAAT AAAGAMAAMG AAGAGAAAAA
     SAMPLE 3       TGATGAAGAA GAAGATGATT TTGTTTATTT TAATTTTAAT AAAGAMAAMG AAGAGAAAAA
     SAMPLE 4       TGATGAAGAA GAAGATGATT TTGTTTATTT TAATTTTAAT AAAGAMAAMG AAGAGAAAAT

     DHFR-TS GENE   TAAAAATTCT ATACATCCAA ATGATTTTCA AATATATAAT AGCTTGAAAT ATAAATATCA
     SAMPLE 2       TAAAAATTCT ATACATCCAA ATGATTTTCA AATATATAAT AGCTTGAAAT ATAAATATCA
     SAMPLE 3       TAAAAATTCT ATACATCCAA ATGATTTTCA CATATATAAT AGCTTGAAAT ATAAATATCA
     SAMPLE 4       TAAAAATTCT ATACATCCAA ATGATTTTCA CATATATAAT AGCTTGAAAT ATAAATATCA

     DHFR-TS GENE   TCCTGAATAC CAATATTTAA ATATTATTTA TGATATTATG ATGAATGGAA ATAAACAAAG
     SAMPLE 2       TCCTGAATAC CACTATTTAA ATRTTATTTA TGATATTATG ATGAATGGAA ATAAACAAAG
     SAMPLE 3       TCCTGAATAC CAATATTTAA ATRTTATTTA TGATATTATG ATGAATGGAA CTAAACAAAG
     SAMPLE 4       TCCTGAATAC CAATATTTAA ATRTTATTTA TGATATTATG ATGAATGGAA CTAAACAAAG

     DHFR-TS GENE   TGATCG.. .......... .......... .......... .......... ..........
     SAMPLE 2       TGATCG.. .......... .......... .......... .......... ..........
     SAMPLE 3       TGATCG.. .......... .......... .......... .......... ..........
(a)  SAMPLE 4       TGATCG.. .......... .......... .......... .......... ..........


     MSA-1 GENE     CAGATGCTAA ATCTTACGCT GATTTAAAAC ATAGAGTTCA AAATTACTTG TTCACTATTA
     SAMPLE 3       CAGATGCTAA ATCTTACGCT GATTTAAAAC ATAGAGTTCA AAATTACTTG TTCACTATTA
     SAMPLE 4       -------TAA ATCTTACGCT GATTTAAAAC ATAGAGTTCA AAATTACTTG TTCACTATTA

     MSA-1 GENE     AAGAACTCA.. .......... .......... .......... .......... ..........
     SAMPLE 3       AAGAACTCA.. .......... .......... .......... .......... ..........
(b)  SAMPLE 4       AAAGACTCA.. .......... .......... .......... .......... ..........


     MSA-2 GENE     ACAAGCTGAA AATTCTGCTC CAACAGCCGA ACAAACTGAA TCCCCCGAAT TACAATCTGC
     SAMPLE 2       ACAAGCTGAA AATTCTGCTC CAACAGCCGA ACAAACTGAA TCCCCCGAAT TACAATCTGC
     SAMPLE 3       ---------A AATTCTGCTC CAACAGCCGA ACAAACTGAA TCCCCCGAAT TACAATCTGC
     SAMPLE 4       ---------A AATTCTGCTC CAACAGCCGA ACAAACTGAA TCCCCCGAAT TACAATCTGC

     MSA-2 GENE     ACCAGAGAAT AAAGGTACAG GA........ .......... .......... ..........
     SAMPLE 2       ACCAGAGAAT AAAGGTACAG GA........ .......... .......... ..........
     SAMPLE 3       ACCAGAGAAT AAAGGTACAG GA........ .......... .......... ..........
(c)  SAMPLE 4       ACCAGAGAAT AAAGGTACAG GA........ .......... .......... ..........
```

Figure 19. Sequence alignments of the three archived *Plasmodium falciparum* samples and corresponding published genes. (a) sequence attained using DHFR-TS3 (b) sequence attained using MSA-1D (c) sequence attained using MSA-2C. Sequence polymorphisms highlighted in red, applying the FASTA format: W represents T or A, R represents A or G and M represents A or C.

Table 5. Resultant polymorphisms of the published gene fragment sequence compared to the experimentally attained gene fragment for the three archived *Plasmodium falciparum* samples.

Gene: DHFR-TS						
	Sample 2		Sample 3		Sample 4	
	91% sequence match to gene		92% match sequence to gene		91% sequence match to gene	
Sequence Polymorphism (published to experimental)	Sequence Possibility	Base Location in Gene	Sequence Possibility	Base Location in Gene	Sequence Possibility	Base Location in Gene
A to W transition	A, A, A	806, 807, 808	-	-	A, A, A	806, 807, 808
A to R transition	A, A, A, A	817, 825, 826, 1006	A, A, A, A	817, 825, 826, 1006	A, A, A, A	817, 825, 826, 1006
A to C transition	A, A	823, 981	A, A, A	823, 839, 1019	A, A, A	823, 839, 1019
A to G transition	A	829	A	829	A	829
TGC insertion		838.1, 838.2 and 838.3		838.1, 838.2 and 838.3		838.1, 838.2 and 838.3
A to M transition	A, A, A	842, 894, 897	A, A, A	842, 894, 897	A, A, A	842, 894, 897
A to T transition	-	-	-	-	T	908
Gene: MSA-1						
	Sample 2		Sample 3		Sample 4	
	-		100% sequence match to gene		100% sequence match to gene	
Gene: MSA-2						
	Sample 2		Sample 3		Sample 4	
	100% sequence match to gene		100% sequence match to gene		100% sequence match to gene	

molecular evolution have demonstrated that *P. falciparum* diverged from the related chimpanzee parasite *Plasmodium reichenovi* at approximately the same time as their respective host lineages diverged between 4 and 10 millions years ago, resulting in co-evolution with hominids (Sallares and Gomzi 2001). Furthermore, debate regarding whether population structure of malaria followed low levels of genetic polymorphisms or high levels of genetic variation are in question (Sallares and Gomzi 2001). The answers to these questions and concerns involving *Plasmodium* species are available through the analyses of archival and archaeological records. Thus, in the advent of paleopathology and the PCR technique, the historical time frame of malaria and evolutionary portrayal could be found.

Here we have designed an improved method of *P. falciparum* DNA detection within archival samples with increased specificity, through the use of multiple gene target analyses, and increased sensitivity, through the use of minimal amounts of DNA template, as to not consume the rare amounts of template that may be available for analysis. Due to the nature of the DNA template analyzed, fragmented DNA from archival samples, specificity and sensitivity are key components. Also, amplicon size and PCR parameters are extremely important, thus each parameter has been designed to attain optimal results.

The results have clearly indicated that three of the five samples analyzed contain one human-malaria-causing agent, *P. falciparum*. The reproducibility of this confirmation and the reliability and the efficiency in the aforementioned key components has been proven by species target DNA amplification and sequencing with no extraneous DNA amplified.

Direct sequencing of PCR-amplified targets from ancient DNA is expected to yield the sequence they possessed at the time of death (Salo et al. 1994). As such, we can expect this to be true of the archival samples analyzed, at the time of sample allocation. Accordingly, the

sequencing results attained from the PCR product analyses produced sequences congruent with the genes available from literature. However, numerous polymorphisms were observed when comparing the sample sequence to the published gene fragment. This was expected as the gene sequence used in primer design is a modern variety and may vary with the species variety discovered in each sample. Furthermore, due to the nature of the study, done in blind, the results have thus confirmed previous findings of *P. falciparum* infection within the same three samples identified by this molecular analysis. The remaining two samples with a non-inclusive result of this parasite via molecular analysis were also previously diagnosed as negative.

In conclusion, the findings in the present study suggest a feasibility of use in other application of *P. falciparum* detection. A system such as this, able to amplify small fragments of DNA from multiple genes, applicable for archival as well as archaeological analyses, is thus important and should find application of malaria confirmation in ancient populations thus increasing our knowledge and understanding of its antiquity.

DEGRADED DNA ANALYSIS II: THE DETECTION AND IDENTIFICATION OF LEISHMANIA IN ARCHAEOLOGICAL SAMPLES

Introduction

Leishmaniasis is a disease that has scourged through the centuries leaving behind facets of its demise. Muco-cutaneous leishmaniasis showed its destruction in pre-Columbian America, while cutaneous leishmaniasis revealed a Mesopotamian antiquity (Brothwell and Sandison 1967). Although these diseases aid in our understanding of leishmaniasis, it is our lack of knowledge concerning the antiquity of leishmaniasis and the origins of visceral leishmaniasis that still puzzles research.

Human leishmaniasis, commonly known as "Kala-azar" in the Old World and "Espundia" in the New World, is a severe chronic disease caused by parasites of the *Leishmania* species (Otero et al. 2000). In the New World agents of the American Leishmaniasis belong the genus *Leishmania*, subgenus *Leishmania*, complex *mexicana* (consisting of *mexicana*, *amazonensis*, and *pifanoi*) and subgenus *Viannia* complexes *braziliensis* and *guyanensis*. It exists as free-living extracellular promastigotes inside their insect vectors, members of the phlebotomine sandfly family, and as intracellular amastigotes within the phagolysosome of macrophages and reticuloendothelial cells of the infected host (Wilson et al. 1991). Differentiation between species may be successfully achieved by microscopy, but this approach is labour intensive, has low sensitivity and due to the morphological similarities among the species (Singh et al. 1999), an expert microscopist is required or confusion may arise.

Analysis of archaeological remains poses additional problems concerning disease identification and species characterization. The recovery of remains with characteristic abnormalities representing specific disease is rare and thus a conclusive diagnosis may not be

favourable. In many instances only skeletal remains are present and therefore hamper the diagnosis of infectious disease, which produces characteristic symptoms dermally. To exclude a misdiagnosis, molecular research has advanced and designed more efficient means of identification via nucleic acids and the PCR technique. This is an attractive option for the detection or parasitic protozoa due to its ease of use and the ability to analyze large numbers of samples, as well as having the added advantage of being able to detect infectious agents directly (Morgan and Thompson 1998).

Aim

The aim of this study is to apply the designed and optimized PCR-based detection and identification system for *L. donovani* (Step I) on archaeological samples.

Materials and Methods

General Laboratory Procedures

To guard against contamination of the sample with extraneous DNA, several precautions were taken: (i) a full-body, hooded Tyvek suit was worn throughout the entire extraction procedure, as well as a face mask, goggles and double gloves that were frequently changed when moving stations; (ii) at an independent laboratory in a separate location, the sampling and extractions were carried out in laminar flow hoods located within a room with no previous history of work ever performed on modern or archaeological leishmaniasis; (iii) PCR preparation and analysis was performed in three separate areas, one area for PCR reagent set-up (hood), one area for PCR itself, and one area for PCR analysis; (iv) sterile disposable laboratory instruments were utilized whenever possible, as well as positive displacement pipettes and barrier-tipped pipette tips, with each area containing its own set of equipment that were not interchanged; (v) all areas prior to and following use were thoroughly cleaned using 10% sodium hyperchlorite,

ddH_2O, 70% ethanol, and hoods were UV irradiated for at least one hour; (vi) extraction and reagent blanks (negative controls) were simultaneously run alongside with the detection PCR.

Primer and PCR Optimization Design

Four sets of species-specific primers were designed optimized previously (Step I). Each set of primers targets a unique gene within the *Leishmania donovani* region, and has been designed with a suitable amplicon size target as research indicates for degraded DNA analyses. PCR optimization had also been previously executed, and parameters are followed as such.

Samples

The sample collection of four skulls (#3984, #3937, #4156 and #5377 designated samples 1 to 4 respectively) was excavated by Gustavo Him Paige at the archaeological cemetery of Coyo Oriente in the Atacama in Chile. Three skulls (#3984, #3937 and #4156) are representatives of the Coyo East, South Group and one skull (#5377) is without group assignment. Each skull exhibited pathological lesions in the face, and the characteristics present caused consideration for the occurrence of leishmaniasis.

Extraction/Purification

Sample preparation began with steps to eliminate contaminating modern DNA. These included UV irradiation, surface removal and pre-wash treatment steps. The samples were then prepared by grinding of the muscle tissue attached to the skull surrounding the nasal and orbital regions, with a pestle in the microcentrifuge tube to be used for DNA extraction. DNA was extracted and purified from this tissue utilizing two methods. The first method employed (E/P 1) a modified silica-guanidinium thiocyanate (GuSCN) extraction procedure (Boom et al. 1990) followed by purification by means of P-30 spin columns. The second method employed (E/P 2) a pK extraction procedure followed by purification via phenol:chlorofom separation and ethanol

precipitation. Application of P-30 spin columns proceeded by 10% Chelex (Walsh et al. 1991) incubation added to the purification of the extract.

PCR and Detection

A 25.0μL reaction volume for PCR containing 1X PCR buffer, 2mM magnesium chloride ($MgCl_2$), 0.2mM of each dNTP, 0.2μM of each forward and reverse primer, 1.0 unit of *Taq* Polymerase and 15.0μL of DNA template. The reactions were conducted in 50 cycles with the following program: initial denaturation of two minutes at 94°C followed by 60 cycles of denaturation, primer annealing and primer extension at 94°C for one minute, 60°C for 30 seconds and 72°C for two minutes respectively. PCR products (5.0μL) were added to 3.0μL of 6X loading dye (produced in laboratory): 12.0μL of 2.5% xylene cyanol, 12.0μL 2.5% bromophenol blue, 432.0μL of 35% Ficoll and 544.0μL of ddH_2O) and electrophoresed in a 6.0% (w/v) polyacrylamide gel in 1 X TBE at 120 volts for 45 minutes. Amplified DNA was visualized by ethidium bromide staining, exposed to ultraviolet light and photographed.

Cloning

PCR products were cloned using T4 DNA ligase to insert the DNA fragment of interest into a pure linear pUC19 vector. The ligated vector was introduced into competent *Escherichia coli* JM109 cells and grown overnight on LB Agar containing X-Gal and IPTG to identify successful pUC19 insertion. The resulting glossy, white colonies were plucked and cultured overnight in LB broth. The isolation and purification of the resulting DNA sequence was accomplished with the QIAprep plasmid purification system and amplified via PCR with sequence specific primers. Sequencing of PCR products occurred in a reaction of 40 cycles of 96°C for 30 seconds, 50°C for 15 seconds and 72°C for four minutes employing the ABI Prism® 3100 Genetic Analysis instrument.

Results

Preliminary testing of the primer sets used in this experiment yielded positive identifications for *Leishmania* through PCR and sequencing. Therefore, we were confident that these amplicons would facilitate a positive identification in the analysis of the archaeological samples of the malformations present.

Tissue sections of samples 1 to 3 (E/P 1) were analyzed using the four primer sets, each specific for a unique species-specific gene. Resulting amplification produced a 116bp fragment of the IMP dehydrogenase gene from sample 2 and a 182bp fragment of the kinetoplast minicircle from sample 1 and 2 (Figure 20) indicating successful amplification. Secondly, tissue sections of all four samples (E/P 2) were analyzed with all four primer sets. Amplification resulted in a 112bp fragment of the IMP dehydrogenase gene and 129bp fragment of the amino acid permease AAP13LD gene from sample 3 and a 218bp fragment of the adenylate kinase gene from sample 1 (Figure 21). Cloning and sequencing of these products confirmed the presence of *Leishmania* DNA (Figure 22). However the sequences differ slightly from the *L. donovani* sequences, both published and experimentally determined, as expected if these were American forms of *Leishmania*.

Discussion

Historical documentation of Old World visceral leishmaniasis, or Kala-azar, dates back to the mid to late 19th century, however, due to similar pathological manifestations it was diagnosed as a virulent form of malaria (Cox 2002). It was not until 1900 that *L. donovani* was characterized as the parasite responsible for the disease (Cox 2002). We can therefore assume that previous findings of this disease were characterized incorrectly in many historical documents. These documents refer to this as a 'virulent form of malaria', but are in actuality

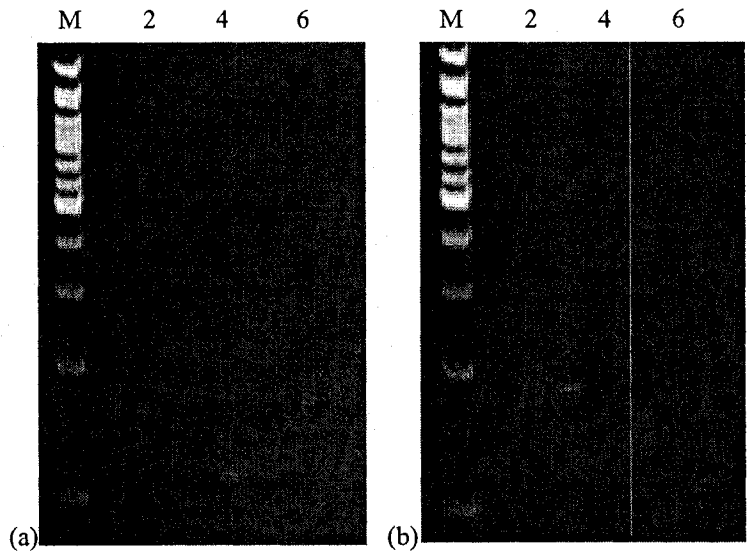


Figure 20. 6% PAGE image of the singleplex PCR results for archaeological samples 1 to 3 (E/P 1). Image schematics as follows: M represents 100bp molecular marker; lanes 1 to 5 represents two extracts of samples 1 and 2 and one extract of sample 3 respectively; lane 6 represents extract negative; lane 7 represents PCR negative. (a) reaction involving primer set LD1F/LD1R (b) reaction involving primer set LD3F/LD3R.

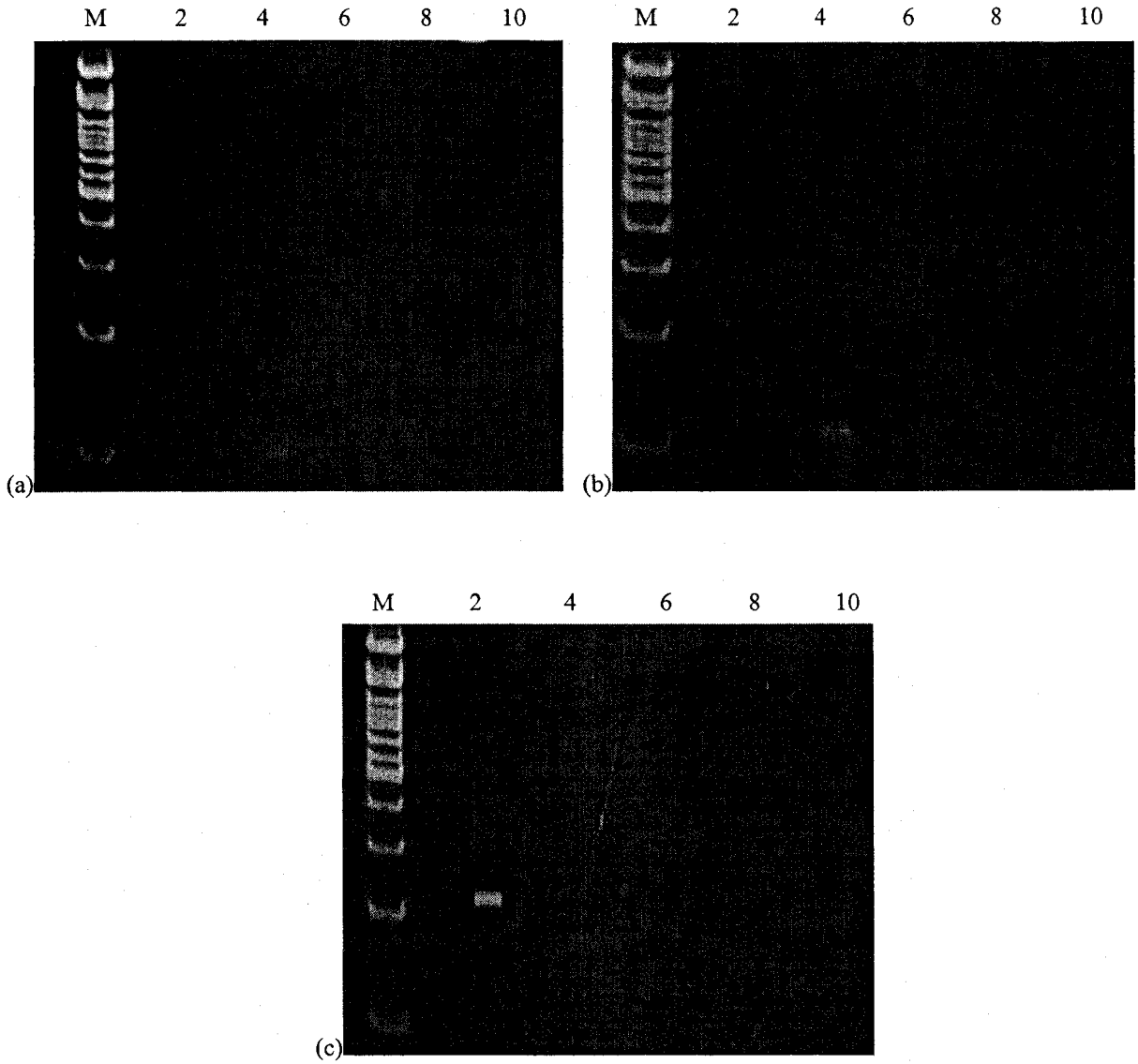


Figure 21. 6% PAGE images of the singleplex PCR results for archaeological samples 1 to 4 (E/P 2). Image schematics as follows: M represents 100bp molecular marker; lanes 2 to 5 represents samples 1 to 4 respectively; lanes 7 to 9 represents extract negative, purification negative and PCR negative respectively. (a) reaction involving primer set LD1F/LD1R (b) reaction involving primer set LD2F/LD2R (c) reaction involving primer set LD4F/LD4R.

```
LD3 GENE      GGAGTAGCCT CAGGACTTTA GGCGGGAGAT ACTATATTAT CGGTAGTATA ATATCATAAG
SAMPLE 1 F    ---------- ---------- ---------- ---------- ---------- ----------
SAMPLE 2 F    ---------- ---------- ---------- ---------- ---------- ----------
LD3F          ---------- ---------- ---------- ACTATATTAT CGGTAGTATA ATATCATAAG

LD3 GENE      TATACGGTAT AGATATATGT TAATTGTAGT ATATTGTAGA TCTATGTTAC AGTGTATAGT
SAMPLE 1 F    ---------- ---------- -----GTAGT ATATTGTAGA TCTATGTTAC AGTGTATAGT
SAMPLE 2 F    ---------- ---------- ---------- ---------- ---------- ---GTATAGT
LD3F          TATACGGTAT AGATATATGT TAATTGTAGT ATATTGTAGA TCTATGTTAC AGTGTATAGT

LD3 GENE      CTATGAACTT ACTAGATATA ATTTG-TAT- --TTGATGCT ATAGTGC-T- ACTGATAGAG
SAMPLE 1 F    CTATGGACTT GCTAGATATA ATTTG-TAT- --TTGATGTT AGAGTGC-T- ACTGATAGAG
SAMPLE 2 F    CTATGAACTT GCTAGATATA ATTTGGTAT- --TTGATGTT ATAGTGC-T- ACTGATAGAG
LD3F          CTATGAACTT GCTAGATATA ATTTG-TAT- --TTGATGTT ATAGTGC-T- ACTGATAGAG

LD3 GENE      TG-TACCTAT CACTAGT... .......... .......... .......... ..........
SAMPLE 1 F    TG-------- -------... .......... .......... .......... ..........
SAMPLE 2 F    TG-TACCTA- -------... .......... .......... .......... ..........
LD3F          TG-TACCTA- -------... .......... .......... .......... ..........
```

Figure 22. Sequence alignments of the archaeological samples, the published genes for *Leishmania donovani* and the experimentally attained sequence. Sample sequence polymorphisms highlighted in red and experimentally attained sequence polymorphisms highlighted in blue.

referring to Leishmaniasis. Therefore, to overcome such inconsistencies in historical diagnosis, new methods of genetic testing for this disease in archaeological material must be employed.

We have designed an improved method of detection for *Leishmania* DNA with increased sensitivity and specificity for the parasite through the use of multiple gene targets. The sensitivity of the assay is made apparent through the use of very little DNA template in the amplification reaction, and its ability to amplify the target DNA in differing tissue types. Due to the nature of the samples used in the analysis, the specificity of detection has proven to be efficient in that the amplified DNA was that of our target species and no extraneous DNA had been amplified. As a result, this system has verified the presence of *Leishmania* from archaeological material through PCR, cloning and sequencing, and thus has confirmed the first detection of the disease in ancient DNA.

Strict contamination controls have been employed in this analysis. The modern optimization of the primers was performed in a different location from the ancient sample extraction. The reagent preparation, sample preparation and extractions are performed in their own separate rooms in a dedicated clean room with an air-shower entry, its own separate air system. Every surface of this clean laboratory is cleaned with bleach once a month and all surfaces are cleaned once a week and before and after every use. Each step in the analysis has its own dedicated equipment and separate hoods. Environmental checks are performed regularly by swabbing the work areas and testing for the presence of DNA via PCR. The analysts wear a full body suit, hood, double sleeves and double gloves, hair net, facemask, hood and additional booties. No *Leishmania* DNA has ever been analyzed in the laboratory nor has any ancient samples suspected as having *Leishmania* ever been analyzed. The analysis also included the verification for endogenous DNA from mitochondrial DNA amplification. None of the

sequences generated were identified as contamination from any of the laboratory handlers or analysts and the profiles generated were consistent with South American haplotypes. No DNA amplification occurred in any of the PCR or extraction controls that were analyzed. The PCR products were cloned and sequenced and one primer set was replicated independently in a different laboratory.

Consequently, the sensitivity, specificity, and the rapid and reproducible nature of this method have proven highly effective in analyzing archaeological material. Verifying the presence of this disease in the past, and comparing them to modern day genetic findings of the species will provide useful information into the evolution of the species, its pathogenesis, and its spread throughout the regions.

CHAPTER 6: SUMMATION AND APPLICATIONS

SUMMARY: DEGRADED DNA ANALYSES

In the past century, detection methods of infectious agents of disease have changed from morphological identification, to immunological-based detection and finally to nucleic acid-based identification systems. Many methods have been used simultaneously for either verification purposes, comparison purposes or to assess the feasibility of a larger study. More recently, molecular detection of infectious diseases have been studied using larger series including specimens with non-specific or even without morphological alterations, suggesting infection with that particular disease (Zink et al. 2002). This approach not only offers the possibility of analyzing modern data but also allows for the comparison of similar situations prevalent in previous times (Zink et al. 2002).

The comparison of modern and ancient species via DNA sequences has become a major factor in describing evolutionary processes. However, the evolutionary processes that generated modern species and populations are commonly inferred through the analysis of morphological and genetic markers, along with studies of abundance and distribution patterns (Willerslev and Cooper 2005). Unfortunately, historical data pertaining to parasites and the diseases they cause is lacking. A great deal of the historical documentation available may have been based on incorrect diagnosis and there is no method to verify the conclusions of the writings. Furthermore, the analysis of contemporary organisms can provide only indirect evidence of this history, and reconstructions remain tentative if they cannot be checked against the fossil record (Willerslev and Cooper 2005). Therefore, in order to attain relevant information on diseases in the past, excavation of archaeological remains and pathological analyses are required. Yet, many diseases resemble one another in pathologies or the presence of the disease may not be clearly

identified in the remains. Thus, the molecular detection and identification of agents of disease must occur to verify the presence of disease.

The invention of the PCR was an extraordinary accomplishment that allowed the molecular research world to expand its horizons. It made it possible to routinely amplify and study even single surviving molecules, allowing the number and range of aDNA studies to diversify with methods now providing a means to record genetic changes in real time, at least over short geological time-scales (Willerslev and Cooper 2005). More specifically, in the field of paleoparasitology, large-scale studies have begun to reveal the true potential of aDNA to record the methods and processes of evolution, providing a unique way to test models and assumptions commonly used to reconstruct patterns of evolution, population genetics and paleoecological change (Willerslev and Cooper 2005). This is true for the parasites themselves, as well as accumulating information pertaining to the host; its life and evolution. For example, if features unique to a parasite's life cycle are known, then the finding of that parasite in an ancient human body often can be translated into prediction of an aspect of the human's behaviour such as diet, occupational activity or domestication of certain animals (Aufderheide and Rodriguez-Martin 1998). Also, documentation of parasitism provides an opportunity to expand cultural or ecological understanding of an archaeological population (Aufderheide and Rodriguez-Martin 1998). Thus, through the comparative analyses of genomes or complete DNA sequences of extant parasitic organisms, a new way of studying the evolution of parasites has been introduced (Sallares et al. 2004).

The initial aim of this study was to design and optimize a system to detect and identify parasites involved in disease. As a result, three independent systems were designed, each of optimum sensitivity and specificity. However, a second major direction of my research focused

on the analysis of medical archived specimens stored in hospitals and museums to study the presence of disease and the causative species in older medical cases.

Application of the singleplex PCR to archived and archaeological samples was performed in accordance with the many criteria put forth by numerous researchers for authentic aDNA analyses. Strict contamination controls had been implemented throughout each step of the analysis. No modern DNA had ever come in contact with the degraded samples as each analysis was performed in different locations with separate equipment and in full laboratory attire. As a result, each analysis has proven successful. The sequence information attained was used to identify the causative agent of disease in these older cases, as either species-specific identification for *P. falciparum* or a genus-specific identification for *Leishmania*. Thus this study leads the way for further analyses involving ancient and/or degraded DNA. Since methodological design remained constant throughout each system developed, degraded DNA analyses involving each of the multiplex PCRs is feasible.

FUTURE APPLICATIONS

The identification of diseases in antiquity is of utmost importance for a variety of reasons. There are numerous questions concerning the evolution of parasites. For example, when examining the history of *P. falciparum*, did this species diverge from related malaria parasite of the chimpanzee *P. reichenovi* approximately four to 10 millions years ago, or does it have a more recent origin (Sallares and Gomzi 2001)? There are also questions concerning mutation rates and the rate of spread through populations, questions about its controversial early history and debate about population structure (Sallares and Gomzi 2001). With *T. cruzi*, its presence does not seem to affect triatomine significantly nor the mammals which have been naturally infected, suggesting a balance between species as a result of long periods of adaptation (Guhl et al. 1999; Guhl et al. 2000). Then, is human Chagas' disease purely an accidental occurrence and when did the adaptation of triatomines to human habitats occur (Guhl et al. 1999; Guhl et al. 2000)? There are also questions concerning the relationship between *A. lumbricoides* and *A. suum* and whether each represents a different species (Loreille and Bouchet 2003).

Many disciplines have devoted an extended amount of time trying to identify and understand the evolution of disease, the distribution of the agent of disease through host, vector and geography and the societal impact. Thus a variety of disciplines may benefit from and contribute to overcome the obstacles of degraded DNA analysis of parasites involved in disease. The implementation of this study would therefore create a new first step in providing the methodology which can be used as a reference guide for further examination of infectious diseases through collaborative efforts between disciplines of archaeology, geology and volcanology, anthropology, paleopathology and paleoparasitology, histology, chemistry, physics, molecular biology, genetics, microbiology, botany, zoology and bioinformatics (Cipollaro et al.

2005). For example, analyzing some diseases that had a significant impact on past human populations that seem to unknown to us today or screening remains for ancient pets and domesticated or wild animals that can act as zoonotic reservoirs or vectors for human diseases (Greenblatt and Spigelman 2003).

The application of this analytical system to unknown samples greatly increases the chances of detecting and identifying an agent of disease, since up to six parasites have been identified by the multiple parasite multiplex PCR. However, inclusion of other parasites into a system such as this is possible with correct optimization techniques. Thus an array of diseases may be detected simultaneously in one reaction. This would then decrease the time required for identification as well as cutting down on cost. The information attained can be used to identify which species caused the past infection in cases where there is more than one parasite that can generate the infection. As well, it can be used to study the parasite's genetic differences between the past and modern varieties and identify evolutionary change over time with the parasite. Finally, by identifying the presence of the disease, epidemiological spread of the disease and the frequency of the disease can be determined with greater accuracy and certainty in the species and the varieties. The results from these and future experiments will allow for inferences to be made on disease antiquity, distribution, and spread, thus allowing for the creation of an evolutionary timeline of the parasite and thus the disease with the potential involvement of disease therapy, treatment and eradication.

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