
**STANDARDISATION AND EVALUATION OF DIFFERENTIAL DIAGNOSTIC
SYSTEMS FOR THE DETECTION OF**
Entamoeba histolytica and *Entamoeba dispar*

**A thesis submitted in fulfilment of
the requirements for the degree of
Doctor of Philosophy
of the University of London**

1999

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ABSTRACT

Entamoeba histolytica is an invasive intestinal amoeba morphologically indistinguishable from *Entamoeba dispar*, a closely related organism that is not able to invade tissues. Differential diagnosis under conventional microscopy is therefore impossible. Reliable tools are needed for clinical diagnosis and for the reevaluation of the prevalence of infection with the invasive species worldwide.

Monoclonal Antibody (MAb) 20/7D exhibited promising results when ascites was used to identify cultured isolates of *E. histolytica* by indirect immunofluorescence assays (IFA), and when used in a Faecal Antigen Capture Enzyme-Linked Immunosorbent Assay (FAC-ELISA) for laboratory diagnosis of amoebic dysentery and colitis. Here, further development of the assay was attempted to increase its sensitivity and use it for detection of asymptomatic carriers of *E. histolytica*. After purification and subsequent titration in ELISA, MAb 20/7D did not adequately distinguish between crude lysates of cultured *E. histolytica* and *E. dispar* trophozoites. MAb 20/7D reacted with a similar soluble antigen of *E. histolytica* and *E. dispar*, which confirmed previous observations in western blot analysis under non-reducing conditions. Therefore, the use of the FAC-ELISA for diagnosis in areas where *E. dispar* is endemic is probably not viable. A nucleic acid detection method was therefore developed. Polymerase Chain Reaction was used to amplify specific tandem sequences in the 24.5 Kb episome of *E. histolytica* and *E. dispar*. After PCR, internal sequences of digoxigenin-labelled PCR products were hybridized to specific biotin-labelled probes for *E. histolytica* or *E. dispar* and detected in Enzyme-Linked Immunosorbent Assay (ELISA). The Polymerase Chain Reaction Solution-Hybridisation Immunosorbent Assay (PCR-SHELA) was evaluated on samples from travellers returning from the tropics to Barcelona. The sensitivity and specificity were 98% and 100% respectively, when results were compared with microscopy. PCR-SHELA was also useful for differential diagnosis in cases of amoebic abscesses, amoebic dysentery, salmonellosis, ulcerative colitis and in asymptomatic carriage of *E. histolytica*.

The new test gives sensitive and specific differentiation between *E. histolytica* and *E. dispar* in clinical specimens and it has proved successful in screening faecal samples in endemic areas for epidemiological purposes.

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LIST OF ABBREVIATIONS

<i>acp</i>	Amoebic Cysteine Proteinase gene
AGD	agar gel diffusion
APC	antigen presenting cells
ATP	Adenosin Triphosphate
BCA	bicinchoninic acid
BSA	Bovine Serum Albumin
CAEP	phosphonolipid ceramide aminoethylphosphonate
CF	complement fixation
CIE	Counterimmunoelectrophoresis
cDNA	Complementary DNA
CIMPAT	Centro de Investigaciones en Microbiologia y Parasitologia Tropical
CP5	Cysteine Proteinase 5
DNA	Desoxiribonucleic Acid
DMSO	dimethyl sulphoxide
EhCP	<i>E. histolytica</i> Cysteine Proteinase
EIA	Enzyme Immunoassay
ELISA	Enzyme-linked immunosorbent assay
FAC-ELISA	Faecal antigen capture ELISA
FAD	Flavin-Adenine Dinucleotide
FCS	Foetal Calf Serum
FeSOD	superoxide dismutase
FMN	Flavin-Mononucleotide
F-1,6-diP	fructose-1-6-diphosphate
FCA	Freund's complete adjuvant
FIA	Freund's incomplete adjuvant
FITC	Fluorescein Isothiocyanate
G-1-P	glucose-1-phosphate
G-6-P	glucose-6-phosphate
G-6-PDH	glucose-6-phosphate dehydrogenase
Gal/GalNac	galactose/N-acetyl-galactosamine
GalNac	N-acetyl-D-galactosamine
GPI	glucose-phosphate isomerase
HK	Hexokinase
HMPS	hexose monophosphate shunt
HRP	Horseradish Peroxidase
ICDDR	International Center for Diarrhoeal Disease Research
IFA	Indirect immunofluorescence assay
IFAT	immunofluorescent antibody test
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IFN	Interferon
IHA	indirect hemagglutination

IL	Interleukin
kb	Kilobase
kDa	Kilodalton
LA	latex agglutination
LPG	lipophosphoglycan
LSHTM	London School of Hygiene and Tropical Medicine
MAb	Monoclonal Antibody
MDR	multidrug resistance phenotype
ME	L-malate dehydrogenase
MHCII	major histocompatibility complex class II
MIF	Merthiolate-Iodine-Formol
NAD	Nicotine-Adenine Dinucleotide
NC	Nitrocellulose
NO	nitric oxide
NP40	Nonidet-P40
OD	Optical Density
OPD	Orthophenylene diamine
PAHO	Panamerican Heath Organisation
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PCR-SHELA	PCR-Solution Hybridisation ELISA
PAGE	Polyacrylamide gel electrophoresis
PGM	phosphoglucomutase
PGE2	Prostaglandin E2
Pi	Inorganic phosphorous
PMS	phenazine methosulphate
PPi	pyrophosphate
Ppiase	pyrophosphatase
PPP	pentose phosphate shunt
PVA	polyvinyl alcohol
RNA	Ribonucleic Acid
rRNA	Ribosomal Ribonucleic Acid
SCID	severe combined immunodeficient
SD	Standard Deviation
SDS	Sodium Dodecyl Sulphate
SEM	scanning electron microscopy
TNF	Tumour Necrosis Factor
TTPase	calcium-dependent thiamine pyrophosphatase
WHO	World Health Organisation

DEDICATION

To

Miguel, Lola, Maria D., Sylvia P.
and Morten, with my deepest
thanks, I love you all

ACKNOWLEDGEMENTS

I would like to thank my supervisor, Dr David Warhurst and Professor Michael A. Miles for opening me the door to the LSHTM, and into the field of *Entamoeba* research. They not only provided financial support, but also contributed with their invaluable advice and encouragement, which were pivotal to attain the objectives we set to achieve during this project. Also, their understanding and solidarity during the difficult passages of the journey taught me what true friendship means.

I am also grateful to Dr Iain Frame, when supervising this project, his input was critical especially during the field work in Colombia, where we shared the task of preparing faecal smears by the dozens in a room with no ventilation at a temperature of over 30°C. Dr Andrew Falconar provided his valuable experience on the characterisation of monoclonal antibodies and discussing with him I also learned one thing or two about dengue. I could not have wished for a better companion to burn the midnight oil at the school. I thank Dr Armando Gonzalez for providing me with hybridoma 20/7D and for allowing me to continue his work on the evaluation of Monoclonal Antibody 20/7D and Dr Rashidul Haque from the International Center for Diarrhoeal Disease Research in Dhaka (Bangladesh) for supplying invaluable cultured material for standardisation purposes.

I must specially thank Professor Felipe Guhl and Professor C. J. Marinkelle from the Centro de Investigaciones in Microbiologia and Parasitologia Tropical at Universidad de Los Andes in Bogota. Twelve years ago Professor Guhl gave me my first part time job nursing the colony of *Rhodnius prolixus*. I had no idea then it would be the start of a career in research. Professor Marinkelle with his example taught me that to be a true parasitologist takes a life time in the making, and that on the way there, hard work can be fun!. Professor Guhl and Professor Marinkelle encouraged and supported me through all my PhD, providing not only their knowledge and experience, but also the laboratory facilities, technical staff and materials for the field work.

Special thanks to Professor Gustavo Vallejo and his family, and to Nancy Vargas, they provided accommodation, laboratory space and technical support at the University of Tolima in Ibague. Also, the Director and the staff of Hospital Pedro Leon Alvarez at La Mesa provided laboratory space and samples, for which I am also grateful.

I must thank the many people who in one way or another assisted me through the project, Mr J. Williams, and the staff at the Malaria Reference Laboratory at LSHTM kindly provided samples, reference strains and advice on diagnosis of intestinal parasites. Dr Stuart Wilson and Ruth McNerney made valuable comments for the standardisation of PCR-SHELA.

At Los Andes University in Colombia I was lucky to receive assistance for the project and the unconditional friendship of Sandra Molina, Carlos Jaramillo, Patricia Fuya, Diana Barrios, and Ana Sierra. All the staff at CIMPAT were always ready to help whatever the task. I could not have done it without them. Also, I thank the many volunteers participating during the screenings on healthy carriers, without them this project would not have been carried out.

I am indebted to Professor Anton Polderman and to Mr Jaco Werveij and all the staff at the Laboratory of Parasitology at Leiden University for their collaboration on the evaluation of PCR-SHELA. They provided interesting samples to be tested blindly, and their enthusiastic approach to new forms of diagnosis of parasitic infections is a continuous source of motivation. Equally important in this project has been the input of Drs Maria E. Valls, Teresa Vinuesa, Maria T. Jimenez de Anta and Manuel Corachan from the Hospital Clinic i Provincial of Barcelona. Their clinic has provided well-characterised samples from travellers as part of a long term ongoing collaboration.

My most special thanks go to my family, for always being there for me, during the good and during the not so good times. Morten with his love and generosity provided the reassurance I needed to finalise this thesis. I thank them with all my heart.

I received partial financial support from the Sir Patrick Manson Bequest for the evaluation of Monoclonal Antibody 20/7D, and a further contribution by the government of Colombia through a scholarship from COLCIENCIAS made possible the completion of my PhD, for which I am grateful. Funding for field work was received from the Commission of the European Communities, the World Health Organisation, through the Programme on Intestinal Parasitic Infections and from the British Council. I thank them for their continuous support and encouragement.

To my friends Rossana, Ana, Angeles, Sarai, Lyda, Claudia and all the fellow students who shared a chat and a coffee, my gratitude.

CHAPTER 1

INTRODUCTION

1.1 Amoebiasis

The term "Amoebiasis" was adopted by the WHO expert committee in 1969 as "The condition of harbouring *Entamoeba histolytica* [(sensu lato)] with or without clinical manifestations" (WHO, 1969). In the past, the fact that many infected people never developed symptoms and spontaneously cleared the infection without treatment originated two hypotheses: one suggested, as originally proposed by Emil Brumpt in 1925, the existence of two species, one invasive and virulent and one morphologically identical and harmless. The second hypothesis pointed at *E. histolytica* as a commensal organism of the human colon with the potential to turn into an aggressive entity. Data collection to clarify the epidemiology and distribution of infection intensified, and in 1984 the WHO meeting of experts on amoebiasis officially acknowledged the magnitude of the world wide prevalence of amoebiasis: 480 million people were carrying the parasite, 10% of them developed invasive disease and from those, about 2% died (WHO, 1985).

Unfortunately, the biochemical and molecular tools needed to confirm either of these hypotheses were unavailable for many decades but finally a redescription of *E. histolytica* Schaudinn, 1903 (Emended Walker, 1911) was published in 1993 by Diamond and Clark reviewing the biochemical, immunological and genetic evidence for the separation of *Entamoeba histolytica sensu lato* into two species and distinguishing the non-invasive species as *E. dispar*. The evidence presented proved that the earliest hypothesis, Emil Brumpt's proposal in 1925 of the existence of two species different only in their ability to produce disease in humans and kittens was correct (Diamond and Clark, 1993). Also, the inability of *E. dispar* to cause necrosis has been tested in hamsters, in which colonisation of the liver by trophozoites of *E. dispar* can cause local inflammatory response

without further invasion, with the development of an effective cellular immune response, that is responsible for the elimination of the trophozoites (Espinosa *et al.*, 1997).

Today, in its new sense, understanding that *Entamoeba histolytica* is the virulent species capable of further invasion, and *Entamoeba dispar* is the commensal species, the original definition of "Amoebiasis" remains and the disease is ranked as the second most important parasitic cause of illness and death after malaria in North and South America, Asia, Africa and Europe (WHO/PAHO/UNESCO, 1997).

1.2 Taxonomy, life cycle and biology of *Entamoeba histolytica sensu lato*

KINGDOM	ANIMALIA
SUBKINGDOM:	<i>PROTOZOA</i>
PHYLUM:	<i>SARCOMASTIGOPHORA</i>
SUBPHYLUM:	SARCODINA
SUPERCLASS:	<i>RHIZOPODA</i>
CLASS:	<i>LOBOSEA</i>
ORDER:	<i>AMOEBIDA</i>
SUBORDER:	<i>TUBULINA</i>
FAMILY:	ENTAMOEBIDAE

Levine *et al.*, 1980

The proposed life cycle for *E. histolytica sensu lato* remains so far unchanged. Dobell (1928) described the complete life history of what was probably a strain of *E. dispar* which had caused asymptomatic infection in primates. Parasites belonging to the complex *E. histolytica/E. dispar* are distributed worldwide, and infect via the faecal-oral route through contamination from one person to another when viable, mature quadrinucleated spherical cysts of 10-16 μm (average 12 μm) are ingested. Whether the details of

excystation from *E. histolytica* are identical to those for (probably) *E. dispar* is not certain, however, Dobell (1928) described excystation by the action of trypsin in the caecum or the lower part of the ileum, producing a quadrinucleate trophozoite from each cyst. The emerging trophozoite lives in close association with the bowel mucosa. After further binary fission, eight uninucleated trophozoites were released, each one ranging between 20 and 40 μm in diameter. The organisms feed by phagocytosis of particulate matter and bacteria and by pinocytosis on liquid nutrients (Proctor, 1991).

Encystation is stimulated when the trophozoites go into the faecal stream with production of a cyst wall, a distinct glycogen vacuole, formation of chromidial bars or chromatoidal bodies and nuclear division to four vesicular nuclei. Cysts are able to survive for long periods in a moist environment. Excretion of cysts by an asymptomatic carrier may reach up to a billion cysts per day, varying day to day in chronic carriers (Warhurst, 1983). According to WHO/PAHO (1992) there are no animal reservoir hosts of *E. histolytica/dispar*, however, the organisms can infect several other hosts and can be found in monkeys (Beaver, *et al.*, 1988), experimental infections have been established in dogs, cats, rats and many other species (Trissl, 1982).

1.3 Morphology and ultrastructure

The plasma membrane of *E. histolytica* trophozoites is trilaminar with a lipid bilayer covered by a glycocalyx (El Hashimi and Pittman, 1970), 20-30 nm thick that contains mucopolysaccharides (Feria-Velasco *et al.*, 1972), lipophosphoglycan (LPG) (Bhattacharya *et al.*, 1992), a polysaccharide containing N-acetylglucosamine repeated units identified as chitin (De la Vega *et al.*, 1997), and cationic iron evenly bound over the surface (Lushbaugh and Miller, 1974). In addition, eighteen peptides and abundant lipids are also found in the membrane. The phosphonolipid, ceramide aminoethylphosphonate (CAEP) was isolated from the surface membrane of *E. histolytica* by Aley *et al.*, (1980). It was also evident in this study that 50% of the total lipid content in the membrane are

ethanolamine-containing lipids. Cholesterol is the principal sterol found though *E. histolytica* is not capable of cholesterol *de novo* synthesis. It has been proposed that cellular debris and cells are the main source of cholesterol through phagocytosis (Lujan and Diamond, 1997).

Trophozoites of *E. histolytica* present a high degree of pleomorphism and the use of scanning electron microscopy (SEM) has made it possible to study surface features such as the presence of lobopodia, endocytic stomata, filopodia and the uroid (Martinez-Palomo, 1987). Trophozoite motility of *E. histolytica* is directional by means of pseudopod formation (lobopodia) or cellular translocation towards chemotactic signals; tubular extensions (filopodia) are associated with the uroid, which forms membrane invaginations of the cytoplasm located at the posterior end of attached trophozoites (Guillen, 1993). This structure accumulates cytoplasmic vesicles for exocytosis (McLaughlin and Aley, 1985) as an efficient way to eliminate capped substances such as toxic chemicals, anti-amoebic antibodies, complement or lectins (Guillen and Sansonetti, 1997). The presence of large amounts of actin that are not polymerized as microfilaments or microtubules has been detected (Martinez-Palomo, 1987). Its distribution in the trophozoite changes according to signal transduction from the environment, being involved in cell traction, capping and adhesion. It is located into the pseudopods (associated to myosin I) and in the posterior regions of motile amoebas (associated to myosin II). Those actomyosin complexes are important in adhesion of trophozoites to target cells (Guillen and Sansonetti, 1997).

The cytoplasm contains the nucleus, vacuoles, membranous vesicles, glycogen and viruses (Diamond *et al.*, 1972). Membrane-associated enzymes have been described but their specific localization is difficult due to the internalization processes constantly carried out in trophozoites. A lysosomal membrane bound pyrophosphatase (PPiase) non-dependent on Mg^{2+} has been demonstrated in *E. histolytica* and its role could be to function as a phosphotransferase in the glycolytic cycle. The presence of calcium-dependent thiamine pyrophosphatase (TTPase) in the membrane of vacuoles and vesicles located in the cytoplasm of *E. histolytica* suggests that the vacuolar system functionally replaces the

Golgi apparatus and the smooth endoplasmic reticulum (McCaul and Bird, 1978). It derives from the internalization of plasma membrane portions by endocytosis or phagocytosis. The existence of different populations of vacuoles, according to the particular characteristics of the ingested materials has been suggested since macropinocytic, micropinocytic and autophagocytic vacuoles have been detected. Also, residual bodies (telolysosomes), primary (phagosomes) and secondary lysosomes (phagosomes containing hydrolytic enzymes) are present (Martinez-Palomo, 1987). Among the lysosomal enzymes detected there are phosphatases (alkaline phosphatase), glycosidases (β -N acetylglucosaminidase), diverse proteinases, and RNAses.

The lack of mitochondria (Rosenbaum and Wittner, 1970), is another remarkable characteristic of *E. histolytica*, with the fact that McLaughlin and Muller (1981) reported the isolation of a membrane-associated Ca-ATPase that suggests a regulatory function in the control of intracellular levels of Ca^{2+} (which in eukaryotic cells is done by the mitochondria). The function proposed for this enzyme is to maintain free high levels of pyrophosphate (PPi) needed for energy-dependent metabolic processes, and which is degraded by membrane-bound pyrophosphatases (McLaughlin *et al.*, 1978). As a calcium-binding protein, calmodulin could be involved in the modulation of its activity (McLaughlin and Aley, 1985).

The nucleus of *E. histolytica* possesses a central spherical karyosome of $0.5\mu\text{m}$ in diameter, surrounded by an achromatic capsule-like structure. It measures between 4 and $7\mu\text{m}$ in diameter, is surrounded by a thin achromatic double layered porous membrane, lined by evenly distributed small granules of chromatin. Those granules are involved in RNA synthesis and are found in contact or very close to each other (Diamond and Clark, 1993). The DNA is distributed and synthesized through the nucleus. It is normally found extensively dispersed until prior to nuclear division, when it condenses around the endosome, arranged in chromosomes with sizes between 300 and 2000 kb (Valdes *et al.*, 1990). Ribosomal RNA (rRNA) or precursor rRNA is found in the periphery of the nucleus, surrounding the DNA, which is more centrally located (Albach, 1989). There are

between 175 to 200 copies of circular extrachromosomal DNA (24.5 kb each) per cell containing two inverted repeats (5.2 kb each) encoding ribosomal RNA (Bhattacharya *et al.*, 1988, Bhattacharya *et al.*, 1989, Huber *et al.*, 1989).

1.4 Metabolism

Entamoeba histolytica is a model for the study of the metabolic processes in primitive eukaryotic cells and as such its metabolism diverges from most typically eukaryotic functions (Wöstmann and Bakker-Grunwald, 1993). Understanding those different metabolic features has been a very important issue for the design of effective strategies for diagnosis, treatment, prophylaxis and control of this protozoan parasite.

It has been established that organisms belonging to the genera *Entamoeba* are not strictly anaerobic and that trophozoites are able to survive in low oxygen tensions and metabolise oxygen. Carbohydrates are the main source of energy for *E. histolytica* and in anaerobic conditions, the products of degradation are ethanol and carbon dioxide, meanwhile in the presence of oxygen, acetate is also formed (Weinbach and Diamond, 1974). Other important features are the absence of cytochromes and the citric acid cycle (Weinbach and Diamond, 1974).

Glucose uptake uses specific pinocytotic temperature-dependent mechanisms (Serrano and Reeves, 1975). Glucose is stored as glycogen and the existence of UDP-dependent enzymes such as phosphorylase, which could be involved in glycogen metabolism has been described. However, the mechanism of synthesis of glycogen is yet not clear since there is no evidence of any glycogen synthase (Takeuchi *et al.*, 1979). The catabolism of glucose in *E. histolytica* differs from the process known for eukaryotic cells due to the presence of uncommon enzymes and the lack of allosteric regulation in glycolysis. Glucose can be converted directly into glucose-6-phosphate (G-6-P) either by glucokinase activity, or may be produced by glycogen degradation via (G-1-P) and

phosphoglucomutase (PGM). Since there is not glucose-6-phosphate dehydrogenase (G-6-PDH) present, it is assumed that the degradation of G-6-P is made through the Embden-Meyerhof pathway, instead of using the pentose phosphate shunt (PPP), also known as the hexose monophosphate shunt (HMPS) (McLaughlin and Aley, 1985). In the same way as glucose, galactose is also used as energy source by two UTP-dependent transferases: glucose-1-phosphate uridyltransferase (specific for glucose-1-phosphate) and hexose-1-phosphate uridyltransferase (Lobelle-Riche and Reeves, 1983). A distinctive feature of glycolysis in *E. histolytica* is the fact that it possesses a fully reversible P_{Pi}-dependent phospho-fructokinase (D-fructose 6-phosphate 1-phosphotransferase), which uses magnesium as a cofactor. P_{Pi} is used as an energy source, enabling the parasite to conserve ATP (Reeves *et al.*, 1974; McLaughlin *et al.*, 1978). Phospho-enolpyruvate is obtained through the standard glycolytic process from fructose-1-6-diphosphate (F-1,6-diP), but its further conversion into pyruvate is different: pyruvate phosphate dikinase, instead of pyruvate kinase catalyses the production of pyruvate with liberation of ATP. It is also important to note that although glycolysis is the main source of pyruvate, it can also be provided through the aerobic deamination of serine by serine dehydratase (Takeuchi *et al.*, 1979).

The absence of lactate dehydrogenase, a means of recovering NAD from pyruvate + NADH (Weinbach, 1981) in the organisms, made it possible to propose another route for the formation of pyruvate from P-enolpyruvate, providing also a source of NADPH to be used in reductive biosynthesis, protection against oxidative damage and cellular detoxification further along in the process by means of a oxaloacetate-malate cycle (Figure 1.1)

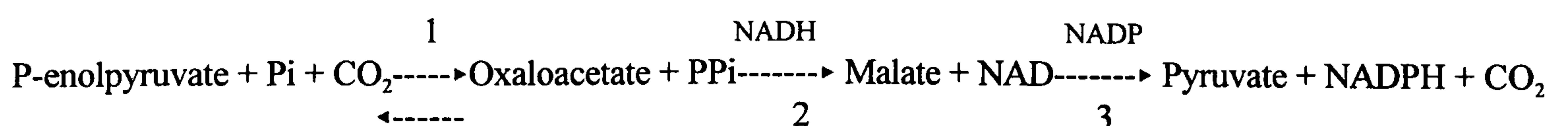


FIGURE 1.1 Oxaloacetate-malate Cycle for Formation of Pyruvate. The enzymes involved in the process are: 1= P-enol-pyruvate Carboxy-transphosphorylase, 2 = Malic dehydrogenase and 3 = Malic Enzyme.

diffusion, meanwhile cytidine, uridine, adenine and guanosine enter via a carrier mediated system. For this system, four transport loci have been identified: two for purine uptake, adenine-adenosine, adenosine-guanosine (Booden *et al.*, 1978). The other two sites are for pyrimidine uptake, uridine-adenosine (purine nucleoside) and uridine-cytidine. Also, hypoxanthine stimulates the uptake of uridine and cytidine (Albach, 1989).

There is polyploidy in *E. histolytica*, with reports of up to 14n (Byers, 1986), with a total amount of nuclear DNA of 0.45 pg/cell, and a genome size of 5×10^4 . G+C ratio for *E. histolytica* has been reported to be 25% (Bhattacharya *et al.*, 1998). The ribosomes (75S and subunits 55S and 35S) of *E. histolytica* are ribonucleoprotein (RNP) bodies grouped (polysomes) in the cytoplasm of trophozoites in an helical array (Kress *et al.*, 1971), when encystation is stimulated, they associate in crystalline aggregates generating the chromatoidal bodies (ribonucleoprotein bodies).

Three species of rRNA, 25S, 17S and 5S are isolated from trophozoites. The 25S RNA is dissociated during nuclear processing into a 17S, a 16 and a 5.8S species. Two DNA-dependent RNA polymerases with specificity for denatured DNA are present, RNA polymerases I and II (Albach, 1989).

1.5 Transmission

The main factors involved in the transmission of parasitic infections in developing countries are poverty, low educational background, lack of home technology, maternal incompetence and ruralism (Mata, 1982). Defaecation near water supplies and indiscriminate squatting are important sources for transmission. Also, faecally contaminated food, and unhygienic irrigation practices result in high infection rates mainly in endemic areas. In developed countries, where good sanitation, education and water supplies are available to the majority of members in the community, increased risk of infection is linked to infected asymptomatic food handlers, institutionalized patients, immigrants and tourists returning from endemic areas.

Like most epidemiological features of amoebiasis, most of the data are now known to refer to "*E. histolytica/dispar*". Mechanical vectors such as flies and cockroaches are important for the transmission of amoebiasis by transporting viable cysts or by contaminating food with their vomit or faeces (Warhurst, 1983). In addition, there is association between prevalence of invasive infections and movement of populations, overcrowding, poverty and ignorance in most developing countries. Intrafamilial spread (Spencer *et al.*, 1981) is seen and transmission in groups living together is very common in mental institutions (Sexton *et al.*, 1974; Spencer *et al.*, 1977; Sargeant *et al.*, 1982a) schools, and refugee camps (Gonzalez-Galvares *et al.*, 1986). Also, contamination of fresh vegetables could account for *E. histolytica* infections, especially among tourists.

Transmission of *E. histolytica* takes place when viable mature cysts are ingested. Trophozoites are not an important stage in transmission because of their inability to survive acid pH in the stomach. However, cyst carriers disseminate infection primarily through direct contamination from the digestive tract of one person to that of another person under poor conditions of personal hygiene. Experimentally it has been established that the infective doses is fewer than 2000 to 4000 cysts, with a prepatent period of 5 days and an average incubation period of six weeks for the development of symptomatology (Knight, 1975). Once ingested, the cysts hatch in the intestine and the freed trophozoites are able to invade and cause dysentery in the susceptible host. Reports on identification of *E. histolytica* cysts during follow-up studies have shown that there are intervals of acute dysentery and cyst shedding among infected or convalescent individuals. During follow-up studies in asymptomatic cyst carriers, three stages were described, the chronic stable cyst shedder, the chronic intermittent cyst shedder and the short term cyst shedder, capable of autoeliminating the infection (Ruiz-Palacios *et al.*, 1992). Those shedding periods are important in dissemination of the disease because cysts may be viable for 10 minutes on the surface of the hands (reviewed by Warhurst, 1983) and up to 45 minutes if they remain moist under the fingernails (Andrews, 1934).

1.6 Clinical Features of *Entamoeba histolytica* Infection

Invasive disease is caused by *Entamoeba histolytica* with invasion of the mucosae by trophozoites which produces clinical manifestations with the potential risk of spreading to extraintestinal foci. Intestinal amoebiasis varies from mild manifestations with diffuse inflammation and microscopical damage, to more grave forms with different levels of ulceration (Trissl, 1982). The most important intestinal syndromes present in invasive amoebiasis are amoebic colitis, acute amoebic colitis (amoebic dysentery), fulminant colitis, amoeboma and appendicitis. However, amoebiasis as a general terminology also involves the asymptomatic intestinal infection by *Entamoeba dispar*, where trophozoites may cause slight irritation of the mucosa surrounding the trophozoite-multiplication area. The asymptomatic cyst passage is the most common presentation of amoebiasis.

Acute amoebic colitis (amoebic dysentery) is characterised by 10-12 bloody stools per day, caused by the haemorrhagic inflammation of the mucosa, where oedema and infiltration by eosinophils and neutrophils is observed (Trissl, 1982), mucus and abdominal pain, without significant faecal leucocytosis. It must be differentiated from bacterial colitis or dysentery caused by *Shigella sp.*, *Salmonella sp.*, *Helicobacter*, *Vibrio sp.*, *Escherichia coli* and *Yersinia enterocolitica* (Reed, 1992). The proctoscopy shows small ulcers on the mucosa and muscularis mucosae, with flask-like extension into the submucosa (Ravdin *et al.*, 1988). Fulminant colitis occurs mainly in children, who suffer bloody diarrhoea, fever and abdominal pain. Appendicitis, colonic perforations, megacolon, and peritonitis are frequent complications of this syndrome with the presence of abundant infiltrates containing lymphocytes, histiocytes, and eosinophils (Trissl, 1982; Reed, 1992). Amoeboma is a complication of intestinal disease presented as a mass lesion in the caecum, ascending colon, rectosigmoid, transverse colon, or descending colon. It mimics colon carcinoma but responds rapidly to antiamoebic therapy and may even resolve spontaneously (Ravdin *et al.*, 1988; Reed, 1992).

Councilman, Lafleur and Osler (1884) confirmed the pathology of amoebiasis and the relationship between intestinal amoebiasis and amoebic liver abscess and its complications (Councilman and Lafleur, 1978). The clinical syndromes present in extraintestinal disease include amoebic liver abscess, pleuropulmonary amoebiasis, peritonitis, pericardial amoebiasis, cerebral amoebiasis, cutaneous amoebiasis and genitourinary amoebiasis. Amoebic liver abscess is the most common of the extraintestinal syndromes and it is the major cause of morbidity and mortality due to amoebiasis worldwide (Ramos *et al.*, 1986). *E. histolytica* probably reaches the liver by hematogenous dissemination from the intestine via the portal system. Once established, contact-dependent lysis of polymorphonuclear neutrophils by amoebae causes destruction of surrounding hepatocytes due to the release of toxic neutrophil constituents. The wall of the cavity consists of fibroblasts, macrophages and lymphocytes and the trophozoites are located within the wall. The cavity is usually filled with tissue debris, odourless and with a characteristic chocolate colour. Amoebae can be isolated with difficulty by culturing material from aspirates. Differential diagnosis with pyogenic abscess and hepatic carcinoma requires serological confirmation of anti-amoebic antibodies and hemoculture to exclude *Escherichia coli*, *Bacteroides spp.*, *Klebsiella pneumoniae*, *Proteus spp.*, *Enterobacter sp.*, *Pseudomonas spp.*, *Citrobacter spp.*, *Staph. aureus*, and *Strep. pyogenes* among others.

Pleuropulmonary amoebiasis is the most common complication of amoebic liver abscess. It is found in 20-35% of patients and is the result of contiguous spread from the right lobe of the liver. The rupture of the liver abscess into the pleural cavity is the most serious pulmonary complication. Peritonitis is the second most common complication of amoebic liver abscess and occurs in 2%-7.5% of patients (Reed, 1992). Pericardial amoebiasis is the most serious complication of an amoebic liver abscess. Despite this, its presentation is infrequent (0.2%), more than two-thirds of patients who have amoebic liver abscess with pericardial involvement have abscesses of the left lobe of the liver (Reed, 1992). Cerebral involvement in amoebiasis is seen in less than 0.1% of amoebiasis patients. In 50% of those patients there is an abrupt onset of symptoms and cerebellar

involvement or rupture within 12-72 hours is the cause of death. Genitourinary amoebiasis in its renal form can be the result of abscess rupture in the liver, hematogenous spread from the lesion in the liver or lungs or extension through the lymphatics. Cutaneous lesions are caused by fistulae from a liver abscess or rectocolitis.

1.7 Invasive Markers in Amoebiasis

Biochemical markers such as the agglutination of trophozoites with concanavalin A (Martinez-Palomo *et al.*, 1973), high rate of erythrophagocytosis (Trissl *et al.*, 1978a) and resistance to complement lysis (Reed *et al.*, 1983) were the markers used to differentiate invasive from non-invasive isolates of *E. histolytica*. The criteria of direct association between the presence of *E. histolytica* and pathogenesis was modified when Sargeant *et al.* (1978) showed that the electrophoretic patterns (zymodemes) of the isoenzymes EC 2.7.1.1 hexokinase (HK); EC 5.3.1.9 glucose-phosphate isomerase (GPI); EC 1.1.1.40 L-malate dehydrogenase (decarboxylating) ME, and EC 2.7.5.1 phosphoglucomutase (PGM) from cultured *E. histolytica* differed depending on the clinical source of the strain (symptomatic or asymptomatic cases). After the analysis of the zymograms of thousands of isolates they characterized 24 zymodemes from which patterns of migration for HK and PGM were an indication of the pathogenic potential of the strains. These observations were corroborated by le Blanc (1992) who, using 12 isoenzymes (Alloenzymes) taxonomically classified pathogenic and non-pathogenic strains of *E. histolytica*. Results in the form of a dendrogram showed the existence of two groups within the "species".

Evidence supporting the existence of only one species, suggesting that virulent behaviour relates to environmental conditions such as variations on bacterial flora in the intestine and in culture has also been produced, and Mirelman *et al.* (1986) and Andrews *et al.* (1990) reported the conversion of strains from non-pathogenic to pathogenic zymodeme and *vice versa* during the axenization process. Attempts to reproduce these

Clark and Diamond (1992) using several cloned strains including the "converted" isolates. Not only was there no conversion since the zymodeme patterns did not change, but the amplification and further digestions of the PCR products of the small subunit RNA genes with *Xba*I (Riboprinting) on the relevant isolates showed the original non-pathogenic restriction pattern (Clark and Diamond, 1991).

Specific probes for pathogenic and non-pathogenic zymodemes based on the non-coding repetitive elements of the 23 kb episomal (extrachromosomal) DNA of *E. histolytica* have been used as invasive markers in clinical and epidemiological studies (Garfinkel *et al.*, 1989). These probes were hybridised directly to the DNA by Bracha *et al.* (1990) and after PCR by Romero *et al.*, (1992) and Acuña-Soto *et al.*, (1993) to identify the amoebal species in clinical isolates and in stool samples. Other gene sequences and antigens used as invasive markers, are the 125 kDa surface antigen (Edman *et al.*, 1990), the 27 kDa cysteine proteinase (Tannich *et al.*, 1991), the 30 kDa nuclear antigen (Tachibana *et al.*, 1991), and the Fe-superoxide dismutase (FeSOD) enzyme (Tannich *et al.*, 1991), which reveal differences not only at genomic but also at the protein sequence level. Many of those markers are currently being successfully used as diagnostic tools and at present, *E. histolytica* and *E. dispar* have been acknowledged as two distinct species by the experts committee of WHO (WHO/PAHO/UNESCO, 1997).

1.8 Mechanisms of Pathogenicity

The cytolytic activity of *E. histolytica* has been studied in cultured cells and it involves four steps: adhesion, cytolysis, phagocytosis and intracellular degradation that combine different chemical and physical processes. After contact and binding, probably via the interaction of host cell carbohydrate residues with N-acetyl-D-galactosamine (GalNac) inhibitable lectin (Ravdin *et al.*, 1988) on the surface of the trophozoites, and introduction of the peptide amoebapore (pore-forming peptide) into the membrane phospholipids of the host cell to form a water filled channel (Leippe *et al.*, 1991; Leippe,

phospholipids of the host cell to form a water filled channel (Leippe *et al.*, 1991; Leippe, 1997), epithelial cells suffer a lowering of transepithelial resistance, detaching, distortion of microvilli and surface blebbing. As soon as the target cells are detached by amoebal filopodia, they are next distributed on the amoebic surface in patches clustering towards the uroid. Engulfment by phagocytic stomata then occurs, thus amoebae lyse target cells prior to phagocytosis (Ravdin and Guerrant, 1982).

Amoebic colonisation and contact-dependent cytolysis of the host tissues involves lectin/adhesin-mediated mechanisms (Chadee *et al.*, 1987; Ravdin and Guerrant, 1981; Rosales-Encina *et al.*, 1987). The lectin-mediated effector has been identified in *E. histolytica* and *E. dispar* as a 260kDa membrane-associated glycoprotein, the galactose/N-acetyl-galactosamine (Gal/GalNac) binding lectin, consisting of a transmembrane heavy subunit of 170kDa and a lipid-anchored light subunit of 35/31 kDa, linked by disulphide bonds (Petri *et al.*, 1989a). The heavy subunit is encoded by a multigene family of five *hgl* genes and the light subunit is encoded by a family of *lgl* genes, both distributed in multiple loci among the genome.

Human colonic mucins, with abundant Gal and GalNac residues are the main receptors for the amoebal lectin (Chadee *et al.*, 1987) and monoclonal antibodies (MAb) raised against the heavy subunit of the lectin detected six different epitopes, with different effects on adhesion to target cells and mucins. Antibodies directed against epitopes 1 and 2, which are present in *E. histolytica* and *E. dispar* enhanced adherence meanwhile monoclonal antibodies directed against epitopes 4 and 5 inhibited adhesion. Another MAb produced against epitope 6 inhibited adhesion to target cells but not to mucins. The enhancement phenomenon may indicate that some anti-lectin antibodies help during the colonisation of the host epithelium by activation of the lectin, thus evading the immune response. During the same study, 56.8% of 44 immune sera from patients with amoebic liver abscess inhibited adherence but interestingly, 36% of the remaining sera enhanced it. (Petri *et al.*, 1990a; Petri *et al.*, 1990b).

Contact-dependent lysis mechanisms involve the secretion of soluble factors that alter membrane stability. A family of pore-forming peptides (amoebapores), are responsible for part of the cytolytic effect of *E. histolytica* by changing the osmotic balance of nucleated cells and bacteria (Leippe *et al.*, 1994; Leippe, 1997). Three isoforms, A, B and C corresponding to mature peptides of 77 aminoacids arranged in four α -helical domains connected by three disulphide bonds (Leippe, 1992) are present in *E. histolytica* and *E. dispar* in a ratio of 35:10:1 (Leippe, 1997), although *E. histolytica* activity is higher due to structural differences (Dodson and Petri, 1994). Domains 1 (residues 1-22) and especially 3 (residues 40-64) possess the pore-forming activity and the other two resemble globular proteins (Leippe *et al.*, 1994).

Amebapores are stored in vesicular compartments and are not released to the environment, unless activated by contact or due to amoebal lysis, however, no specific receptors are required (Leippe *et al.*, 1995; Leippe, 1997). Together with the amoebapore, the mechanical aggressive mechanisms of the trophozoite's cytolytic action are complemented by the presence of several proteolytic enzymes that are probably used in the penetration of tissues, in degradation of exogenous substrates and in inflammation processes. Several cysteine (Thiol) proteinases have been isolated from extracts of axenically cultured trophozoites ranging between 16-96 kDa in molecular weight, which seem to be gene products in both *E. histolytica* and *E. dispar*, sharing a similar structure and function to those of mammalian cathepsins (McLaughlin and Faubert, 1977; Lushbaugh *et al.*, 1984). As opposed to amoebapores, cysteine proteinases are secreted by exocytosis (Leippe *et al.*, 1995) and their secretion is triggered and increased by the contact of the mannose-containing glycoproteins in the plasma membrane of trophozoites with receptors in bacteria or target cells (McKerrow, 1992).

The proposed role of cysteine proteinases in invasion, (observed in cultured cells *in vitro*), is to reversibly round and detach cells from monolayers, without any lytic effect (Keene *et al.*, 1986), resembling the function of cysteine proteinases found in macrophages and cancer cells. Studies of liver abscess formation in severe combined immunodeficient

(SCID) mice confirmed their role in amoebic abscess formation (Stanley *et al.*, 1995). Also, their function in limiting the host's immune response seems to be related to their capacity to degrade specific IgG and cleave complement factor C3 into C3a and C3b and to degrade IgA, C3a, and C5a (Que and Reed, 1997). The major neutral proteinase, with a molecular weight of 56kDa was able to break down extracellular matrix, laminin, fibronectin and type I collagen (Keene *et al.*, 1986; Li *et al.*, 1995). Luaces and Barrett (1988) purified histolysin, a cysteine proteinase of Mr 26kDa by SDS/polyacrylamide gel electrophoresis or 29kDa by gel chromatography. Later, three different cysteine proteinases were identified by Reed *et al.* (1989) on gelatin substrate gel electrophoresis, with molecular weights of 56, 40 and 30 kDa, the latter identical to the one identified by Luaces and Barrett (1988). Also a cysteine proteinase of 60kDa reported by Spinella *et al.*, (1997) with different physicochemical characteristics to those reported previously could be the same 70kDa surface protease found by Avila and Calderon (1993). The 60kDa protease also resembles the CP5 isolated by Benkert *et al.*, (1997) which seems to be present only in *E. histolytica* and that is membrane associated (Avila and Calderon, 1993; Spinella *et al.*, 1997; Benkert *et al.*, 1997).

At the molecular level, a multigene family of cysteine proteinases (*acp* or *ehcp* genes) have been identified in *E. histolytica* (Reed *et al.*, 1993; Bruchhaus and Tannich, 1996; Mirelman *et al.*, 1996). Six genes (*ehcp1-ehcp6*) were identified by Bruchhaus *et al.* (1996) and the products of three of those genes (EhCP1, EhCP2 and EhCP5) were found to account for 90% of cysteine proteinase expression in *E. histolytica*. The sequences of three of those genes, *acp1/ehcp3*, *acp2/ehcp2* (histolysain=histolysin) and *acp3/ehcp1* (amebapain) were first reported by Reed *et al.*, (1993). The sequence of *acp1* was only 35% and 45% homologous to *acp2* and *acp3* respectively, although *acp2* and *acp3* share 85% homology between their sequences. Homologous sequences to those three genes have also been identified in *E. dispar* (Reed *et al.*, 1993; Bruchhaus and Tannich, 1996), however, the level of their expression is 10 to 100 fold higher in the mRNA from *E. histolytica* (Tannich *et al.*, 1991).

The conserved gene sequences of cysteine proteinases in *E. histolytica* and *E. dispar* resemble those of papain-cysteine proteinases and are translated in a similar way, as preproenzymes. Thus, the multiple number of gene products isolated suggest the possibility of different structures due to post-transcriptional processes during protein synthesis. However, it is not clear if the differences are due to different specific functions or activities (Que and Reed, 1997).

Synthetic inhibitors of cysteine proteinases targeting intestinal forms have been proposed as possible chemotherapeutic agents for the treatment of amoebiasis. Also, the potential use of cysteine proteinases in diagnosis was highlighted by Reed *et al.* (1989) with an ELISA test using the 56 kDa proteinase which detected antibodies in 83% of patients with invasive amoebiasis and none in *E. dispar* infected subjects (Reed *et al.*, 1989). Luaces *et al.*, (1992) standardised an histolysain detection system in stool samples (ENZYMEBA) with a sensitivity of 87.5%, although it doesn't differentiate between *E. histolytica* and *E. dispar* infections (Luaces *et al.*, 1992). Histolysain was also used in an ELISA for detection of circulating specific antibodies in 72.7% of patients with amoebic abscess, but also detected antibodies in 18.1% asymptomatic cyst carriers and 2.5% of healthy controls (Luaces *et al.*, 1993).

Among other important enzymes found in *E. histolytica* and *E. dispar*, a metalloproteinase, the Ca^{2+} dependent collagenase is located in electrodense bodies of 80-200 nm in diameter which are not surrounded by any membrane. This enzyme is not liberated into the culture medium and is activated only after contact with the collagen type I and Ca^{2+} , with the intervention of calmodulin (Muñoz *et al.*, 1984). Other components of the electrodense granules are $\text{Fe}^{2+/3+}$, Na^+ , Mg^{2+} , K^+ , P, S, Cl⁻; Pi and PPI. Phospholipases are also involved in the cytolytic processes in invasive amoebiasis, Said-Fernandez and Lopez-Revilla (1982) detected haemolytic activity related to phospholipase A in sonicates and homogenates of *E. histolytica*. Degradation of phospholipids produce free fatty acids, and lysophospholipids such as lysophosphatidylcholine, product of hydrolysis of phosphatidylcholine from membranes of the parasite (Said-Fernandez and Lopez-Revilla,

1982). Also, coding of haemolytic peptides has been reported within the ribosomal DNA inverted repeats in the extrachromosomal circular DNA from clones derived from *E. histolytica* genomic DNA (Jansson *et al.*, 1994), and amplification of the 2.6 kbp haemolysin structural gene has been carried out from amoebic liver abscess samples (Zindrou, 1997).

Two soluble components involved in the aggressive behaviour of the trophozoites are found in used culture medium, the first is a β -N-acetyl-glucosaminidase whose role seems to be the disruption of glycoprotein holding the mucosal epithelial cells and also helps in the breakdown of ingested erythrocytes. The second is a chemotaxis inhibitor for monocytes (macrophage migration inhibition factor, MIF) which could be responsible for the local transient anergy present at the beginning of amoebic invasion (Kretschmer *et al.*, 1980). Studies in gerbils have shown that by modulation of the host immune system, trophozoites survive inflammatory reactions at infection sites affecting cell mediated immunity, namely macrophages and T-cells. Cell mediated activation of immunity during amoebic invasion induces T cell derived cytokines (IFN- γ , IL-2 and TNF- β) which in turn induce macrophage mediated cytotoxicity, involving the production of TNF- α to increase nitric oxide (NO) levels with O_2^- and H_2O_2 as cofactors (Campbell and Chadee, 1997).

Trophozoites produce serotonin and neurotensin which promote loss of tissue fluid and red blood cells, generating vascular thrombosis, ischemia and necrosis of the intestinal epithelium. The trophozoites that are able to reach the blood stream and make contact with complement cleave C3 (by action of their cysteine proteinases), activating the alternative pathway by C3b. During the process, C3a is degraded, limiting anaphylaxis reactions and affecting the host inflammatory response (Reed, 1995). At the same time, at the extraintestinal site, the inflammation is absent or mild but necrotic processes do occur (Trissl, 1982).

Delayed hypersensitivity is retarded and local down regulation of macrophage function is observed, either by inhibition of TNF- α production (via Prostaglandin E_2 (PGE $_2$))

from the parasite and macrophages) or by modulation of T cell functions by amoebic proteins which suppress mitogenesis and induce production of interleukins 4 and 10 (IL-4, IL-10), known by their inhibitory effect on macrophage functions. Campbell and Chadee have recently proposed that soluble amoebic proteins induce production by macrophages of PGE₂ and LTC₄ (a pro-inflammatory mediator) and whose function is to reduce the ability of antigen presenting cells (APC) to present amoebic antigen to T cells, by reducing the expression of major histocompatibility complex class II molecules (MHCII), so no further macrophage activation occurs and formation of amoebic granulomas may be favoured by low levels of TNF- α (Campbell and Chadee, 1997).

Although serum antibodies rise during amoebic invasion, they are not protective and frequent reinfections occur in endemic areas (Krupp, 1970; Ravdin and Guerrant, 1982). Many of the molecular mechanisms involved in the proposed downregulation of the immune system by *E. histolytica* are not yet clear mainly because the information available is fragmented, the identification of the specific amoebal components involved is required and more *in vivo* studies are needed to construct a complete picture of the inhibitory processes.

1.9 Diagnosis

The laboratory plays a key role in the diagnosis of invasive amoebiasis since, together with imaging diagnosis and colonoscopy, it confirms clinical findings in cases of fulminating colitis, amoeboma, peritonitis or liver abscess. It is also valuable when diagnosis by exclusion is carried out in entities such as inflammatory bowel disease (ulcerative colitis and Crohn's disease), carcinoma, and dysenteric syndromes caused by bacterial agents involving *Shigella spp.*, *Salmonella spp.*, *Campylobacter jejuni*, or *Clostridium difficile* among others (Healy and Kraft, 1972; Tucker *et al.*, 1975; Sanderson and Walker-Smith, 1984; Mhlanga *et al.*, 1992; Chan *et al.*, 1995). However, the frequent misdiagnosis due to lack of skilled personnel and delay in obtaining and providing

laboratory results are contributory factors to morbidity and mortality in amoebiasis (Krogstad *et al.*, 1978; Yolken, 1982). Improvement in sensitivity and specificity in differential diagnostic tests is urgently needed and ideally, those diagnostic tests should be available in a flexible format for their application in large scale studies, especially in endemic areas.

1.9.1 Microscopy

The most common method for the diagnosis of amoebiasis is the direct examination of smears of freshly shed stools for the identification of trophozoites and/or cysts. Wet-mount examinations using physiological (0.85%) saline are useful for the identification of *E. histolytica*/*E. dispar* trophozoites according to their motility and the presence or absence of ingested erythrocytes (Gonzalez-Ruiz *et al.*, 1994a). The size, nuclear characteristics and disposition of the chromatin in the cyst are diagnosed using Lugol's iodine. However, the difficulty in estimating the presence of the amoebic infection arises most commonly from low sensitivity due to failure to detect an organism during microscopical identification.

Measurement by calibrated eyepiece micrometer permits the differentiation of cysts of *E. histolytica*/*dispar* from cysts of other related amoebas (*E. hartmanni*, *E. coli*, *E. nana*, and *Iodamoeba butschlii*) and from cells and other parasites (Gonzalez-Ruiz *et al.*, 1991, Gonzalez-Ruiz and Bendall, 1995). Furthermore, in order to give an accurate result in the study of intestinal amoebiasis it may also be necessary to use several techniques in conjunction, including preservation in polyvinyl alcohol (PVA) or other fixatives such as 10% formalin and Merthiolate-Iodine-Formalin (MIF). Also permanent staining, which detects the presence of trophozoites that are missed in concentration methods, are complementary diagnostic schemes. However, for successful identification, it is important to avoid the presence of substances such as antibiotics, laxatives or anti-diarrhoeals in the samples. These substances damage parasite morphology (Proctor, 1991).

The examination of trophozoites and cysts is the most important means of distinguishing between the different species of amoeba present in the gut when microscopical diagnosis is required. However, there are few key distinctive morphological features to be taken into account. For instance, trophozoites of *E. hartmanni* are similar in morphology and motility to those of *E. histolytica/E. dispar*, although the differential feature is their smaller size, measuring between 5 and 12 μm with an average between 8-10 μm . Also, if trophozoites with ingested red blood cells are present in the sample, this gives certain identification of *E. histolytica* (Gonzalez-Ruiz *et al.*, 1994a). *Endolimax nana* trophozoites, with a similar size (6-12 μm) are easily confused with those of *E. hartmanni*, however, their large, variably-shaped karyosome is the distinctive feature. The cyst stage in *E. hartmanni* measure between 5-10 μm , spherical in shape with up to four nuclei identical to those of *E. histolytica/E. dispar*, however an overlap in size exists between cysts of *E. histolytica/dispar* and *E. hartmanni* in the 10-12 μm population. Cysts of *Endolimax nana* are 5-10 μm in size (average of 6-8 μm), with spherical or ovoidal shape and up to 4 nuclei with a large central karyosome each.

E. coli trophozoites are large in size (15-50 μm), with rough vacuolar cytoplasm with blunt pseudopods and a nucleus that can be distinguished without staining. They give rise to spherical cysts with 8 nuclei when mature; abundant peripheral nuclear chromatin in granules of irregular size and uneven distribution are present. A cyst size of 15 μm and upwards and the number of nuclei are the main morphological distinctive features.

In the case of *I. buetschlii*, trophozoites are 8-20 μm in size, with one nucleus containing a characteristic very large, centrally located karyosome. Cysts vary in size from 5-20 μm , with an average of 10-12 μm , and this is the reason why they are sometimes confused with those of *E. histolytica/E. dispar*. However, their shape is usually irregularly ovoidal and in fresh specimens contain a single nucleus with a large karyosome without peripheral chromatin. In fresh specimens the cytoplasm usually contains a large, well defined glycogen vacuole which stains dark brown with Lugol's iodine.

Technical and parasite life cycle factors such as skill of the examiner and intermittent cyst excretion influence the sensitivity of microscopy. Those factors were first indirectly measured by Kershaw as early as 1946 when reviewing the diagnosis of amoebiasis on the basis of microscopical detection of positives. His results showed that when one stool sample was examined only 50% of the cases were diagnosed whereas after the examination of 8 to 12 samples it was possible to cover about 70-90% of the cases. He concluded that after the examination of three specimens, a high proportion of positives will remain undiagnosed (Kershaw, 1946). Stamm *et al.*, (1957) studied the positivity of microscopical stool examination for *E. histolytica/dispar* cyst excretion in Pakistan, where 45% of the positive individuals were detected in the third sample, reaching 67% in the sixth sample. Similarly, during a recent study, Hiatt *et al.*, (1995) concluded that 10.4% of *E. histolytica/dispar* carriers required between 4 and 9 stool examinations before being diagnosed. Those studies support the use of auxiliary techniques such as the incorporation of concentration methods in order to improve diagnosis of most intestinal protozoa and helminth infections, especially when low numbers of parasites are present in the samples.

Flotation with zinc sulphate is often used for the diagnosis of amoebiasis. The organisms are concentrated in the surface after centrifugation because the solution has a higher specific gravity (1.18) than the parasites. This technique is particularly useful in developing countries where costs and restrictions to the use of certain chemicals are important. A key advantage of this method is that after washing, the concentrated samples are suitable for culture since there is no fixative in the solution. However, the samples must be examined promptly in order to avoid cyst distortion and trophozoites are missed. Alternatively, a formol-ether method is also useful for concentration of eggs and cysts (Allen and Ridley, 1970), and the fixation facilitates the further staining of the sample for species differentiation. However, PCR amplification of samples concentrated by this method is very difficult, unless the pellet is washed immediately after concentration, and culture requires omission of the formalin fixative.

In the particular case of the diagnosis of intestinal amoebic infection, even after detection of cysts resembling *E. histolytica/dispar* by microscopy, discriminating between these species is now recommended, thus a specific detection system is required. To fulfill this need, a number of techniques are available, including *in vitro* culture of clinical specimens, from which the isolation of microorganism is attempted for further characterisation of strains in zymodemes. Also, a range of highly specific antigen, antibody and nucleic acid detection systems have recently been developed.

1.9.2 *In vitro* Culture

One of the most useful laboratory aids for the diagnosis of amoebiasis is the cultivation of stool samples and for future reference the term "Strain" indicates a laboratory grown population of amoebas isolated from a well characterised source (Trissl, 1982).

Although cultivation is a time consuming procedure because the complex ecological relationships in the gut are difficult to mimic in the laboratory, the isolation of amoebic strains in culture permits their identification and provides protein extracts and nucleic acids for their classification into zymodemes and for molecular studies at the antigenic and genetic level respectively. Isolation of amoeba cultures directly from faeces requires the presence of the accompanying intestinal flora in the culture medium (xenic culture), where composition is not completely defined. Also, the addition of starch is necessary as a carbohydrate source, and the use of antibiotics keeps the balance between bacterial and amoebal populations allowing further subcultures. The most commonly used culture medium for isolation and maintenance of strains is Robinson medium (Robinson, 1968). It was devised as a polyxenic biphasic medium mainly based on *Escherichia coli* culture, enriched with horse serum, for the isolation and maintenance of *Entamoeba spp.*

Intensive research in amoebiasis at all levels was stimulated when axenic cultures (no associated organisms required for growth) were developed in 1961 by Dr L. Diamond.

His TTY-S-CEEM25-diphasic medium containing a cell-free chick embryo extract and horse serum was the precursor of the TP-S-1 (Diamond, 1968) monophasic medium for mass cultivation of trophozoites. Chick embryo extract was not needed and adult bovine serum replaced horse serum. Once cultivation was available, the cryopreservation of strains became possible, and with it, the first reference strains were characterised. Growth efficiency in axenic cultures was improved by substitution of the ox-liver digest in TP-S-1 by a mixture of yeast extract, iron, vitamin B12, thiocetic acid and polyoxyethylenesorbitan monooleate (Tween 80) in a new medium, TYI-S-33 (Diamond *et al.*, 1978; Diamond, 1980). However, gradual transfer from polyxenic to axenic conditions is necessary, since the process involves a long adaptation period and only virulent strains are likely to survive long term culture (Robinson, 1968). Although some axenic *E. histolytica* and a few *E. dispar* well characterised reference strains are available thanks to the process of axenisation, the problem of selection of "culturable" population remains. This could affect the many processes clarified by molecular studies, since their results could be an expression of phenomena due to the adaptive processes of the organism to culture and which might not represent the population composition in the host during the natural life cycle. An alternative approach to solve this problem from the molecular biological aspect is the study of uncultivated cyst-derived material. For this, it is necessary to establish a bank of samples or extracted nucleic acids from a wide range of geographical areas for further investigation.

1.9.3 Zymodeme Characterisation

The term "zymodeme" refers to a specific standard electrophoretic pattern of amoebic enzymes by which it is possible to group isolates. Reeves and Bischoff (1968) first studied electrophoretic patterns of five amoebic enzymes: Glucokinase (HK) EC 2.7.1.1; Glucose-phosphate isomerase (GPI) EC 5.3.1.9; Phosphoglucumutase (PGM) EC 2.7.5.1; Malate dehydrogenase (ME) EC 1.1.1.40; and NADP diaphorase (unclassified) to classify strains of *Entamoeba* species.

Comparison of the electrophoretic patterns of three enzymes: Glucose-phosphate isomerase (GPI EC 5.3.1.9), Phosphoglucosmutase (PGM EC 2.7.5.1) and L-malate: NADP⁺ oxidoreductase (ME EC 1.1.1.40) permitted the grouping of *E. histolytica sensu lato* into four groups according to the patterns found and based on those patterns, an association between the presence of a fast band in PGM and isolates from clinically symptomatic patients was established (Sargeant and Williams, 1978; Sargeant *et al.*, 1978). Also, different patterns were found for *E. hartmanni*, *Endolimax nana*, *Iodamoeba buetschlii*, *Dientamoeba fragilis*, *E. moshkovskii* (isolated from a crude sewage) some of which were known as the "Laredo" strains (isolated from man), *E. invadens* (isolated from snakes) and *E. chattoni* (isolated from monkeys). All these strains showed completely different patterns to those seen in strains of *E. histolytica* (Sargeant and Williams, 1979; Sargeant *et al.*, 1980a).

The use of zymodeme characterisation as a tool to describe the epidemiology of amoebiasis was first attempted by Sargeant *et al.*, (1980b) in Mexico City. During this study the enzyme hexokinase (HK EC 2.7.1.1) was incorporated (Farri *et al.*, 1980), where two bands of rapid migration were characteristic in pathogenic strains. Eleven zymodemes were described, and association of zymodemes II, VI, and XI to pathogenicity was established. Zymodemes were then formally defined as "a population of amoebae differing from a similar population in the electrophoretic mobilities of certain specified enzymes".

Further studies of the epidemiology of amoebiasis in a rural area in Mexico (Sargeant *et al.*, 1982b) revealed two new zymodemes: one pathogenic, XII and one non-pathogenic, XIII. A review of isoenzyme characterization of *E. histolytica* stated the presence of 14 zymodemes and established the isoenzyme patterns for other amoebae (Sargeant *et al.*, 1982c). Also, new zymodemes (XI, XIX, and XX) were reported to be produced experimentally by Blanc *et al.*, (1989) by mixing cultures of non-pathogenic with pathogenic zymodemes, and recently zymodeme XIX has been isolated from West Africa (Jackson and Suparsad, 1997) and Brazil (Barral de Martinez *et al.*, 1995). The mixture of two pathogenic zymodemes (II and XIV) produced zymodeme XX with a γ band in GPI

and PGM, also recently isolated by Gatti *et al.*, (1997) from two institutionalised patients in a psychiatric hospital in northern Italy. All the patterns found were different for the different species and were recorded as standards for zymodeme determinations on isolates. However, Blanc and Sargeant (1991) found variability in the expression of the γ and δ bands of GPI and PGM depending on the amount of starch used in Robinson's medium. When strains from zymodemes III, V, XI, XII and XX were cultured with low amount of starch (2-4 mg), the γ band in PGM disappeared. Also, strains corresponding to zymodemes IV, V, VI, X, XII, and XVIII when cultured with 2-4 mg of starch lost their δ band in the gels. The bands were expressed again in the gels when cultured with 12-15 mg of starch in Robinson's medium. In addition, intensity of the bands was directly proportional to the amount of starch in the culture medium, although α and β bands of PGM and GPI never showed any variation.

Later, Jackson *et al.*, (1992) also reported a reduction from multiple to single banding patterns in the same two enzymes during axenisation of strains belonging to zymodeme XI, which changed to zymodeme II, both pathogenic, and strains belonging to zymodeme III became zymodeme I, both non-pathogenic, thus, there was no conversion from pathogenic zymodeme to non-pathogenic zymodeme or vice versa. Interestingly those results added to the controversy on the stability of zymodemes, which originated from the description of changes in isoenzyme patterns of non-pathogenic isolates during axenisation attempts by Mirelman *et al.*, (1986) and Andrews *et al.*, (1990). During the process, the associated bacterial flora from amoebic cultures was suppressed and replaced by irradiated bacteria. Changes in PGM and HK electrophoretic patterns were demonstrated (conversion from zymodeme III patterns to zymodeme II pattern), and a concomitant increment in virulence was observed. The converted strain reacted with the *E. histolytica* specific probe P145, in contrast to the original culture which reacted with *E. dispar* specific probe B133 (Mirelman *et al.*, 1986, Bracha *et al.*, 1990). Reverse conversion was achieved by reassociating the original bacterial flora. The authors speculated about the probability that all *E. histolytica* strains contain copies of the same sequences in their genomes, but changes in conditions of growth may cause the amplification of different elements and express

modified amoebic components and behaviours, for example making them develop pathogenic characteristics. Those observations seemed to imply that sources of stress, such as adaptation to a new medium, exposure to antibiotics, and alterations in the bacterial flora in culture could induce expression of repressed genes or a post-translational process in proteins responsible for aggressive behaviour. However, the probability of selection of a previously existing but undetectable subpopulation in the original cultures was considered.

Failure of several authors attempting to reproduce conversion following the methodology described in the original papers suggests artifactual results in the original studies (Clark *et al.*, 1992, Jackson *et al.*, 1992). A possible explanation for the switch in aggressive behaviour was cross-contamination of cultures and further experiments by Clark and Diamond (1993) demonstrated that the converted isolates were identical to the corresponding reference cultures and that as few as 10 pathogenic trophozoites were able to overgrow a non-pathogenic culture. This phenomenon was demonstrable by riboprinting after 72 hours in non-pathogenic cultures where bacterial flora was suppressed. Changes of isoenzyme patterns were evident after 11 subcultures. Thus, contamination of the non-pathogenic culture with pathogenic forms during the axenisation process seems to have been the source of such conversions and the doubts about the stability of zymodemes were dismissed.

The relevance of the culture technique with zymodeme analysis for the differential diagnosis of amoebiasis was highlighted during a survey on the prevalence of amoebiasis in Mahé, an island belonging to the Seychelles. The results of this study confirmed the problem of both overdiagnosis and misdiagnosis of amoebiasis by microscopy when using culture and zymodeme analysis as gold standards. During a first screening, 24 samples were reported positive for *E. histolytica* by the resident laboratory, however, after quality control, only 50% were *E. histolytica/E. dispar* infections and after further culture and zymodeme analysis, only 8.3% (2/24) were true *E. histolytica* and 41.6% (10/24) were *E. dispar*. The lack of measurement of cysts was reflected in the report of the resident laboratory which included 20.8% (5/24) samples with *E. hartmanni* that were originally

identified as *E. histolytica* positive specimens by the microscopist (Sargeant, 1992). During a second screening, a different laboratory on the island reported 12 samples as positive for *E. histolytica*, from them, isoenzyme analysis identified 5 (41.6%) *E. dispar* infections, 6 (50%) *E. hartmanni* strains and one was *D. fragilis*. The results from the Mahé study revealed that from 313 stools cultured, 121 gave a positive culture, from which 6.6% were identified as *E. histolytica* isolates, 33% were classified as *E. dispar* and 60.3% were positive for other intestinal amoebas. At this point it is necessary to note that the sensitivity of the culture technique is variable and when a culture does not grow it should not be necessarily assumed that it reflects a misdiagnosed sample. Nevertheless, the samples containing *E. histolytica/dispar* in the Mahé study (48/313) revealed a prevalence of 2.5% (8/313) for *E. histolytica* infections and 12.7% (40/313) for *E. dispar* infections after their confirmation by zymodeme analysis on the relevant cultures. Those 48 *E. histolytica/E. dispar* infections identified by microscopy masked 16.7% (8/48) true *E. histolytica* infections and, importantly, 83.3% *E. dispar* carriers, that would have been overdiagnosed as having *E. histolytica*.

Polyxenic culture of specimens with zymodeme analysis is a very valuable tool and it is currently the gold standard technique for the classification of isolates as invasive or non-invasive strains. However, because zymodeme analysis is not a technique of routine use during clinical diagnosis of amoebiasis, it is only available in few reference laboratories through the world. The technique is expensive and not ideal, taking more than a week, the failure of some cysts to grow, and the loss of cultures before lysates can be made. Moreover, as mentioned above, selection of amoebic population occurs in the process of initial cultivation and subsequently in continuous culture. Added problems are the experience and skill required for the careful standardisation of cultures, manipulation of the protein lysates and interpretation of the electrophoretic patterns.

1.9.4 Antibody Detection

Serology is one of the most useful tools for the diagnosis of extraintestinal amoebiasis and seroprevalence is a good global indicator of endemicity of invasive infection, especially when data on differential diagnosis are not available. The importance of techniques such as Enzyme-Linked Immunosorbent Assay (ELISA), radioimmunoassay (RIA), indirect immunofluorescence (IFAT) and indirect haemagglutination (IHA) arises from the possibility to examine a large number of samples rapidly, with small amounts of serum and with reasonable sensitivity and specificity (Maddison, 1991). Although a wide variety of serological tests are currently successfully used in developed countries to detect extraintestinal amoebiasis, in 95% or more cases (Krogstad *et al.*, 1978), the distinction between background levels of antibody in uninfected persons and raised levels in persons suffering from intestinal disease in endemic areas is difficult to assess due to the fact that antibodies can persist for 2 to 5 years or more after therapy (Krupp and Powell, 1971).

Indirect haemagglutination uses erythrocytes sensitized with axenically cultured antigen. If present, antibodies react against and agglutinate the red cells. It has been reported to be positive in 87% to 95% of amoebic liver abscess and in 85% to 95% of patients with acute amoebic dysentery (Proctor, 1991). For indirect immunofluorescence (IFAT) methanol or acetone fixed trophozoites are incubated with patient's serum on a slide. Antibodies will bind to the trophozoites and the reaction can be detected with anti-immunoglobulins labelled with fluorescein. Vijayamma *et al.* (1981) evaluated this technique for the diagnosis of invasive amoebiasis. The test was positive in 96% of the patients with hepatic amoebiasis and in 73% of those with amoebic dysentery. The test was not useful in cases of amoebic colitis, in which only 15% of the cases had positive reaction with low titres. When compared to indirect haemagglutination, Carvalho *et al.* (1991) found IFAT to be equally sensitive to IHA in individuals with *E. histolytica* trophozoites. The test was positive in 75% of the individuals, confirming previous reviews (Healy, 1971).



ELISA is one of the most sensitive and specific techniques used in the diagnosis of hepatic amoebic abscess. The test can be performed without sophisticated technology and can be read visually or by colorimeter. Highly skilled personnel are not necessary and the test can be adapted to use microquantities of reagents and serum. Crude extracts (Aguirre *et al.*, 1991a, Restrepo *et al.*, 1996; Kraoul *et al.*, 1997), the purified adhesin from axenic cultures of *E. histolytica* reference strains (Abd-Alla *et al.*, 1992, Braga *et al.*, 1996) and recombinant proteins (Stanley *et al.*, 1990, Lotter *et al.*, 1993) are among the different sources of antigen used.

One of the ELISA techniques that has incorporated the use of a novel antigen is the detection of IgG raised against the heavy subunit of the galactose-inhibitable adherence lectin for the diagnosis of invasive amoebiasis. The protein was affinity purified from axenic cultures of *E. histolytica*, using a mixture of five mouse monoclonal antibodies. Although sensitivity was found to be 94% and specificity is 90%, it does not differentiate between past and present infections (Abd-Alla *et al.*, 1992). Also, as opposed to the use of crude antigen where a greater number of epitopes are exposed to the sera tested, using one antigen limits the response measured. Nevertheless, the advantage of systems using recombinant technology is that such systems eliminate the need for massive culture of this fastidious organism. The problem could be solved by the use of a mixture of different recombinant antigens.

Commercial tests for the detection of anti-*E. histolytica* antibodies are available. Kraoul *et al.*, (1997) evaluated an indirect hemagglutination (IHA Amoebiasis kit, Fumouze Diagnostics, France), a latex agglutination (LA Bichro-latex kit, Fumouze Diagnostics) and a rapid enzyme immunoassay kit based on the standard ELISA format (EIA, Amibiase Serologie, LMD, California). A population of 143 sera were screened, including 43 sera from patients with diagnosis of hepatic amoebiasis, 33 sera from patients diagnosed with a hepatic disease different from amoebiasis and 67 healthy individuals. The highest sensitivity was achieved with the IHA kit (97.6%), with a specificity of 97%; the positive predictive value was 93.3% and the negative predictive value was 98.9%. However, EIA

was 100% specific and sensitivity was 93%, with a positive predictive value of 100% and a negative predictive value of 97.1%. LA specificity was compared against the EIA results and it was found to be 95%, with a sensitivity of 90.7%. The lowest positive and negative predictive values were scored by this test, 88.6% and 95.9% respectively (Kraoul *et al.*, 1997).

The use of EIA was considered of advantage for sample processing (20 minutes) and simplicity. Although costs are higher than IHA and LA, they are reduced when the number of samples to process increases, making it ideal for its use in endemic areas for the diagnosis of hepatic amoebiasis. Dot-ELISA, is a variation of the standard ELISA microwell format that was developed by Kumar *et al.*, (1983). This test uses nitrocellulose sheets as the matrix to which the antigen is applied. The advantages of this technique are the reduction of incubation times compared to conventional ELISA and that predotted sheets can be stored for up to three months making them available for field use.

The comparison of two recently standardised serological tests (including a recombinant antigen) with an EIA in which the antigen was a standard crude soluble extract (SA-EIA) of *E. histolytica* was carried out in South Africa by Lotter *et al.*, (1995). The P1-EIA test is based on a recombinant surface protein of *E. histolytica* and the latex agglutination test (M-LA) uses a membrane fraction as the antigen covering the polystyrene beads. After testing 76 patients with proven invasive amoebiasis and 91 control subjects with no amoebiasis but other conditions comprising lung and liver disease, cancer, gastroenteritis, and other abdominal or general diseases, the sensitivity of M-LA and P1-EIA were 96.1% and 86.1% respectively whereas specificity was 92.3% and 96.7% respectively. The SA-EIA assay had the best sensitivity at 97.4% although its specificity was lowest at 62.5% due to detection of high antibody titres in the control group.

Persistence of antibodies after treatment was measured with the P1-EIA test in 16 patients with amoebic liver abscess and high antibody titres at admission. All patients except one tested negative or slightly positive after a 5 month follow up period (Lotter *et*

al., 1995). The clearing of anti-P1 specific antibodies could indicate the possibility of using this test in endemic areas, however, it is necessary to test considerably more samples against other crude antigen preparations such as whole lysates of cultured trophozoites.

1.9.5 Antigen Detection

The difficulty of sensitive and specific detection of *E. histolytica* in faecal samples has led to the development of many antigen detection methods using solid phase immunoassays. Root *et al.*, (1978) used an immunochemical method involving a rabbit polyclonal IgG conjugated to enzyme able to detect as little as 0.22 µg/ml of amoebic protein. However, freezing resulted in the loss of reactivity of the sample and non-specific background was increased after storage of the samples for 24 hours at 4°C. Some non-specific reaction was due to the presence of peroxidase activity in the sample, pigments masking the enzyme reaction and unidentified cross reacting components. Additionally, the long incubation times with the sample and with the conjugate were perceived to be disadvantageous for mass screening of samples. Later, during a survey in Mexico city, the test revealed a prevalence of infection of only 4.4%, compared to 10.6% detected by microscopy (Randall *et al.*, 1984). With hindsight, however, no doubt a proportion of the infections diagnosed microscopically as "*E. histolytica*" were *E. hartmanni*, or *E. dispar*.

The use of a multilayer ELISA by Grundy (1982) brought a new possibility for the development for the detection of *E. histolytica* antigens in stool samples. This ELISA used a rabbit anti-*E. histolytica* capture antibody and a human anti-*E. histolytica* immunoglobulin as the detecting antibody. The sensitivity of the test was 0.15-0.3 µg/ml *E. histolytica* protein, equivalent to 1 trophozoite. The test did not react with faeces containing *E. histolytica* cysts. This was probably due to the antigenic differences between cysts and the trophozoites used to produce the capture antibody. In 1987 the system was modified in order to detect cyst passers excreting low numbers of parasites (Grundy *et al.*, 1987). The system used specific sheep anti-*E. histolytica* immunoglobulins as the capture

step and rabbit anti-*E. histolytica* IgG as the detecting antibody. The conjugate was sheep anti-rabbit immunoglobulin coupled to horseradish peroxidase. A slightly different approach with the capture ELISA format was followed by Baumann and Gottstein (1987) with a double-antibody sandwich ELISA for the detection of detergent-solubilized *E. histolytica* antigen in stool samples using rabbit hyperimmunoglobulins for the capture and the detection steps. This assay was reported to have a sensitivity of 93% and a specificity of 93%. The results of this test were not affected by the repeated freezing and thawing of the samples nor by their storage for several months. However, the assay was able to detect only trophozoites, probably due to the different antigenic determinants released during the solubilizing procedure, with the cysts being more resistant than trophozoites.

Among the currently commercially available tests for antigen detection in faeces, ProSpecT EIA (Alexon Inc., Sunnyvale, CA) is widely used for detection of *E. histolytica*/*E. dispar* infections by detecting an "*E. histolytica*-specific" antigen (EHSA), with a sensitivity of 78% and a specificity of 99% when compared to microscopical analysis (Ong *et al.*, 1996). Further evaluation of this test revealed a 35% sensitivity when compared to microscopy of faecal samples (Benzeguir and Kettis, 1997). The test was also evaluated with samples from 469 German travellers returning from endemic areas. In this study, the sensitivity of the test was 90.3% while by microscopy only 87.1% of cases were detected (Jelinek *et al.*, 1998). However, although the sensitivity of the ProSpecT EIA is better than microscopy, this test does not differentiate *E. histolytica* from *E. dispar* and as such is useful for screening purposes only.

Antigen detection using monoclonal antibody technique is an attractive approach for the diagnosis of amoebiasis. One of the advantages of using these antibodies is the fact that they can be selected against a group of antigens to achieve a high level of specificity and sensitivity. Once the cell line secreting the antibody is obtained, it should be immortal and a constant source of monoclonal antibody. However, monoclonal antibodies often have low affinity constants rendering assays insensitive. It is therefore necessary to choose for affinity as well as for specificity. The use of enzyme immunoassays incorporating

antibodies for the detection of antigens in body fluids is becoming more widespread. The increased specificity provided by the monoclonal antibody is linked to higher sensitivity due to the enzyme-substrate amplification stage.

Ungar *et al.*, (1985) developed an indirect enzyme-linked immunosorbent capture assay for the detection of *E. histolytica* in human faeces using a monoclonal antibody for the capture step and immune rabbit sera for detection. The test had a 82% sensitivity and 98% specificity. This capture assay could detect both cysts and trophozoites even if the samples were frozen. The use of monoclonal antibodies has the advantage of bulk production but the structural lability of the antibody produced makes it difficult to keep them attached to solid phase surfaces.

Strachan *et al.*, (1988) reported a technique using monoclonal antibody in immunofluorescence. The advantage of this technique was the possibility to distinguish between pathogenic and non-pathogenic *E. histolytica* quickly (2 days) compared to diagnosis by isoenzyme tests. The disadvantage of this assay was the necessity to establish cultures from the stools in order to obtain trophozoites to be tested. Only 50% of the stools cultured in this study were positive. Tachibana *et al.*, (1990) produced the monoclonal antibody 4G6 that reacts only with pathogenic strains of *E. histolytica* and identified an invasive-specific antigen with a molecular weight of 30 kDa which is used for diagnosis in cultures.

Gonzalez-Ruiz *et al.*, (1992) produced a monoclonal antibody capable of identifying invasive strains of *E. histolytica* by immunofluorescence. When assayed by western blot, it reacted with an 84 kDa antigen in pathogenic strains and with an 81 kDa antigen in non-pathogenic strains. The fact that this antibody recognises only invasive strains by IFA on methanol fixed amoebas suggests that this epitope is detected only under non-denaturing conditions in invasive strains and it is present but non exposed in non-pathogenic strains. Also recently, detection of the galactose and N-acetylgalactosamine inhibitable adherence lectin of *E. histolytica* and *E. dispar* has been made available in the form of two

commercially available tests, *Eh/Ed* CELISA with Mabs detecting epitopes 1 and 2 and the specific *E. histolytica* CELISA test uses Mabs against epitopes 3 to 6 to detect the adhesin of *E. histolytica* (Cellabs, Cat. Ref. KE1 and KE2 respectively).

1.9.6 Nucleic Acid Detection

Molecular biological technology has been used to distinguish pathogenic from non-pathogenic strains of *E. histolytica* by means of techniques involving the use of DNA probes and the Polymerase Chain Reaction (PCR) in the same assay. The advantages of these techniques are their sensitivity (<200 trophozoites) and specificity since with appropriate stringency conditions, no cross reactions with other amoebae are present (Bracha *et al.*, 1990). Moreover, Samuelson *et al.*, (1989) reported direct detection of *E. histolytica* in faeces using a DNA probe. Specific applied molecular biological techniques in faecal material provide more accurate information of the organisation in the infecting population because the problem of culture media selection pressure is avoided. Garfinkel *et al.* (1989) isolated non-coding repetitive elements from the 23 kb extrachromosomal episome of *E. histolytica* and reported two DNA probes: one (P133), specific for non-pathogenic forms and the second (P145) specific for pathogenic strains. Comparison of the DNA sequences showed only 44% identity, giving support for high genomic differences between pathogenic and non-pathogenic *E. histolytica*.

At approximately the same time, the detection by two single copy cDNA probes representing homologous genes in two strains of *E. histolytica*, one pathogenic (cEH-P1) and the other non-pathogenic (cEH-NP1) was reported by Tannich *et al.*, (1989). DNA sequence analysis on these particular sequences together with the comparison of their predicted amino acid sequences showed 12% evolutionary divergence in structure. These findings support the hypothesis of 2 genetically defined species in *E. histolytica*.

Studies on homologous cysteine proteinases of *E. histolytica* by Tannich *et al.*, (1991) revealed a 16% divergence in the nucleotide sequence when pathogenic and non-pathogenic subspecies were compared. Support for the hypothesis of the existence of two morphologically indistinguishable species in *E. histolytica* was also provided by Clark and Diamond (1991). When studying the multicopy ribosomal RNA genes (on episomal DNA) of the two forms they found that they were distinct with an estimated genetic distance between the small subunit rRNAs of pathogenic and non-pathogenic strains of 2.2%, comparable to differences found between human and mouse rRNA. Also, 15 pathogenic strains of *E. histolytica* isolated worldwide had identical riboprints. Cruz-Reyes *et al.* (1992) confirmed the hypothesis of Clark and Diamond (1991) when performing a fingerprint analysis of total DNA from *E. histolytica* isolates by probing with recombinant ribosomal DNA sequences amplified by PCR.

1.10 Treatment

Amoebicides (drugs for the treatment of amoebiasis) are divided into three categories namely luminal, systemic and mixed amoebicides. Luminal amoebicides are characterised by their low absorption in the lumen: diloxanide furoate (Furamide), the halogenated 8-hydroxyquinolines diiodohydroxyquin (Iodoquinol) and iodochlorohydroxyquin (Clioquinol or Enterovioform); also antibiotics such as erythromycin, tetracycline and paromomycin (Humatin) are able to eliminate luminal forms. Systemic amoebicides, which include emetine, dehydroemetine and chloroquine (Aralen) are used to eliminate tissue forms (invasive trophozoites) and are less effective against luminal forms. Mixed amoebicides such as the nitroimidazole derivatives metronidazole (Flagyl), tinidazole (Fasigyn), and ornidazole (Tiberal) are effective against both luminal and systemic forms, however because their rapid absorption, their effectiveness against luminal forms is reduced and they are normally administered in association with a luminal amoebicide (Webster, 1990; Saavedra-Lira and Perez-Montfort, 1996).

The most widely used drug is metronidazole (1-[2'-hydroxyethyl]-2-methyl-5-nitroimidazole), a 5-nitroimidazole whose broad spectrum of protozoal and antimicrobial activity includes the treatment of trichomoniasis, amoebiasis and giardiasis as well as serious entities caused by anaerobic bacteria (*Bacteroides spp.*, *Clostridium spp.*, *Fusobacterium spp.*, *Peptococcus spp.*, *Peptostreptococcus spp.* and *Eubacterium spp.*). The mode of action of the drug is based on the reduction of the drug by pyruvate:ferredoxin oxidoreductase to give a toxic free radical. The capacity of the intermediate products to oxidize the DNA causes strand breakage, loss of the helical structure by interaction with guanines and cytosines and cellular death in bacteria (Coulson, 1995).

Acute amoebic colitis is treated with 750 mg of metronidazole three times daily for 5-10 days, orally or intravenously. Side effects of metronidazole are nausea, vomiting and abdominal discomfort. Alcohol ingestion must be avoided when taking metronidazole because of the uncomfortable side effects caused by inhibition of the enzyme alcohol dehydrogenase (disulfiram effect). The possibility of mutagenic and carcinogenic effects has been proposed after positive results were seen in the Ames *Salmonella* mutant test and in animal models. Early reports from Legator *et al.*, (1975) detected mutagenic activity (using the Ames test) in urine of patients after 6 days of treatment. Studies by Rustia and Shubik (1979) where metronidazole induced hepatomas, mammary and pulmonary tumours in noninbred rats after prolonged treatment seemed to confirm those results. In addition, studies from Ostrosky-Wegman *et al.*, (1994) used biomarkers of cell proliferation in lymphocyte cultures of individuals treated with metronidazole and found an increase in lymphocyte proliferation after treatment. However, Beard *et al.*, (1979) found no appreciable increase of cancer in humans after the use of metronidazole, and Friedman & Shelby, (1989) did not notice a rise in the incidence of tumours.

The controversy remains today since implication of metronidazole as a dangerous agent is difficult due to the large latency periods between exposure, clinical manifestations and detection bias in the cancer studies.

During treatment of amoebic colitis it is important to continue the course of treatment with luminal agents since metronidazole reaches lower concentrations in the lumen. Although its mechanism of action is not yet known, the most common luminal agent is diloxanide furoate, with an efficacy rate higher than 90%, given 500 mg orally three times daily for 10 days. Among the 8-hydroxyquinolines used as luminal amoebicides, Iodoquinol (Diodohydroxyquin) is also used, 650 mg three times a day orally for 20 days but must be avoided in patients with hepatic failure or iodine allergy. Doses exceeding 2 g per day for long periods are associated with subacute myelo-optic neuropathy (SMON) (Webster, 1990). Aminoglycoside antibiotics such as Paromomycin are administered over 5-10 days in doses of 1.5 g per day or three doses of 25-35mg/kg/day in children.

In cases of extraintestinal amoebiasis, metronidazole remains the treatment of choice. Cure rates greater than 95% are reached when the drug is given for 5-10 days. Other drugs such as chloroquine, emetine and its less toxic derivative, dehydroemetine are used for the treatment of severe invasive intestinal and extraintestinal amoebiasis but their use is restricted to cases where nitroimidazoles are ineffective or contraindicated. Emetine blocks the synthesis of proteins in the trophozoite by inhibiting the transfer of peptidyl-tRNA in the ribosome from the aminoacyl to the peptidyl site. Some of its adverse effects involve the cardiovascular and neuromuscular systems, the central nervous system and the gastrointestinal tract (Webster, 1990).

Although little is known about resistance to drug treatment in amoebiasis and there are only a few reports on treatment failure, emetine resistant strains have been produced *in vitro*. Those strains exhibit the same characteristics found in mammalian tumour cells with multidrug resistance phenotype (MDR), which are resistant to multiple chemotherapeutic drugs and overexpress a P-glycoprotein which increases drug efflux. Cross-resistance to hydrophobic anti-amoebic drugs such as colchicine, iodoquinol and diloxanide is one of the features of the amoebic emetine-resistant mutants, and six P-glycoprotein-like genes with 40-41% homology to human *mdr* genes have been characterised (Descoteaux *et al.*, 1992; Gomez *et al.*, 1996). Therefore, the emergence of wild populations of *E. histolytica*

carrying *mdr* genes must be considered as a potential problem for the future control of invasive amoebiasis.

New anti-amoebic compounds based on the energy metabolism of *E. histolytica* are under development. Inhibitors such as gossypol, particularly targeting alcohol dehydrogenase and malic enzyme, and several bisphosphonates, inhibitors to the PPI-dependent enzymes phosphofructokinase and pyruvate phosphate dikinase are being tested. Compounds, with few toxic effects, could offer a safer alternative for the treatment of invasive amoebiasis (Saavedra-Lira and Perez-Montfort, 1996; Byington *et al.*, 1997).

1.11 Epidemiology and Control

Assessing the epidemiology of amoebiasis in the world is a difficult task due to the technical difficulties and because many of the previous studies on the transmission dynamics of amoebiasis were based on the observation of cyst excreting individuals, the majority of whom were probably harbouring *E. dispar*. Problems in the estimation of prevalence are related to the lack of clear case definitions and homogeneous methodology for the diagnosis in the different studies, selection bias in the populations sampled, and inability to easily identify invasive strains. For this reason, previous reports on prevalences of cyst excretion are to be re-evaluated using new differential diagnostic tools in order to separate *E. histolytica* from *E. dispar* infections. Based on the review of the prevalences and incidences of amoebiasis from 1970 to 1985, Walsh (1986) concluded that despite the fact that in the population infected with *E. histolytica sensu lato*, prevalence remained the same during this period of time, the growth of the population reflected an increase in the incidence of infection. From 500 million people infected, 38 million developed invasive disease and from those, about 40 thousand died. Simplistically, we can deduce that 90% of infections were probably due to *E. dispar* and the remaining 10% of infections were due to *E. histolytica*. This remains to be confirmed using modern techniques.

Prevalences of amoebiasis in endemic areas remain stable and mortality is low. However, morbidity causes a great economic impact in poor populations in terms of production and it is directly related to their daily subsistence income. The patterns of infection and disease for *E. histolytica* differ from other intestinal protozoa, for example, it has been observed that susceptibility to clinical amoebiasis increases with age, so that adults are ten fold more susceptible to develop invasive disease compared to children, and males are more likely to develop invasion than females (Bundy, *et al.*, 1992). However, because of their role in the household, females seem to be the source of infection to children (Bray and Harris, 1977; Ruiz-Palacios *et al.*, 1992).

Improvement in water supply availability, together with sanitary education and adequate excreta disposal may reduce transmission but longitudinal studies with homogeneous methodology and good control of confounding variables must be designed. The presence of *E. histolytica* cysts in the environment is difficult to demonstrate although cysts are able to survive moist environments, and, remain viable in water supplies, seawater, faeces, night soil, sewage and crops contaminated through irrigation with contaminated water or night soil. However, cysts are sensitive to desiccation, which reduces their viability on the surface of vegetables to three days in dry conditions or less than one day in the environment under dry, hot weather. Vegetables are made safe by soaking them in water at 55°C, vinegar for 30 minutes (Warhurst, 1983).

The most effective method for the control of the transmission of parasitic diseases is the sanitation of water at high temperature. Heating water at 60°C for 1 minute or at 55°C for 10 minutes ensures total destruction of cysts, however, cysts survive longer in water at lower temperatures. Cysts are also eliminated by chlorination of water in the form of 3 mg/L of free chlorine residual ie. after the specified contact time of 30 minutes, there is 3mg/L free chlorine remaining. However, the process is again temperature dependent and normally requires higher concentrations or longer contact times than those used to eliminate faecal bacteria (Warhurst, 1983). For the latter, only 0.5 to 1 mg/L residual chlorine is needed for 30 minutes.

Treatment of cyst excretors is one of the proposed approaches for the control of amoebiasis. Mass treatment has been applied in some endemic areas, with a rapid reduction in prevalence. However, reinfection rates are high and this approach must be carefully considered under the knowledge that 90% of infections are due to *E. dispar* and the risk of invasive amoebiasis in those carriers is very low (Ruiz-Palacios *et al.*, 1992). Drug pressure is also an important factor for the emergence of drug resistant populations of parasites, which could create greater public health problems.

The improvement of quality of life (housing, sanitation and education) is the holistic approach suggested for the success of parasitic control programmes. However, those measures are only effective with an adequate infrastructure for primary health care at the community level (Mata, 1982). Mathematical modelling in amoebiasis is useful to predict the effect of potential intervention. A theoretical model proposed by Knight (1975) suggests that an improvement in hygiene will halve transmission in a population with a 50% prevalence of amoebic infection if applied for more than 10 years, meanwhile mass treatment will not be effective if transmission remains constant.

The identification of virulent organisms is an important target to improve the cost-effectiveness of treatment and to propose prophylactic measures and control strategies for *E. histolytica* infection. New diagnostic strategies and estimation of infection prevalences are needed for the evaluation of the epidemiology and distribution of *E. histolytica* and *E. dispar* in endemic and non-endemic areas. Also, only after carefully planned long term prospective studies will it be possible to establish effective intervention measures for the control of amoebiasis. Other intervention strategies such as a vaccine have been proposed for the control of amoebiasis and protection studies with the galactose-specific adherence lectin of *E. histolytica* suggest that a recombinant vaccine is likely to be available in the future (Petri and Ravdin 1991, Zhang and Stanley, 1997, Stanley, 1997)

As in many other parasitic infections, the factors influencing the worldwide distribution of amoebic infection remain unclear. From a universal point of view, it is

known that the interaction of factors such as the environment, human behaviour and parasite biology affect the epidemiology and transmission of parasites. For instance, limiting faecal-oral transmission by encouraging improvements in hygiene habits in food handlers, specially women and girls, and linking the hygiene campaigns to availability of good quality water supplies and soap would lower the rate of parasite transmission. However, it is also desirable to readily identify reinfections through epidemiological surveillance programmes in endemic areas. Research on the environmental, sociocultural and behavioural factors relevant to amoebiasis is needed.

1.12 Aims of the Project

1. To confirm the data on prevalence of intestinal amoebic infection in two areas believed to be endemic in Colombia, using routine microscopy of stool samples, culture and serology.
2. To evaluate the potential of MAb 20/7D to be incorporated as a tool for the differential diagnosis of amoebiasis in endemic areas.
3. To standardise a PCR system for the sensitive and specific differential diagnosis of amoebiasis in clinical samples.
4. To evaluate PCR-SHELA as a tool for differential diagnosis of amoebiasis in endemic and non-endemic areas.
5. To propose effective research targets in endemic and non-endemic areas according to the new data obtained from the evaluation of new differential diagnostic tools.

CHAPTER 2

GENERAL METHODS

2.1 Microscopy of Stool Samples

Two smears of approximately 2 mg of each fresh stool sample were prepared on a microscope slide. The smear at one end of the slide was made in isotonic saline solution (Appendix 1) and the smear at the other end was stained with one drop of Lugol's-iodine solution (Appendix 1). Each smear was covered with a 22x22 mm glass cover slip. The sample was examined by microscopy with 10X and 40X objectives for the presence of trophozoites in the saline side of the slide and for the presence of cysts and ova on the Lugol's iodine half. All cysts of amoebas were measured with an ocular micrometer to ensure the quality of the diagnosis.

2.2 Zinc Sulphate Concentration Method

Three serial samples from each subject were pooled and approximately 1g was diluted in 14 ml of a saturated zinc sulphate solution prepared in water (density 1.18) and centrifuged at 1500 rpm for 15 minutes. A cover slip was then carefully placed over the liquid surface on each centrifuge tube and after one minute it was transferred to a glass slide with a drop of Lugol's iodine (Faust, *et al.*, 1938) and each sample was examined for the presence of cysts.

2.3 *In Vitro* Culture of Trophozoites

2.3.1 Axenic Culture of *Entamoeba histolytica* Trophozoites

Axenic trophozoites of *Entamoeba histolytica*, (strain HM1-IMSS) were grown in TYI-S-33 medium (Diamond *et al.*, 1978a. See Appendix 1) in 12 ml flat sided Nunc

culture tubes (Nunc 1-56758A) at 37°C and growth was checked every 24 hours using an inverted microscope until confluent growth was achieved. These cultures were then chilled on an ice bath for 20 minutes in order to detach the trophozoites, centrifuged at 200xg for 10 minutes at 4°C, and the pellet was resuspended in fresh medium and distributed into 25 cm² cell culture flasks. The medium in each flask was replaced every 48 to 72 hours until the growth became confluent. For antigen preparations, the trophozoites were harvested by centrifugation, and the pellets were washed in chilled PBS pH 7.2 (Appendix 1), repeating the procedure 3 times. These cells were then counted using an improved Neubauer haemocytometer, adjusted to 1x10⁶ trophozoites/ml and the pellets were stored at -70°C.

2.3.2 Polyxenic Culture (Robinson, 1968)

About 50 mg of each faeces was added to a culture bottle containing a saline agar slant, 0.1 ml of 0.5% erythromycin (Sigma E-6376), approximately 10 mg of rice starch (BDH), and 2 ml of primary Robinson's medium (Appendix 1). Each culture was incubated for 24 hours at 37°C before the supernatant was removed and replaced by secondary Robinson's medium (Appendix 1). After a further 24 hours incubation, each supernatant was discarded and an aliquot of the sediment was examined microscopically for the presence of trophozoites after addition of Lugol's iodine. When positive, subcultures were made three times a week in secondary Robinson's medium and an average of 5 sediments per isolate were pooled before expanding. Each pool was transferred into a 25 cm² cell-culture flask and filled with liquid Robinson's medium (Appendix 1), incubated at 37°C for 48 hours and chilled in ice-water bath for 20 minutes. After centrifugation at 200xg for 10 minutes at 4°C, the trophozoites were washed 3 times in PBS pH 7.4 and cells were counted in haemocytometer. Pellets were processed according the corresponding antigen preparation.

2.4 Isoenzyme Characterisation of Isolates

Starch gel electrophoresis was used to separate specific isozymes (hexokinase, HK EC 2.7.11; glucose-phosphate isomerase, GPI EC 5.3.19; L-malate dehydrogenase, ME EC 1.1.1.40; and phospho-glucomutase, PGM EC 2.7.5.1) by size and charge and therefore to classify different isolates of *E. histolytica* and *E. dispar* as described by Sargeant *et al.* (1978). The variation in protein structure of these enzymes indirectly reflects the presence of genetic differences among organisms. The advantage of using enzymes for the classification of isolates is the specificity of their substrates, yielding specific migration patterns.

2.4.1 Lysates for Starch Gel Electrophoresis

Lysates were prepared from a minimum of 1×10^5 trophozoites per ml. After centrifugation at 200xg for 5 minutes, the supernatants were discarded and the pellets were diluted with 20 μ l of lysis buffer which contained protease inhibitors (Appendix 1) and then immediately transferred to liquid nitrogen.

2.4.2 Starch Gel Electrophoresis

4.3 g of electrophoresis starch (BDH) was dissolved in 43 ml of the corresponding tank buffer (Appendix 1) in a round-bottomed flask. This mixture was stirred while being heated to boiling temperature. The mixture was then degassed and 40 ml was spread onto each glass plate and allowed to cool for 20 minutes. Meanwhile, each of 4 tanks was filled with 800 ml of the corresponding tank buffer (Appendix 1) and two sponge (plastic) wicks were soaked in the same buffer. The cooling plate was put in the tank and connected to the tap. Embroidery cotton boiled in water and dried on filter paper was cut into threads

for sample application. When ready, slots were made in the gel and the lysates were thawed. Threads were saturated in these samples and placed with forceps in the slots (one thread for one sample) ensuring that they were below the gel surface. Each gel was then placed in the tank, and the wicks were then adjusted to ensure that a good contact between the buffer and the gel was obtained. A thin glass plate was placed crosswise onto each gel plate and a thick glass cover was placed covering the gel plates. Each gel was subjected to a constant current of 20 mA (240 V) for 3 hours while each gel was cooled by the circulation of tap water at approximately 15 °C.

2.4.3 Substrate Preparation for Isoenzymes

After electrophoresis, each gel was stained with a specific substrate in the dark. ME was developed by incubation with a mixture containing malate, NADP, phenazine methosulphate (PMS) and the tetrazolium salt, MTT. The NADPH₂ formed by the enzymatic reaction, in the presence of PMS (hydrogen carrier), reduced the MTT to a blue insoluble formazan which was deposited at the sites of ME activity.

GPI was stained by a similar method by employing fructose-6-phosphate, glucose-6-phosphate dehydrogenase (G6PD) (linking enzyme), NADP, PMS and MTT. Glucose-6-phosphate formed at the sites of GPI activity, then reacted with the NADP in the presence of the G6PD to form NADPH₂ and then reacted with PMS to reduce the MTT to formazan (Bagster and Parr, 1973).

PGM was developed using glucose-1-phosphate, glucose 1-6-diphosphate, NADP and glucose-6-phosphate dehydrogenase (see above). HK was identified by its ability to convert glucose to Glucose-6-phosphate with conversion of added Adenosine 5'-Triphosphate into Adenosine diphosphate. Glucose-6-phosphate dehydrogenase is the linking enzyme to convert G6P into 6-phosphogluconate. This reaction produces NADPH₂ and MTT is converted into formazan through the link with PMS.

2.5 Cryopreservation of Trophozoites

Trophozoite cultures were centrifuged at 700 g for 5 minutes and most of the supernatant was discarded and the pellet was resuspended (in the remaining amount of supernatant) to a concentration of 2×10^6 trophozoites per ml. 0.5 ml aliquots were distributed into 1.2 ml cryovials (Nalgene, Sigma V-4757) and an equal volume of cryoprotectant solution (15% v/v Sigma D-5879 dimethylsulphoxide, in MRC Ringer) was added and mixed. After equilibration at room temperature for 15 minutes, these vials were then transferred to a Union Carbide BF5 freezing plug in position F and then placed in the neck of a LR-35-9 cryobank overnight, to slowly cool (at 1°C per min.) the samples to -50°C. The vials were then transferred into liquid nitrogen.

For retrieval, each vial was placed in a water bath at 37°C until thawed and its content was immediately transferred into a 12 ml flat-sided plastic test tube filled with pre-warmed complete culture medium and incubated at 37°C for 24 hours. Then, the used medium was decanted carefully and replaced with fresh complete medium. Observation under the inverted microscope to confirm confluent growth was done every 24-48 hours.

2.6 Estimation of the Protein Concentration of Lysates

Protein concentrations were estimated according to the bicinchoninic acid (BCA) protein Assay Reagent (Pierce 23223) into microtiter plates. BCA reagents A and B were mixed at a proportion of 1/50 and 200 µl volumes were added to wells in an ELISA plate. Duplicate wells with standards of 1, 0.5, 0.25, 0.125, 0.0625 and 0 mg/ml of Bovine Serum Albumin (Sigma A-4503) in PBS were used and 10 µl of either standard or culture lysate were added to the freshly prepared BCA working reagent. Plates were sealed with cellotape, incubated at 37°C for 30 minutes and Optical densities (ODs) at 570 nm were determined with a Dynatech MR 700 microplate reader and protein concentrations were calculated from the standard curve.

2.7 Trophozoite Antigen Preparations

2.7.1 Antigen Preparation for Rabbit Immunisations

Entamoeba histolytica HMI-IMSS trophozoites were grown axenically in TYI-S-33 (TPS-1) medium as described in 2.3.1. Approximately 1×10^6 trophozoites were washed three times at 200xg for 10 minutes in sterile PBS pH 7.2 (Appendix 1). After the third wash, the supernatant was discarded and the pellet was immediately resuspended in 0.25 ml of PBS and diluted 1:1 in complete Freund's adjuvant (Sigma F-5881). The antigen was homogenised with the help of a 1 ml syringe, 21 gauge needle.

2.7.2 Antigen Preparation for ELISA Plates

Trophozoites of the strain HMI-IMSS were axenically cultured as described in 2.3.1. The trophozoites were diluted 1:10 in sterile distilled water (hypotonic shock), frozen and thawed three times and finally centrifuged at 700 g for 10 minutes. The protein content of the supernatant was determined by the bicinchoninic acid (BCA) Protein Assay (Pierce 23225) using dilutions of bovine serum albumin (BSA Sigma A-4503) as standards. The protein concentration of lysates was adjusted to a stock concentration of 6 mg/ml, dispensed in aliquots of 0.2 ml, lyophilized and stored at -20°C until used.

2.7.3 Antigen Preparation for IFAT Slides

Control slides for immunofluorescence were prepared from axenically grown *E. histolytica* reference strain (HMI-IMSS) and from polyxenic *E. dispar* reference strain (SAW 1734). The axenic cultures were placed in ice-cold water bath for 20 minutes and centrifuged at 200 g for ten minutes. The culture medium was discarded and the pellet was washed for 10 minutes twice with PBS at 200 g. The pelleted amoebae were resuspended

in PBS, giving a final concentration of 10-20 trophozoites per field when a drop of 20 μ l was observed under the microscope using the 40X objective. The slides were air dried and fixed in methanol for five minutes and stored at -20°C with desiccant until used.

Supernatants of polyxenic Robinson's cultures were collected avoiding disturbing the starch layer, they were centrifuged and washed as previously described for the axenic strain. Then, the same protocol followed with the invasive trophozoites from axenic cultures was applied to the non-invasive trophozoites derived from polyxenic cultures.

2.8 Detection of Anti-*E. histolytica* Specific Antibodies in Human Sera by ELISA

The ELISA methodology for detection of anti-*E. histolytica* antibodies was the following:

Immulon II ELISA plates were coated with 100 μ l of a 1:5000 dilution of stock antigen in carbonate-bicarbonate buffer pH 9.6 (coating buffer, Appendix 1), for a final protein concentration of 1.2 μ g/ml. The plates were incubated overnight at 4°C and the unbound antigen was eliminated by three washings with PBS- 0.1% Tween 20 during 3 minutes each time. Then, the plates were dried and stored at -20°C with desiccant until used.

For the test, 200 μ l of 0.5% Casein in PBS were used to block the plates for 1 hour at 37°C and the excess was eliminated by washing with PBS-T as previously described. 100 μ l of serum diluted 1:100 according to previous chequerboard were added to each well and incubated for 1 hour at 37°C. The unbound antibodies were washed 3 times for 3 minutes with PBS-T. 100 μ l per well of Goat anti-Human Polyvalent Immunoglobulins-peroxidase conjugate (1/5000 in PBS-T) were incubated for 1 hour at 37°C. The excess of conjugate was washed with PBS-T as previously described. The substrate was prepared in citrate-phosphate buffer pH 5.0, 20 mg of orthophenylene diamine (OPD) and 20 μ l of

30% hydrogen peroxide were added to 50 ml of buffer. 100 μ l were added to each well and the plate was incubated 20 minutes in the dark at room temperature. The enzymatic reaction was stopped by addition of 50 μ l of 2.5N HCl. The Optical Density was read at 492 nm.

2.9 DNA Extraction Using Phenol-chloroform Method

The protocol described below has been modified from Romero *et al.*, 1992:

1. Approximately 1g of faeces were resuspended in 3 ml of PBS (liquid faeces should not be diluted).
2. The samples were lysed using three freeze/thaw cycles by immersion in a mixture of dry ice/ethanol followed by immersion in a water bath at room temperature.
3. 1 ml of the lysate was centrifuged for 1 minute at 6500 rpm in a microcentrifuge and the supernatant was transferred to a new tube to which an equal volume of lysis buffer and 10 μ g of Proteinase K were added and the mixture was incubated overnight at 37°C (this incubation was later changed to 2 hours at 55°C).
4. The mixture was then divided in 500 μ l aliquots, to which an equal volume of water-saturated phenol (Cat. No. RP3024 Rathburn, UK) was added and mixed by inversion. The suspension was then centrifuged at 14000 rpm in a microfuge for 5 minutes. The upper layer of each was transferred to a new tube and the phenol extraction was repeated.
5. The supernatants were then extracted with a mixture of 1:1 phenol:chloroform. The mixtures were centrifuged at 12000 rpm for 5 minutes and the upper layer of each was taken.
6. Extraction with chloroform was followed by centrifugation at 12000 rpm for 5 minutes. The upper layers was then transferred to an eppendorf tube.
7. Then, two volumes of 100% ice-cold ethanol plus 50 μ l of 3M sodium acetate solution were added, followed for an incubation at -70°C for 10 minutes.
8. Centrifugation for 30 minutes at 14000 rpm followed, and the supernatant was discarded.

9. Then, 1 ml of 70% ice-cold ethanol was added, followed for a centrifugation at 14000 rpm for 5 minutes. The supernatant was discarded and the DNA was air dried.
10. The DNA was then redissolved in 50 μ l of sterile distilled water.
11. Concentration of DNA was determined by spectrophotometry at 260 nm and the DNA was checked by electrophoresis using 0.8% agarose gels.
12. The DNA stocks were stored frozen at -20°C. Dilutions for use were stored at 4°C.

CHAPTER 3

AMOEBIASIS IN COLOMBIA

3.1 History of the Problem

Intestinal protozoal infections such as amoebiasis and giardiasis are particularly endemic in some Latin American countries due to their socioeconomical conditions and cultural features, which are linked to a high frequency of transmission. Amoebiasis is an important cause of morbidity and mortality in Mexico and as a result, Mexico is one of the countries with pioneering and accurate studies on the biology and epidemiology of amoebiasis in Latin America. An average of 10-20% of the Mexican population are asymptomatic carriers of *E. histolytica*/*E. dispar* (Martinez-Palomo, 1986) and according to Walsh, approximately 5% of the population develop symptoms and produce an antibody response yearly (Walsh, 1986). Antibodies in the Mexican population can be detected in all age groups, predominantly in school children (Sepulveda, 1980), with high frequencies of amoebic rectocolitis in children younger than 5 and in adults older than 20 years (Pardo-Gilbert, 1971). In the case of Colombia, fewer reports on the prevalence of amoebic infection are available; the first national survey on morbidity carried out by the Ministry of Health between 1965 and 1966 identified 3656 infected subjects out of a sample population of 15426 individuals, with an overall prevalence of 23.6% for *E. histolytica*/*E. dispar* infection (MINSALUD,INS, 1969).

After a second national survey carried out between 1977 and 1980, the overall prevalence had dropped to 12.1% (Corredor, 1980). No differential diagnosis between *E. histolytica*/*dispar* and *E. hartmanni*, nor serological tests were included in either study. Those reports reflect the prevalences of intestinal infection with parasites in the population, but the degree of morbidity caused is difficult to assess when indirect but specific tools such as antibody measurement or antigen detection systems are not incorporated in the studies.

Investigations carried out previously in different geographical areas of Colombia reveal common characteristics to those reported for other countries such as variable prevalence according to geographical area and variation with the health status of the population studied, with mental hospital based-studies recording higher prevalences than those reported for other hospitals (Tables 3.1 and 3.2).

TABLE 3.1 *E. histolytica/E. dispar* INFECTIONS DIAGNOSED BY MICROSCOPY IN COLOMBIAN HOSPITALS**

STUDY	HEALTH FACILITY	PREVALENCE (%)	LOCATION
Botero, 1957	Outpatients Clinic	8	Medellin (Antioquia)
Botero <i>et al.</i> , 1958	Mental Hospital	18.4	Medellin (Antioquia)
Botero and Restrepo, 1959	Mental Hospital	13	Medellin (Antioquia)
Faust, 1958	Outpatients Clinic	11.1	Cali (Valle)
Faust, 1958	Mental Hospital	40.3	Cali (Valle)
Krupp, 1970	Outpatients Clinic	62	Cali (Valle)
Valencia and Alban*, 1960	Outpatients Clinic	34.4	Bogotá (Cundinamarca)
Los Andes University, 1989 [§]	Outpatients Clinic	7.4	Ibague (Tolima)

* Diagnosed by rectosigmoidoscopy sample

**Modified from Duque and Zuluaga, 1962

[§] Unpublished data

TABLE 3.2 PREVALENCE OF CLINICAL AMOEBIASIS IN COLOMBIA BY SEX

STUDY	DIAGNOSIS	SEX RATIO (% M:F)	AGE GROUP (YEARS)	CASES
Salazar and Valencia, 1962	Amoebic Colitis	46:54	NR	846
Bravo, 1964	Hepatic Amoebic Abscess	80:20	13-62	88
Bravo, 1965	Hepatic Amoebic Abscess	50:50	0-12	22
Garcia and Acuña, 1966	Hepatic Amoebic Abscess	93:07	4-70	44
Duque*, 1968	Hepatic Amoebic Abscess*	42:31	0-80	220
Zuliani and Garcia*, 1970	Amoebic Colitis*	50:50	0-12	20
Saravia <i>et al.</i> , 1978	Hepatic Amoebic Abscess	75:25	16-80	20
Varela <i>et al.</i> , 1990	Hepatic Amoebic Abscess	94:06	24-65	16

*Autopsy Material

Unfortunately, methodological differences in the screening, including number of samples examined, age of the sample, intermittent cyst excretion, overdiagnosis by confusing *E. hartmanni* with *E. histolytica/dispar*, and the use or not of different concentration methods, are also confounding factors that complicate interpretation of results (Table 3.3).

TABLE 3.3 *E. histolytica/E.dispar* PREVALENCE* OF INFECTION IN THE GENERAL POPULATION IN COLOMBIA

STUDY	PREVALENCE (%)	LOCATION
Duque and Zuluaga, 1958	24.6	Medellin (Antioquia)
Duque and Zuluaga, 1958	50	Chocó (Chocó)
Faust, 1958	33.5	Cali (Valle)
Carvajal <i>et al.</i> , 1995	36	Buenaventura (Valle)
Restrepo, 1962	55	Leticia (Amazonas)
Diaz and Ospina, 1961**	28.1	Bogotá (Cundinamarca)
Agudelo, 1987	12.9	Bogotá (Cundinamarca)
Agudelo <i>et al.</i> , 1993	3	Bogotá (Cundinamarca)

* Prevalences reported include *E. hartmanni*

**Bravo, 1964

Host and parasite related factors both contribute to the epidemiology and distribution of the disease and greatly influence the seroepidemiology of invasive amoebiasis. Among the host related factors, age, sex and nutritional status of the carrier determine morbidity (Kretschmer, 1990). Although invasive intestinal involvement is the first step required for extraintestinal amoebic infection, the failure of circulating antibodies produced during previous infections to protect against reinfection, and the initial delay in immune response during the invasive extra-intestinal stage make the establishment of new foci possible (Sepulveda, 1982; Gilter and Mirelman, 1986). Once invasive trophozoites attach to the host cell by means of the galactose-inhibitable lectin (Petri *et al.*, 1989b), cysteine proteinases degrade the extracellular matrix producing the typical "flask shaped ulcer", surrounded by an acute initial inflammation, where polymorphonuclears, mast cells

and monocytes liberate lysosomal enzymes, proteases, histamine and cathepsins (Martinez-Palomo, 1987). Local anaphylactic reactions seem to contribute to the pathology of intestinal amoebiasis. The increase in permeability observed during intestinal invasion could in part be due to the release of neuroactive substances by mast cells, that degranulate when activated by IgE-amoebic antigen complexes (Trissl, 1982).

As previously described (Chapter 1), untreated acute intestinal amoebiasis often evolves to extraintestinal involvement, particularly hepatic abscess. Invasive amoebic infection in children (Tables 3.2 and 3.4) does not exhibit sex predilection but is often mortal due to perforation (Bravo, 1964; Zuliani and Garcia, 1970; Trissl, 1982). In contrast, adult males (Table 3.2) suffer more frequent hepatic involvement, but mortality rates are low (Alvarez-Alva and Loza-Saldivar, 1971). Pregnancy and malnutrition predispose to depressed immunological status, which, together with inoculum size and virulence of the strain are also implicated in the extent of the damage in the host (Trissl *et al.*, 1978b; Kretschmer, 1990).

Although no differential diagnosis between *E. histolytica* and *E. dispar* was carried out, a parasitological survey by Faust in a poor ward in Cali (Valle, South West Colombia, Figure 3.1) highlighted the relationship between high prevalence of infection and a predominantly carbohydrate based diet (yucca, plantain, rice and potatoes) in the population studied (Faust, 1958). Diamond reviewed the influence of iron and nutritional immunity and relates the high prevalence of amoebiasis to increased ingestion of iron in the diet and malnutrition, which enhances susceptibility to invasion by depletion of the immune system. In his review, Diamond suggests that the observation of a higher prevalence of disease in adult males also indicates the involvement of an hormonal component (Diamond, *et al.*, 1978b; Diamond, 1982).

TABLE 3.4 *E. histolytica/E. dispar* PREVALENCES OF INFECTION FOR CHILDREN IN COLOMBIA

STUDY	AGE RANGE	PREVALENCE (%)	LOCATION
Cardona and Raad*, 1987	0-14	8.1	Manizales (Caldas)
Los Andes University* [§] , 1989	0-12	22.6	Medellin (Antioquia)
Los Andes University* [§] , 1989	0-18	18.3	Ibagué (Tolima)
Trujillo and Vargas, 1990	0-7	2.4	Ibagué (Tolima)
Aguirre <i>et al.</i> , 1991a	0-12	7.5	Bogotá (Cundinamarca)
Duque <i>et al.</i> , 1994	0-18	6.2	Bogotá (Cundinamarca)

* Prevalences including *E. hartmanni*

[§] Unpublished data

Recent epidemiological studies are not available in Colombia and amoebiasis was reported to be endemic in the country based in observed prevalences of infection covering different geographical areas (Table 3.3). According to the reports on invasive disease (Table 3.2), between 1960 and 1965 Garcia-Torres and Acuña studied 44 clinical records corresponding to hepatic amoebiasis cases from the Hospital San Juan de Dios and the Santa Marta clinic (Instituto de Seguros Sociales) in Santa Marta, located in the North Atlantic coast (Figure 3.1). In this study, the distribution of cases according to sex was 93% of males and 7% of females with only 4.5% of patients under 20 years of age and 61.3% of cases in the age range between 20 and 49 years. Alcoholism and malnutrition were present in 38.6% of the patients and 52.3% suffered dysentery previous to the abscess (Garcia-Torres and Acuña, 1966). Also, a report on fatal amoebiasis by Duque (1968) reviewing 220 cases of invasive amoebiasis on autopsy material collected in three university hospitals in the country (153 cases in Medellin, 38 cases in Bogota, 25 cases in Cali and 4 cases from Bucaramanga) shares many common features. The ratio of males to females in this study was 65%:35% and the higher frequencies of infection were 20.9% among children between 2 and 5 years of age, and 31.4% among adults between 21 and 40 years.

The author describes malnutrition and immunosuppression-associated diseases (digestive candidiasis and pulmonary aspergillosis) in 7.7% of patients, 5.4% of them were children. All the children in the study (73) and a third of the adults (49) were malnourished and 15.6% of the female cases were related to pregnancy, puerperium or abortion (Duque, 1968).

At the same time as the introduction of metronidazole as the therapeutic agent against amoebic infection, serological tests were included in the diagnosis of invasive amoebic infection at most of the university hospitals in the country, and the first reports are those from the University Hospital in Cali. Indirect haemagglutination test (IHA) was used to evaluate antibody levels before and after treatment of amoebiasis by Krupp (1970) in asymptomatic carriers and patients with invasive disease. From 168 subjects with amoebic colitis, 81% had positive titres of antibodies which persisted for six months, although reinfections (40%) made it difficult to continue the follow-up study. In the group with extraintestinal amoebiasis (31 patients), 87.1% were positive with titres that persisted for the six month follow up study and in this group also, the high titres of antibody did not prevent reinfections. The serological study of cyst passers (75) revealed 9% positives, and among 118 healthy controls 7% had antibodies. No relation was found between antibody level and age, sex and concurrent bacterial or parasitic infections (Krupp, 1970). Some time later, Saravia *et al.* (1976) collected a series of 40 cases with probable diagnosis of hepatic amoebic abscess in patients attending at the same hospital from 1972 to 1974. The comparison of serological diagnosis by indirect hemagglutination (IHA), agar gel diffusion (AGD), latex agglutination (LA), and counterimmunoelectrophoresis (CIE) techniques indicated that although IHA was the most sensitive (92%) and specific (97%) technique, AGD with a sensitivity of 89% and a specificity of 97% was simple and easily accessible to small laboratories, with the only disadvantage of the time delay in obtaining the results. An alternative proposed was the counterimmunoelectrophoresis, with a sensitivity of 75% and a specificity of 97%. The authors highlighted the simplicity and rapid results of LA that together with its high sensitivity (92%) and specificity (94%), make the kit ideal for the hospital format, however, the costs were excessive for the hospital budget.



FIGURE 3.1 POLITICAL DIVISION OF COLOMBIA

Also during 1976, a different evaluation of serology was carried out by Restrepo *et al.* in Medellin using complement fixation (CF), AGD, IHA, and LA in patients with hepatic abscess, acute amoebic colitis, and asymptomatic cyst carriers. Complement fixation was positive in 93% of asymptomatic cyst passers, 94% of patients with amoebic colitis and 90% of patients with probable amoebic abscess. Although sensitivity in cases of hepatic abscess was 90% and in colitis cases was 94.6%, specificity was only 50%, detecting asymptomatic cyst passers and healthy controls as positive. Clearly complement fixation is of limited use in endemic areas for invasive amoebiasis (Restrepo *et al.*, 1976).

Results with AGD revealed a better specificity (100%) sacrificing sensitivity, which was 65% for hepatic abscess cases and 54% for amoebic colitis, detecting antibodies in only 3% of asymptomatic cyst passers. In this study LA detected antibodies in 60% of the cases of acute amoebiasis, 70% of cysts passers, 83% of abscess cases and 20% in healthy controls. Sensitivity was 72% and 86% for hepatic abscess cases, with a specificity of 83%. Specificity for IHA was 83%, with 84% sensitivity in cases of colitis and 83% in patients with hepatic abscess.

The specific detection of IgG anti-amoebic antibodies by the ELISA technique using a crude lysate of axenically cultured trophozoites permits the serological evaluation of a great number of samples in short time, with the possibility of automation in hospitals. A retrospective study of 19 cases of clinically diagnosed hepatic amoebic abscess collected from 1987 to 1990 in two university hospitals in Bogota (Varela *et al.*, 1990) is the first report of its use in diagnosis by exclusion in Colombia. Invasive amoebic infection was confirmed by a significant rise in antibody response measured by the ELISA test in 16 cases. The use of ELISA was a valuable tool for the differential diagnosis in the 3 remaining patients, who were believed to have pyogenic abscess, where clinical manifestations and echography were not clear. Those three patients had a negative amoebic ELISA result and good response to treatment with antibiotics, confirming the pyogenic origin of the abscesses.

A further valuation of the ELISA technique for the diagnosis of hepatic amoebic abscess was carried out by Aguirre *et al.*, (1991b) with 71 sera collected during the four previous years in Bogota. This study reported a sensitivity of 95% with 100% specificity when analysing 59 sera from patients with amoebic abscesses and 12 sera from patients with other aetiologies such as cancer, pyogenic abscesses, and cysts of the liver. Positive predictive value was 100% and negative predictive value was 80%. It was recommended to test at least two samples from different days in cases in which the first result is negative. A comparison between ELISA and CIE was published by Restrepo *et al.*, (1996) with sera from 30 patients with suspected hepatic amoebic abscess of the liver, 30 patients with symptomatic amoebic colitis and 50 healthy controls. ELISA detected all cases of amoebic liver abscess (27) and did not react in 3 cases of abscess later classified as from bacterial origin. In comparison, CIE detected 20 cases of amoebic liver abscess, with no cross reactivities in pyogenic cases. Both tests were highly sensitive in cases of amoebic abscess (100% and 88% respectively) and 100% specific when evaluated against abscesses from bacterial origin. However, for amoebic colitis CIE was negative in all cases, only three had a level of antibodies detectable by ELISA and none of the healthy controls was positive by either test.

Serology has been shown in Colombia and other endemic areas (Treviño *et al.*, 1997) to be valuable in the hospital environment as a confirmatory test for the evaluation of extraintestinal amoebiasis, when used together with other diagnostic aids such as X rays, ultrasonography, gammagraphy, and fluoroscopy. However, the results of the previous studies confirm observations that only low and non-specific levels of antibodies are present in the early stages of intestinal invasion, although serology can be a good measure of exposure to amoebic invasion. Microscopy is however not useful for the detection of carriers of *E. histolytica sensu stricto*, emphasising the need for antigen detection systems or nucleic acid detection systems able to make a differential diagnosis between carriers of *E. dispar* and individuals carrying *E. histolytica*.

3.2 MATERIALS AND METHODS

The first step in the study is to identify and confirm some endemic areas (Ibague in the Tolima department and La Mesa, in Cundinamarca) for intestinal infection with *E. histolytica/dispar*, with the further purpose of collecting material to be used during the different stages of the project.

3.2.1 Description of the Study Area at Ibague

The first step of the project was to determine the exact situation of amoebiasis in places that in the past have been designated as endemic for *E. histolytica/E. dispar*. The pilot study was developed at Ibague, capital of the department of Tolima (Figure 3.1), which covers a population of 1,150,080 inhabitants. This city is situated 4°27' latitude North and 75°15' longitude West, in the Andean zone, 1285 m above sea level, with an average temperature of 24.1°C, a mean Relative Humidity of 73.7% and a bimodal rain pattern corresponding to April-May and October-November.

This part of the research project was carried out at the biology laboratory in the Department of Biological Sciences at the University of Tolima, with the agreement of the academic authorities for a study which included obtaining blood samples on a voluntary basis. Subjects were asked their consent for venipuncture.

The area of Ibague was selected for the study because a previous screening on school children in 1989 exposed a prevalence of 15.6% for *E. histolytica/E. dispar* by microscopy of stool samples (Universidad de los Andes, 1989 unpublished data). Also, according to the university health authorities, an outbreak of invasive amoebiasis was observed in the student's residency at the university in 1987, which was understood to indicate that invasive strains were circulating among the population and a new parasitological and serological survey in the form of a pilot study was necessary to

determine prevalence of infection. Also, further parasitological surveys were carried out in La Mesa, in the department of Cundinamarca and in different areas of Santafé de Bogota, the capital to the same department applying the modifications to the sampling methodology which were the result of the pilot study.

3.2.2 The Community and the Population in the Study at Ibague

3.2.2.1 Subjects

At Ibague, two groups of subjects, one consisting of 127 students (Group I), with ages between 5 and 18 years at secondary school "Carlos Lleras Restrepo" (Figure 3.2), chosen because of the precarious sanitary facilities present. The school is located in El Salado ward, one of the poorest semirural wards outside Ibagué. The second group of subjects consisted of 181 university students and staff from Tolima University (Group II), with age ranging between 19 and 85 year olds.

3.2.2.2 Sample Collection

A short talk covering basic information on what intestinal parasites are and simple hygiene routines to avoid their transmission was given to each class prior to requesting volunteers for the study. Also the methodology for the collection of the sample was explained, with extra marks being offered by the biology teacher to those who completed the study. The same information was given at a meeting with parents of students at the school, explaining the arrangements made with the local health authorities for the treatment of those students carrying pathogenic parasites.

Each volunteer was given a sample container, together with simple worded instructions on how to collect the material. Immediately after the collection of the first

sample, volunteers were issued a new container labelled with the corresponding number of the subject in the study, until completion of three samples per subject was achieved. At the school, stool samples were collected from 6:30 am to 7:30 am in every class room during two weeks and transported to the laboratory at the university within the following hour.



FIGURE 3.2 SCHOOL SANITARY FACILITIES AT IBAGUE

Advertisement for the collection was done two weeks before through the welfare office at Tolima University, consisting of posters being displayed throughout the university campus. The questionnaire and methodology for sample collection were the same as used for the school but the results were reported to the welfare office and those students requiring treatment were referred to the university health services. Samples from the university population were collected from 8 am to 11 am at the laboratory during two weeks. The processing of the samples started immediately after arrival in batches of 10 for microscopy. A pool of the three samples from each volunteer was concentrated with zinc sulphate and examined by microscopy with Lugol's iodine. Results were registered into an Epi Info 5.1 database.

All *E. histolytica/dispar* microscopically positive samples were cultured in primary Robinson's medium (Appendix 1), which was replaced by secondary Robinson after 24 hours. Sediments were examined for positivity using Lugol's iodine after 48 hours (Figure 3.3) and cultures with a negative sediment were discarded after 96 hours.

Blood samples were taken from volunteers when the third stool sample was obtained. Standard radial vein puncture was carried out using 10 ml syringes. After removing the clot, centrifugation at 700 g for 10 minutes separated the serum, followed by inactivation of the complement at 56°C for 30 minutes in a water bath. Sera were stored at -20°C until transported to the reference laboratory in Bogota, where serology was carried out during the following weeks. Sera were tested by ELISA test in duplicate wells and the cut off for each plate was calculated by adding two standard deviations to the mean of three duplicated pools of negative sera.

3.2.3 La Mesa, Department of Cundinamarca. Description of the Area

La Mesa is situated in the department of Cundinamarca (Figure 3.1) 4°38' latitude North and 74°28' latitude West, with an average temperature of 22°C and an altitude of

1,198 m. Being located in the Tequendama province of the Department of Cundinamarca, the total population of the municipality was estimated to be 19,132 persons in 1993 from whom, only 2,639 had potable water and from those, 1,954 had a proper sewage system (Departamento Administrativo de Planeacion, Gobernacion de Cundinamarca, 1995).

3.2.4 The Community and the Population Screened at La Mesa

3.2.4.1 Subjects

At La Mesa, 138 subjects were tested in three different locations: "Futuro Juvenil", the local charity which runs an orphanage housing 56 children and granted permission for the parasitological survey but restricted information on age and clinical background of the children. A voluntary collection was carried out at the "Departamental" Secondary School, which is one of the 4 secondary schools at La Mesa covering a total population of 1,935 students. Students in their fifth year of secondary school were asked to provide samples, from which 53 provided stools. The remaining 29 subjects tested were patients attending the Hospital Pedro Leon Alvarez and from whom at least one stool test each was submitted.

3.2.4.2 Sample Collection

Authorities were contacted at the orphanage "Futuro Juvenil" in order to obtain stool samples from the children residing in their location at La Mesa. An informal talk on basic hygiene technique to avoid parasite transmission, stressing the need of an adequate sample collection was given to the staff in charge. Written instructions and the corresponding containers were provided, and three consecutive samples were obtained and further transported to the Hospital Pedro Leon Alvarez, where all the samples were processed as described in 3.2.2

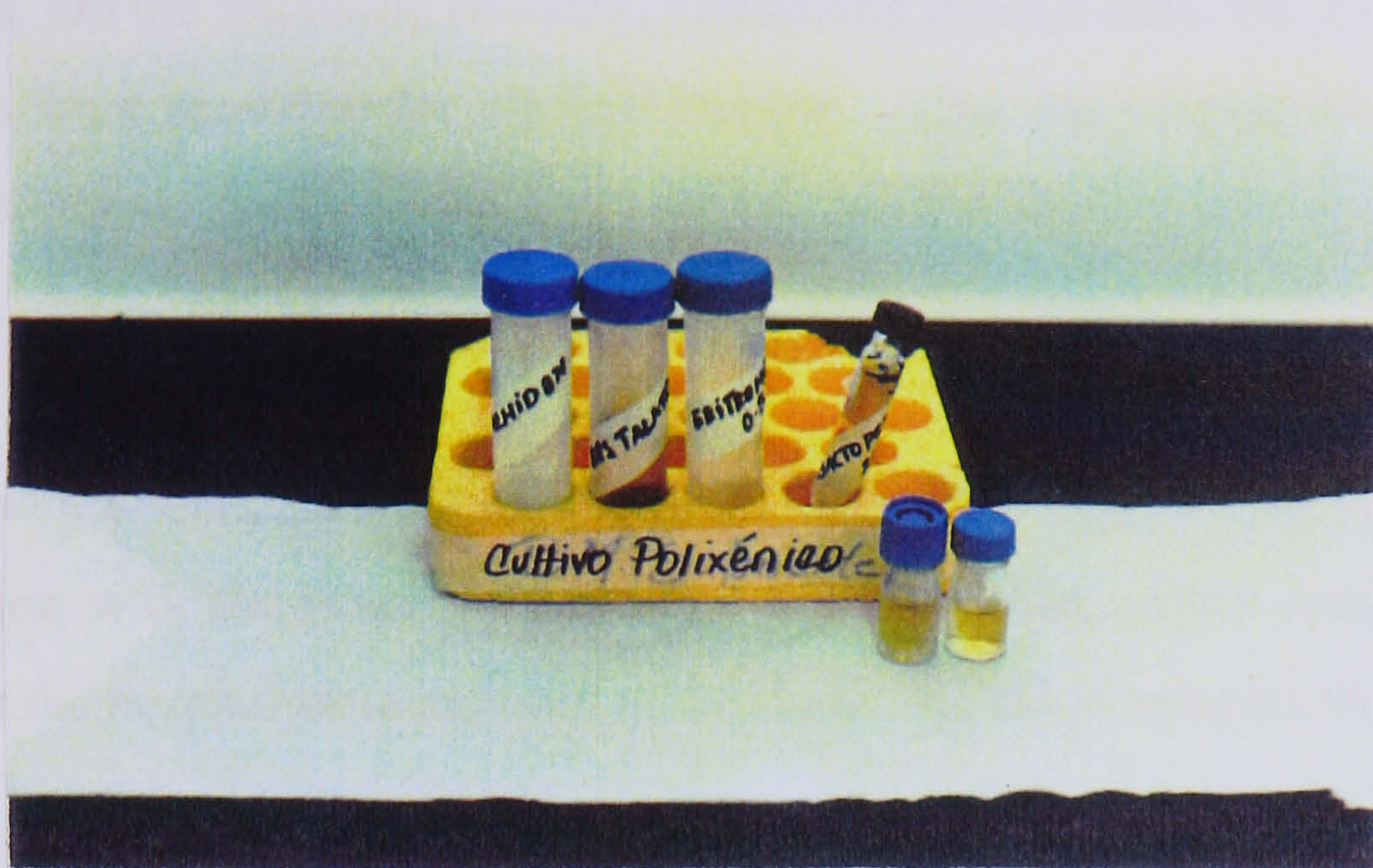


FIGURE 3.3 A. POLYXENIC ROBINSON'S CULTURE SYSTEM.

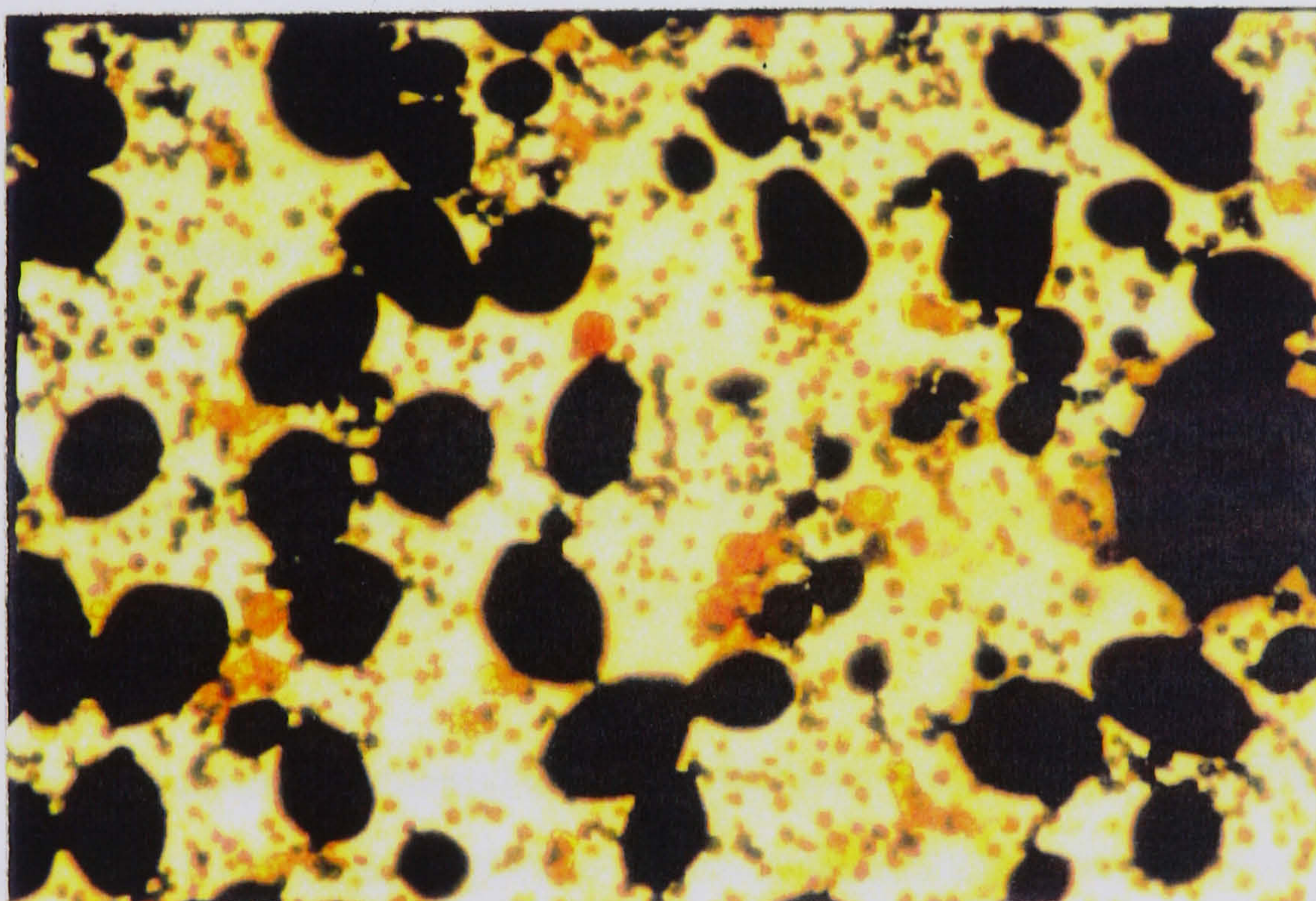


FIGURE 3.3 B. POSITIVE CULTURE (Lugol's iodine)

Parental permission was requested at the Colegio Departamental prior to sample collection for which a talk was given during the previous Saturday to the start of the collection. On the following Monday, a short talk to each class and instructions with the containers were provided for the first sample. After the first sample was obtained, a labelled container for the sample was given, and the same system applied for the third container.

Results were reported in writing to the staff in charge for the arrangement of a consultation with the general practitioner at the hospital. Stool samples produced by patients at the hospital were included in the study. No blood samples were collected at La Mesa.

3.3 RESULTS

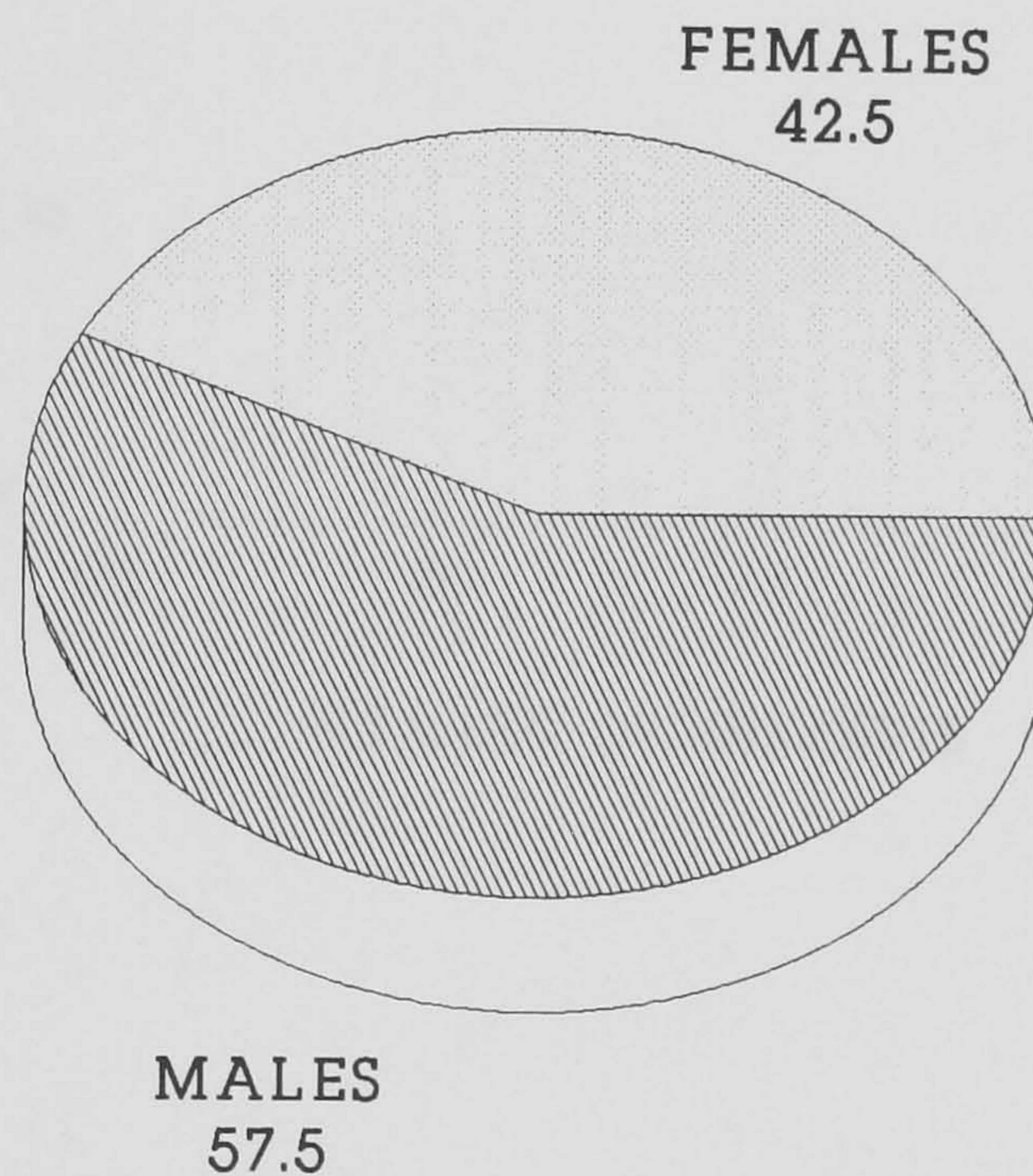
3.3.1 Ibague, Department of Tolima

As the pilot study, this screening was to provide information not only on the prevalence of amoebiasis in the population, but in the feasibility of the application of the methodology proposed.

A total of 308 serial stool samples were collected and examined by microscopy during the field trip to Ibague (Tolima). 127 samples were collected in the secondary school (Group I) and 181 were collected at the university (Group II).

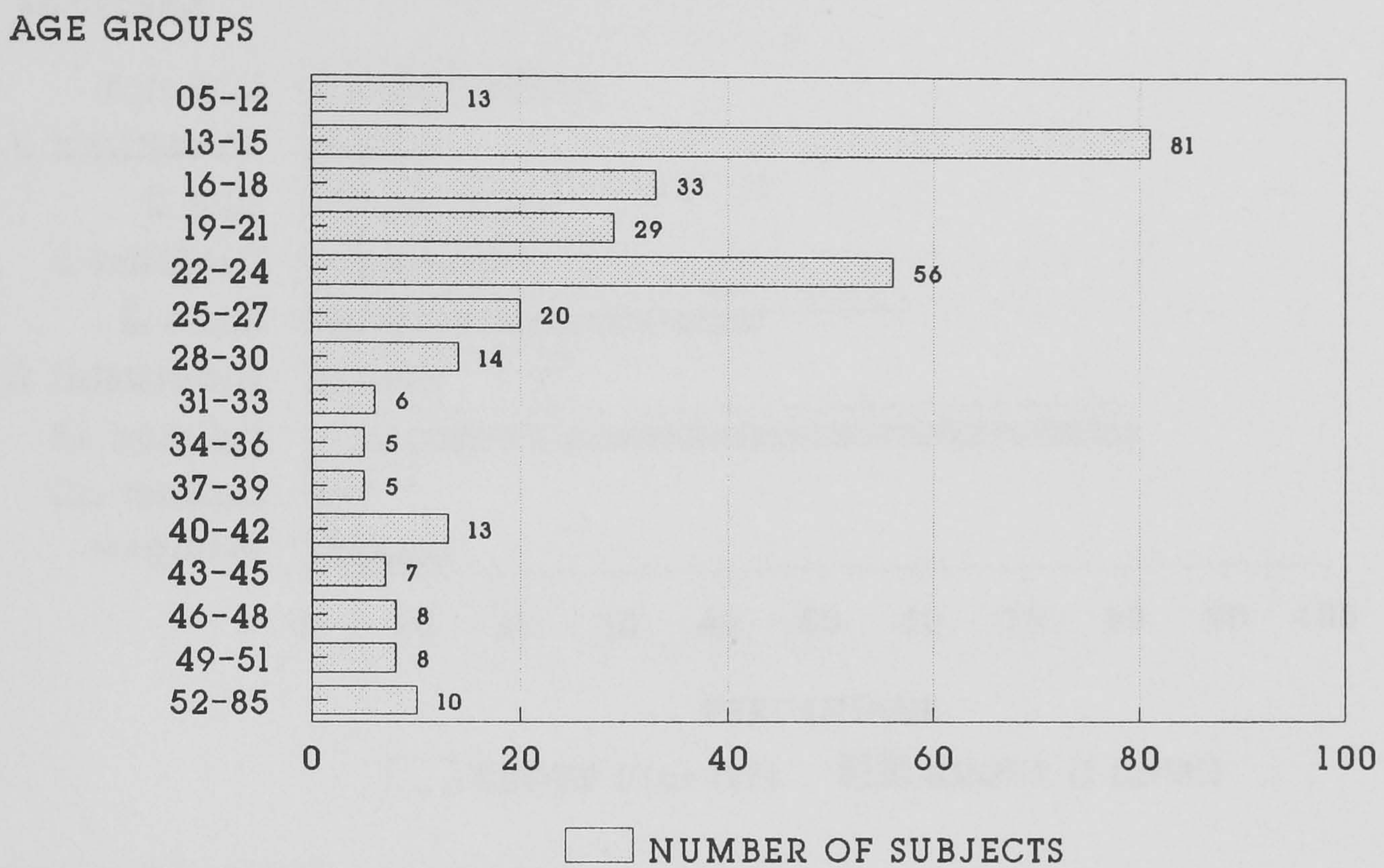
The population consisted of 42.5% females and 57.5% males (Figure 3.4), with a distribution of 50.4% females (64) to 49.6% males (63) in group I and 37% females (67) to 63% males (114) in group II, for a total of 131 females and 177 males in the study.

FIGURE 3.4 SEX RATIO(%)
IBAGUE SAMPLES



In relation to age distribution, group I consisted of individuals between 5 and 18 years of age, and group II covered individuals between 19 and 85 years of age. The most densely populated age groups were the 13-15 year olds in group I (81/127), 63.8% of the school population tested, and the 22-24 year olds in group II (56/181), corresponding to 30.9% of the university population (Figure 3.5).

FIGURE 3.5 AGE DISTRIBUTION OF THE POPULATION AT IBAGUE

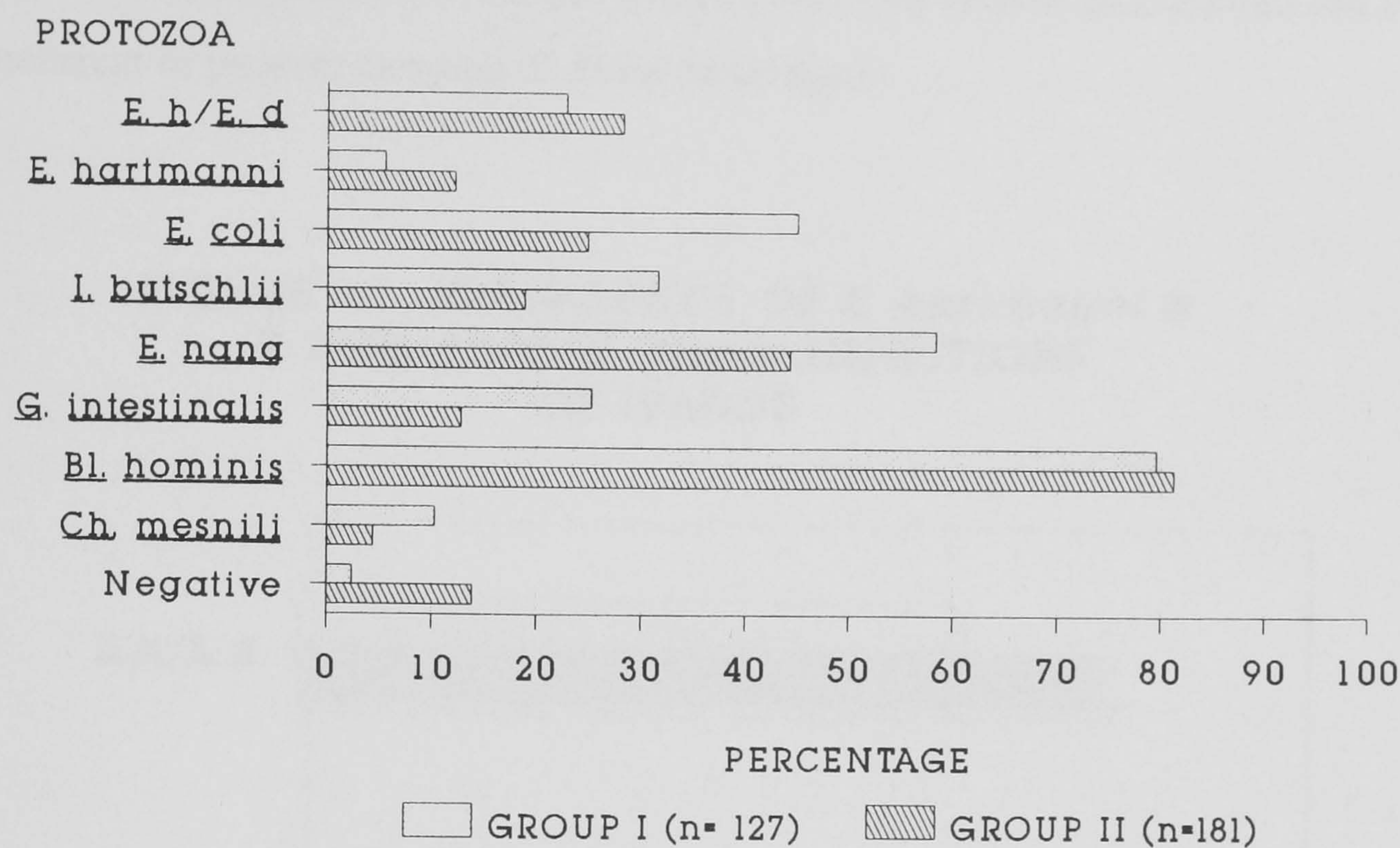


n= 308

3.3.1.1 Microscopy

High prevalences of intestinal parasitism were found in the two groups studied at Ibague, where *Blastocystis hominis* was the most common protozoan found, with a prevalence of 80.5% in the overall population, 79.5% subjects (101/127) from group I and 81.2% subjects (147/181) from group II. Only 8.4% of persons were found negative for all of the parasites investigated. From them, 3.1% (4/127) were in group I and 12.1% (22/181) belonged to group II (Figure 3.6).

FIGURE 3.6 MICROSCOPY OF STOOLS (%)
GROUPS *I AND **II IBAGUE

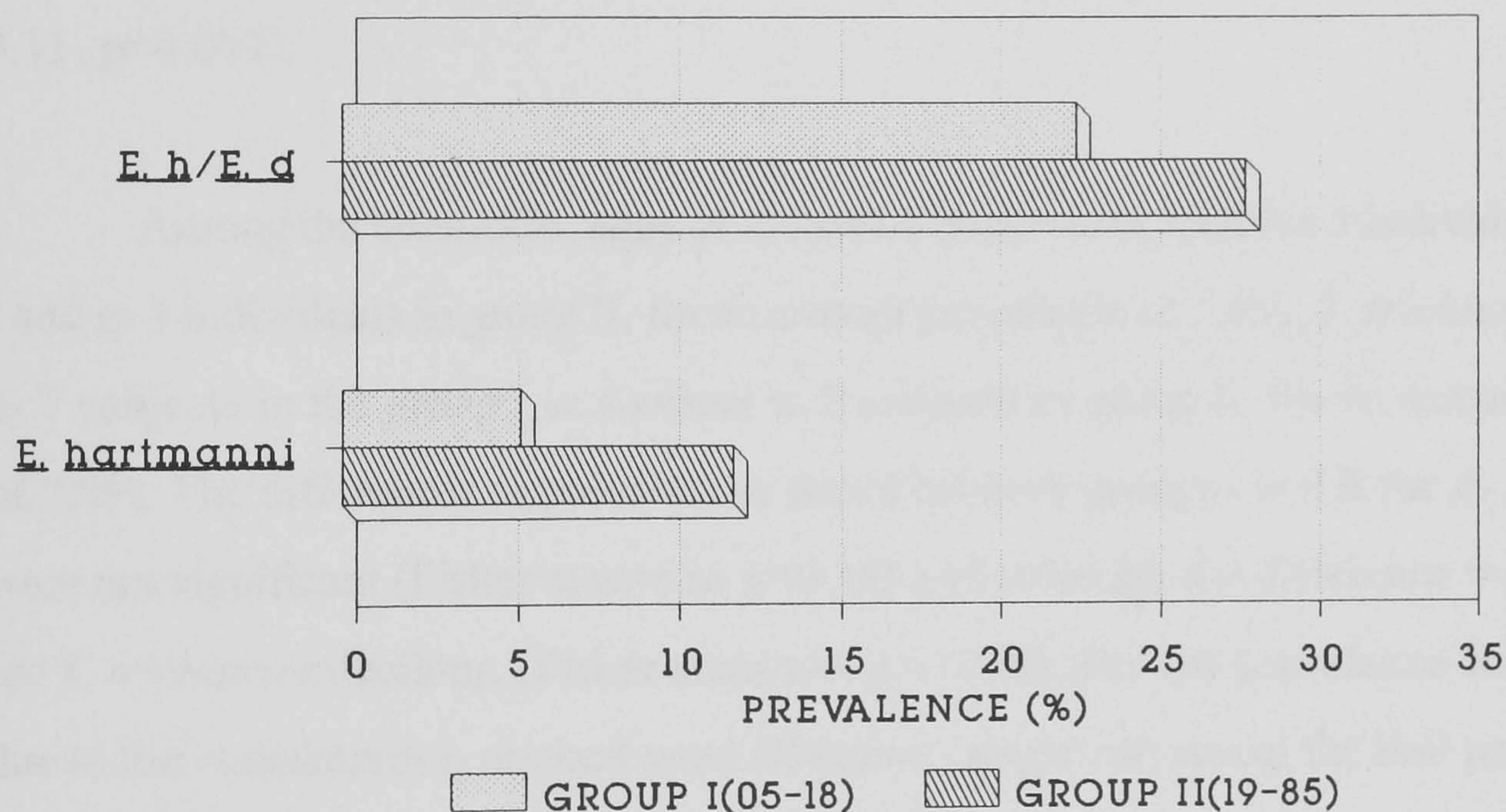


GROUP *I: 05-18 YEARS
GROUP **II: 19-85 YEARS

The result of prevalence presented for *E. histolytica/E. dispar* was 22.8% (29/127) in group I. From them, 20.7% (6/29) had trophozoites without red blood cells on direct examination with isotonic saline. Similarly, the prevalence of *E. histolytica/E. dispar* in group II was 28.1% (51/181), and from them 9.8% (5/51) were carrying trophozoites only but also without red blood cells. No significant difference in prevalence was found between groups I and II (Chi square, Yates Corrected 0.85, $p=0.35$). The overall prevalence of *E. histolytica/E. dispar* infections was 26% (80/308), from which, 13.7% (11/80) had only trophozoites without ingested red blood cells. Because no haematophagous trophozoites were observed, and all the subjects were asymptomatic, none of those infections was classified as amoebic colitis, however, some of the subjects were found later

to have high titres in the serology test and those results were reported to the University. The overall prevalence of *E. hartmanni* in the population was 9.4% (29/308), with 5.5% of those infections in Group I (7/127) and 12.1% (22/181) in Group II (Figure 3.7). Again, no significant difference in the prevalence was found between both groups (Chi square 3.12, Yates corrected, P=0.077). However, 13 out of the total of 29 infections (44.8%) were concurrent in patients carrying *E. histolytica/dispar*.

FIGURE 3.7 PREVALENCE OF *E. hartmanni* & *E. histolytica/E. dispar* INFECTIONS AT IBAGUE



E. h: *E. histolytica*
E. d: *E. dispar*

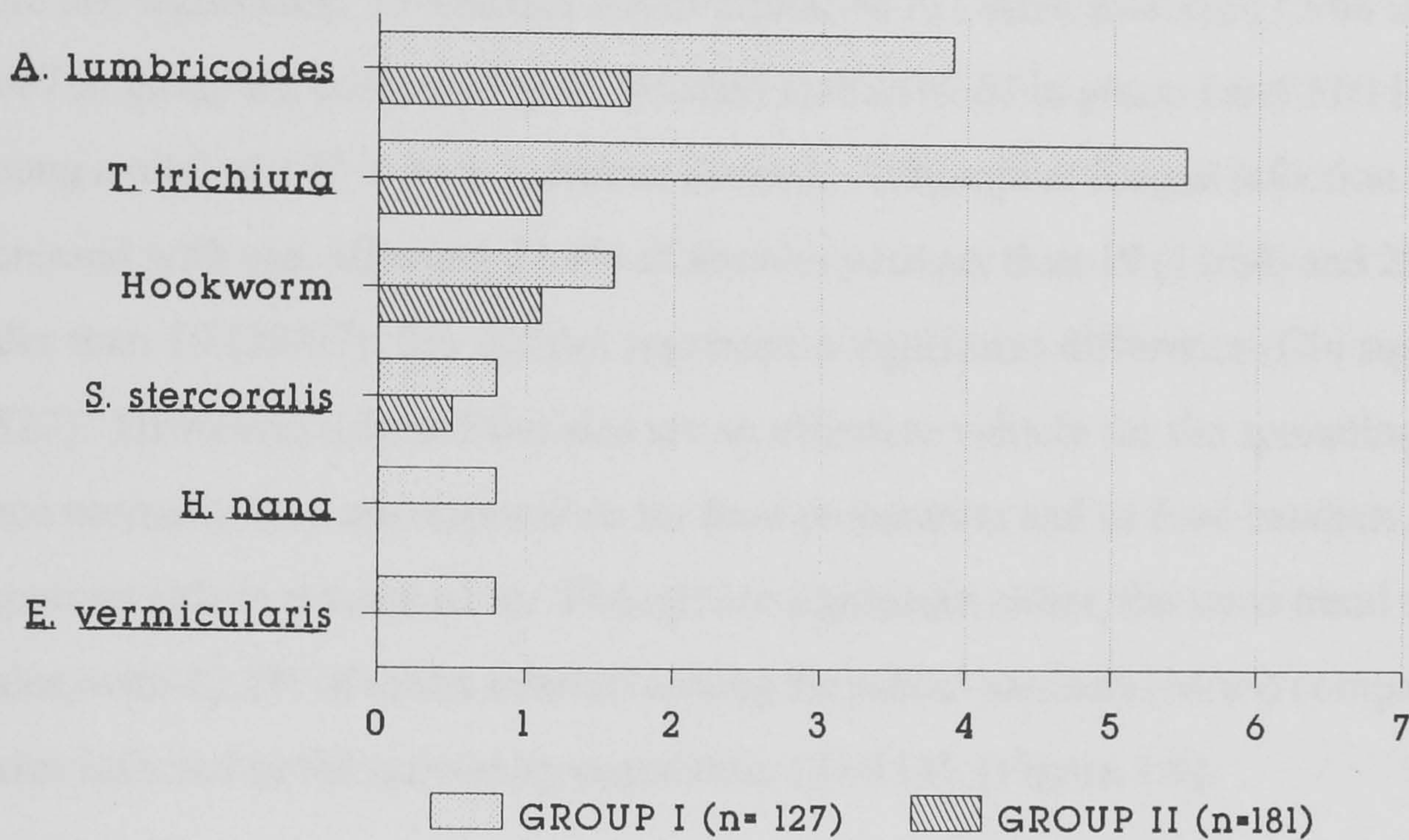
Giardia intestinalis was found in a high proportion of samples (17.9%), however, prevalence in group I was doubled at 25.2% (32/127) when compared to 12.7% (23/181) in group II, which represented a significant difference between both groups (Chi square 7.11, Yates Corrected, p= 0.0076). Of those infected with *Giardia intestinalis*, 10.9% individuals (6/55) had trophozoites in their faeces during the direct exam with isotonic saline.

Other protozoal infections in descending order of prevalence were 50% overall for *End. nana*, represented in 74 infections in the group I (58%) and 80 infections (44%) in group II, with a significant difference between both groups (Chi square 5.36, Yates corrected, $p=0.02$). *E. coli* was present in 45% of the individuals in group I (57/127) and in 25% of individuals in group II (45/181), where a significant difference was also found (Chi square Yates corrected 12.6, $p=0.0003$), for an overall prevalence of 33.1% for in the population. 24% of the population tested was infected with *I. buetschlii*, 40 individuals (31.5%) in the group I, and 34 (19%) individuals in group II, with a significant difference between group I and group II (Chi square Yates Corrected 5.93, $p=0.014$). *Ch. mesnili* was present in 6.8% of the population, 13 in group I (10%) and 8 in group II (4%), but no significant difference in prevalence for both groups was found (Chi square Yates corrected 3.11, $p=0.077$).

Among the helminths, eggs of *A. lumbricoides* were found in 5 individuals in Group I and in 3 individuals in group II, for an overall prevalence of 2.6%; *T. trichiura*, was found in 7 subjects in the group I in contrast to 2 subjects in group II, for an overall prevalence of 2.9%. The differences in prevalences found between groups I and II for *A. lumbricoides* were not significant (Fisher exact test $p=0.19$) and although the difference was significant for *T. trichiura* infections, (Fisher exact test $p=0.028$), the low prevalence found probably due to the concentration method used (flotation) might not reveal the real prevalences for helminth eggs.

Hookworm infections were found in two individuals in group I and in two individuals in group II, for a global prevalence of 1.3%. Eggs of *E. vermicularis* were found in one individual in group I only, which corresponds to a prevalence of 0.3% (Figure 3.8).

FIGURE 3.8 HELMINTH INFECTIONS
IBAGUE SAMPLE



• SECONDARY SCHOOL
 **UNIVERSITY

The retrospective analysis of the microscopy results at Ibague showed a high degree of polyparasitism when comparing the overall results between groups I and II. In addition, only 8% of the population (3 individuals in group I and 25 individuals in group II) shed three negative samples. Faecally transmitted parasites are evident in both groups, with prevalences for *G. intestinalis* significantly higher in school children than in university students. *E. coli*, *I. buetschlii*, and *End. nana* were also found in high prevalence. A visit to the sanitary facilities at the school, where some toilets did not flush, floors were contaminated with urine and no toilet paper, paper towels or soap were available, may be relevant. The housing conditions in El Salado area varied from inadequate with no sanitary facilities to rudimentary with basic sanitary facilities and partially treated drinking water. It is likely that lack of basic hygiene education contributes to intrafamilial transmission of

parasites, with people failing to wash their hands after visiting the toilet. Also, a contributory factor is the fact that although health authorities advise boiling any water used for drinking and food preparation purposes, these guidelines are seldom followed.

Differences in prevalence of infection with *E. histolytica/E. dispar* among sexes were not significant, 35 females out of a total of 131 were infected (15/64 in group I and 20/67 in group II), compared to 45 infected males (14/63 in group I and 31/114 in group II) among a total of 177 in both locations studied. Although at Ibagué infection rates slightly increased with age, affecting 23.4% of females younger than 19 (15/64) and 29.8% females older than 19 (20/67), this did not represent a significant difference (Chi square 0.40, $p=0.527$). However, infected females are an effective vehicle for the spreading of infection since normally they are responsible for food preparation and as food handlers, they play an important role in transmission. Though not significant either, the same trend was noted for males, with 22.2% of males infected among the school students (14/63) compared to 27.2% males infected in the university population (31/114). (Figure 3.9).

FIGURE 3.9 *E. histolytica/dispar*
INFECTIONS ACCORDING TO GENDER
IBAGUE SAMPLES

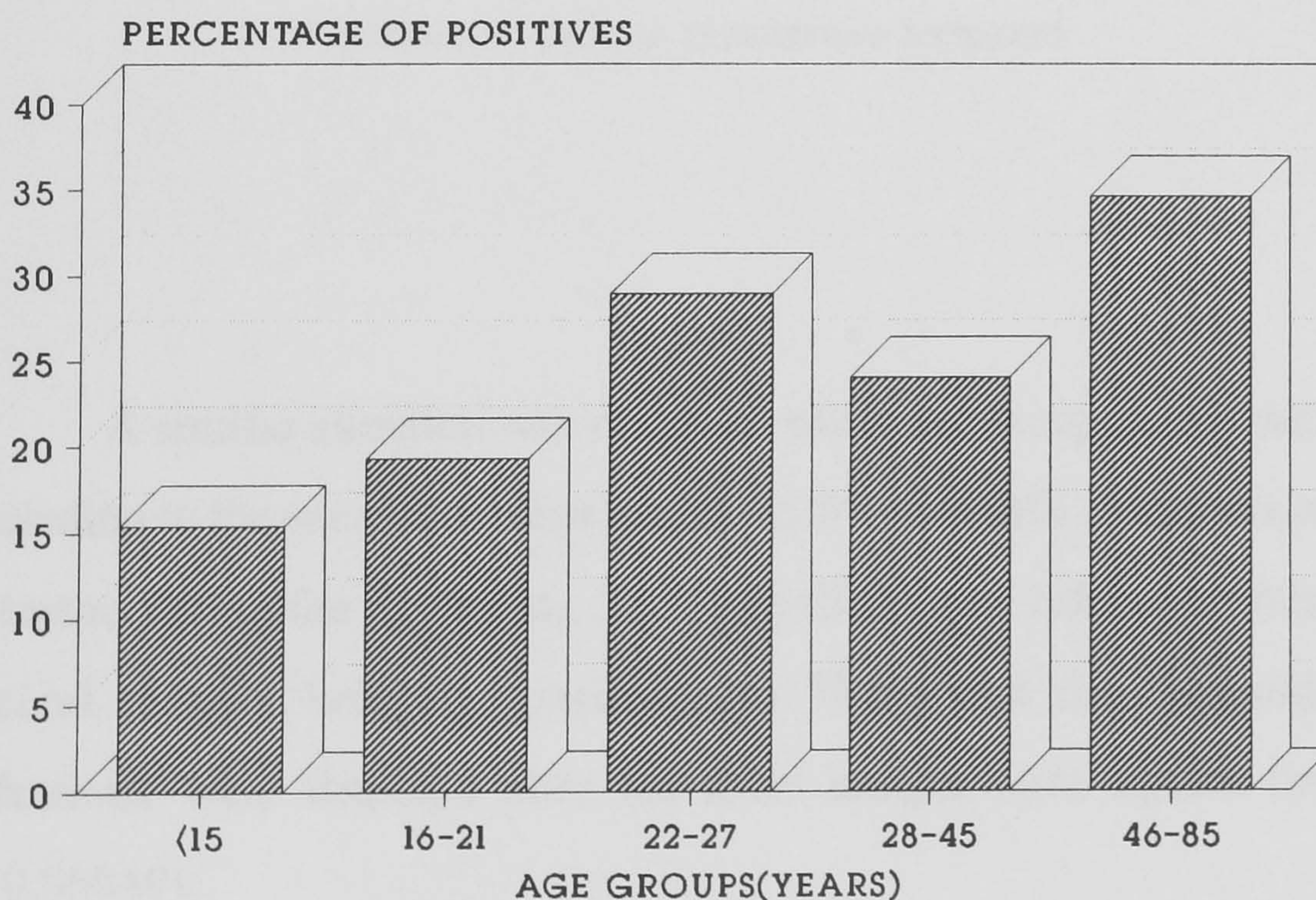


GI:05-18 YEARS
GII:19-85 YEARS

Similarly, no increment in the prevalence in relation to age is evident when analysing infections in the different age groups (Chi square for linear trend 0.413, $p=0.520$; Figure 3.10). When three groups including the individuals up to 27 years of age (5 to 15 year olds, 16 to 21 year olds and 22 to 27 year olds) were compared, no significant differences in prevalence were found (Chi square 0.078, $p=0.78$). When a second comparison was carried out with the individuals over 22 years of age (22-27 year olds, 28-45 year olds and 46-85 year olds), no significant differences were shown either (Chi square 0.075, $p=0.78$). The analysis after separating the population in two groups, from 5 to 27 year olds and from 28 to 85 year olds confirmed that there was no significant difference in prevalence of infection with *E. histolytica*/*E. dispar* among this population in relation to age (Chi square 0.05, $p=0.818$).

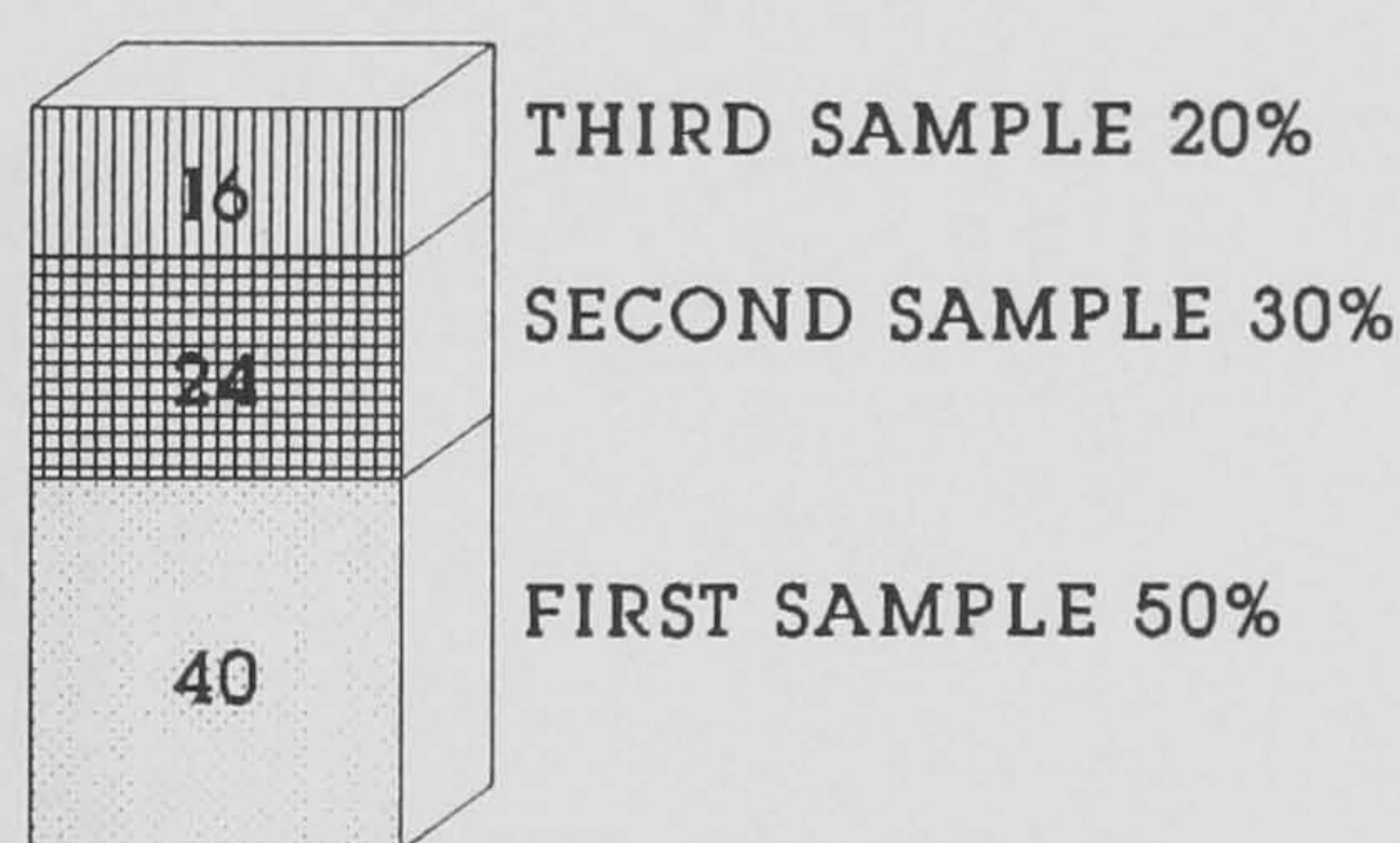
Retrospective studies on the influence of the number of samples examined in the detection of new parasitic infections by microscopy were carried out for the two potentially most prevalent pathogenic protozoa found, *E. histolytica*/*dispar* and *G. intestinalis* with the results from Ibagué.

FIGURE 3.10 *E. histolytica* PREVALENCE OF INFECTION BY AGE GROUP AT IBAGUE



After the first stool sample the prevalence of infections with *E. histolytica* was 13%, with detection of 50% of the carriers (40 positives). After the second examination, the prevalence rose to 20.8%, thus a further 30% (24 new infections) was detected and with the third examination (16 new infections, Figure 3.11), the "final" prevalence detected by microscopy reached 26% (80/308). This change was statistically significant (chi square for linear trend=16.269, p=0.00005) and justifies the screening of consecutive samples from the same subject.

FIGURE 3.11 INFLUENCE OF SAMPLE NUMBER FOR *E. histolytica*/*E. dispar* DETECTION BY MICROSCOPY. IBAGUE SAMPLE

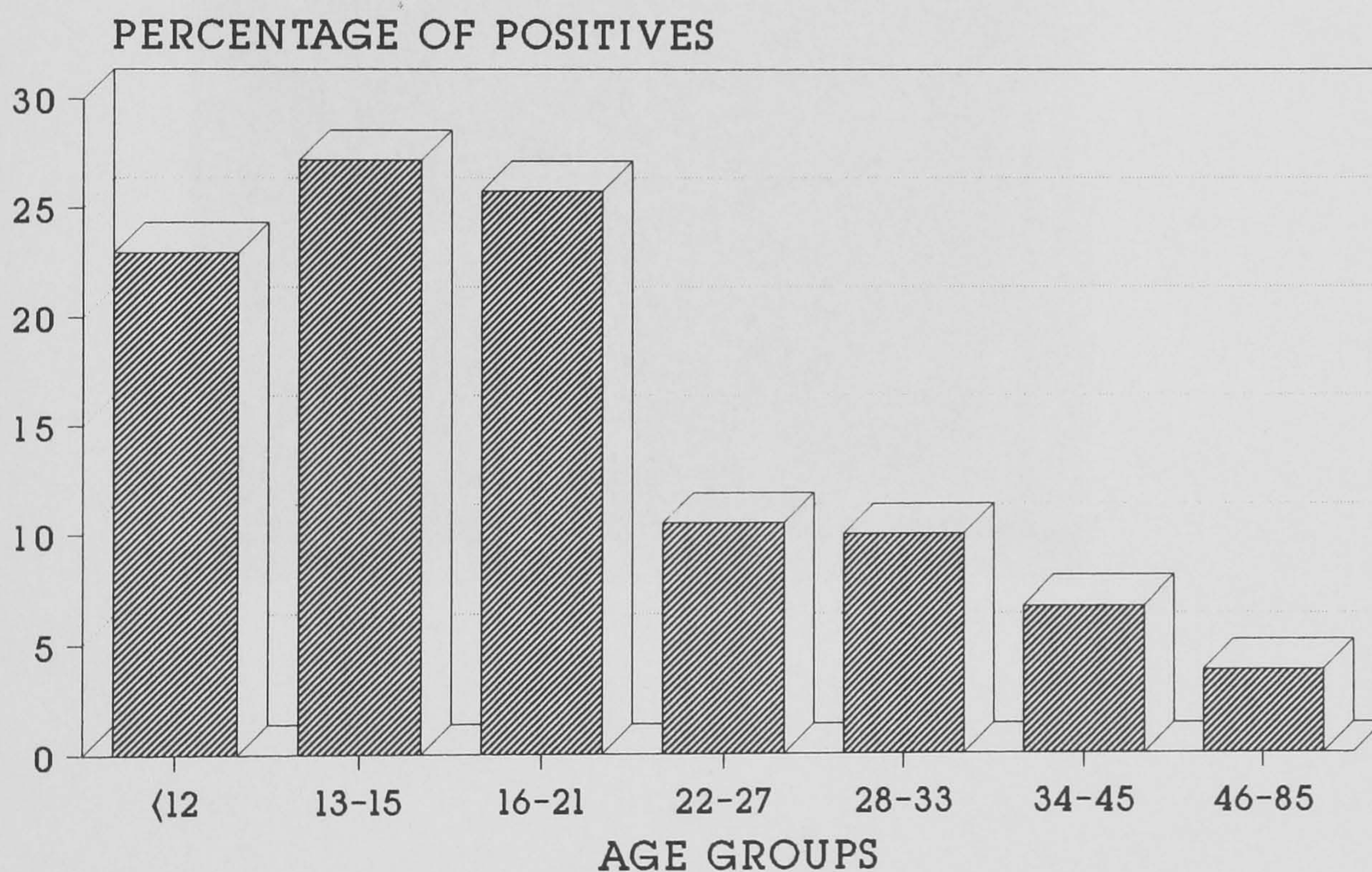


PROPORTION OF NEW INFECTIONS DETECTED

A similar situation was observed when assessing the prevalence of *G. intestinalis* according to the sample number tested, 45.4% (25/308) of carriers of *G. intestinalis* were detected during the first exam, 25.4% (14/308) new infections were detected during the second sample, bringing sensitivity to 70.8% and the "remaining" 29.1% (16/308) infections were detected after the third sample (Chi square for linear trend 12.15, p=0.00049).

G. intestinalis infections were concentrated in the population younger than 22, with 41 (74.5%) infections of the total of 55 in the overall population (Figure 3.12). When this prevalence was compared to the prevalence found in the group of people older than 22 (13/152), the difference in prevalence was significant (Chi square 15.53, $p=0.00008$). However, no significant differences (Chi square for linear trend 0.001, $p=0.971$) were found when comparing the prevalences between the first three groups (5-12 years old, 13-15 years old and 16-21 years old). Similarly, no significant difference in prevalence (Chi square for linear trend 1.233, $p=0.266$) was found among the groups where individuals older than 22 were classified (22-27 years old, 28-33 years old, 34-45 years old and 46-85 years old).

FIGURE 3.12 *G. intestinalis* PREVALENCE.
POSITIVE SUBJECTS ACCORDING TO AGE GROUP
IBAGUE SAMPLE



The former results indicate that the sensitivity of the microscopical analysis is directly proportional to the number of samples examined (Kershaw, 1946; Stamm, 1957; Mathur and Kaur, 1973). However, the possibility of missing some recently acquired infections must be taken into account because zinc sulphate, as other concentration methods, fails to concentrate trophozoites. Also, large, dense structures are missed, therefore, the possibility is that some light helminth infections were not detected, which may explain the relatively low levels of helminth infections reported.

3.3.1.2 Cultures

Eighty samples found positive for *E. histolytica/dispar* by microscopy were cultured and although 48 sediments were positive after the first 24 hours, 20 survived to the sixth subculture and only 8 were characterised by hexokinase. All strains showed slow banding patterns for hexokinase, characteristic of non-invasive strains (Figures 3.13A and B).

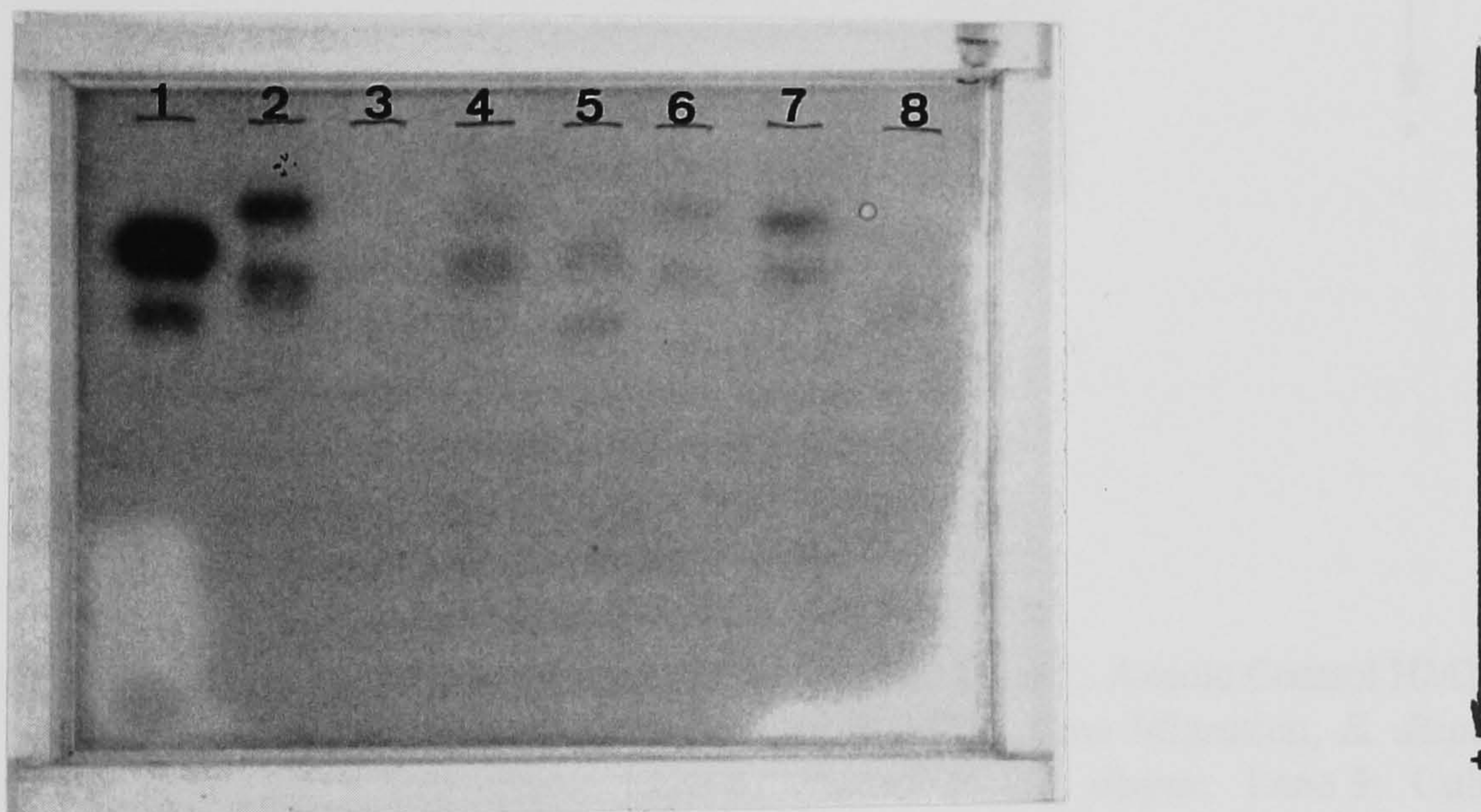


FIGURE 3.13 A. Hexokinase Profiles on Cultures from Ibaguè. Lane 1: Axenic Control HM1-IMSS, Fast Migration, *E. histolytica*. Lane 2: Polyxenic Control SAW 1734, Slow Migration, *E. dispar*. Lane 3: Culture 28, Negative. Lane 4: Culture 32, *E. dispar*. Lane 5: Control CIMPAT Culture LFS, *E. histolytica*. Lane 6: Culture 72, *E. dispar*. Lane 7: Culture 160, *E. dispar*. Lane 8: Control CIMPAT Rosa, *E. histolytica*.

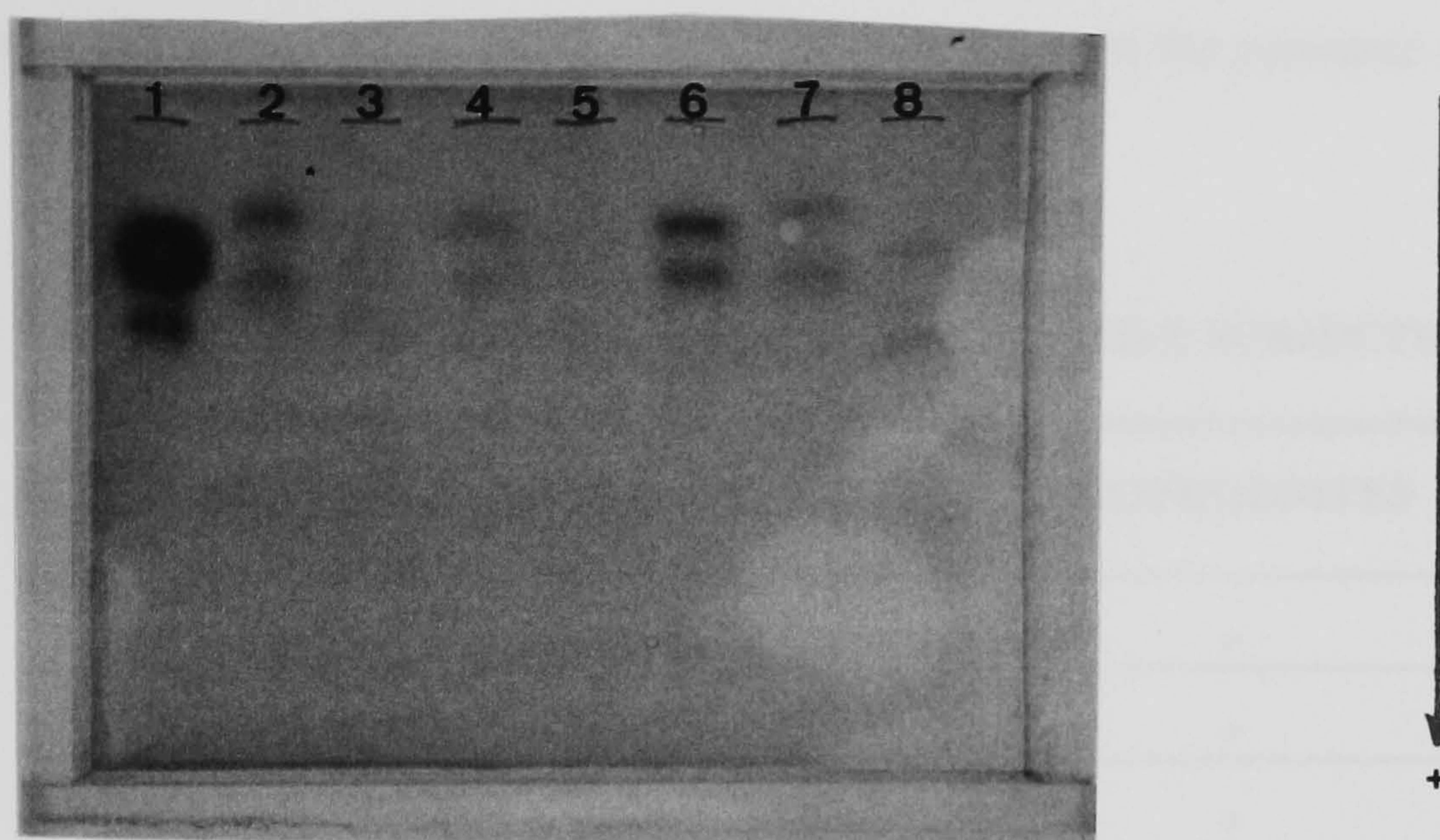


FIGURE 3.13B. Hexokinase Profiles on Cultures from Ibague. Lane 1: Axenic Control HM1-IMSS, Fast Migration, *E. histolytica*. Lane 2: Polyxenic Control SAW 1734, Slow Migration, *E. dispar*. Lane 3: Control CIMPAT culture LFS, *E. histolytica*. Lane 4: Culture 169, *E. dispar*. Lane 5: Culture 186, *E. dispar*. Lane 6, Culture 189, *E. dispar*. Lane 7 Culture 28, *E. dispar*. Lane 8: Control CIMPAT Rosa, *E. histolytica*.

3.3.1.3 Serology

Serum samples were obtained from 114 out of 127 subjects in Group I and from 140 individuals out of 181 in Group II, giving a total 254/308 (82.5%) subjects tested by serology. In group I, 16/114 (14%) subjects had antibodies against *E. histolytica* in serology by ELISA. In group II, 28/140 (20%) subjects were positive by ELISA for anti-*E. histolytica* antibodies. No significant difference of the positivity for anti-*E. histolytica* antibodies between the two groups was found (Chi square 1.17, $p=0.2789$). The overall positivity at Ibague was 44/254 (17%) individuals by ELISA, which indicates that this

proportion of the population is or has been in contact with invasive strains. From 80 individuals infected with *E. histolytica/E. dispar*, 14 had positive serology and in 6 of them trophozoites were present, although without phagocytosed red blood cells (Table 3.5). These findings do not guarantee that they are *E. histolytica sensu stricto* carriers, but the association of trophozoites and high ELISA titre could suggest the presence of an invasive strain.

TABLE 3.5 SEROLOGY RESULT ON *E. histolytica/dispar* POSITIVE SUBJECTS AT IBAGUE

SAMPLE ID	SEROLOGY OD 492 nm	CYSTS	TROPHOZOITES
41	0.586	-	+
131	0.441	-	+
145	0.596	-	+
150	0.300	+	+
153	0.270	+	-
162	0.339	+	-
181	0.306	+	-
199	0.380	+	-
206	0.513	+	-
216	0.300	+	-
224	0.333	-	+
249	0.398	+	-
272	0.637	+	-
306	0.329	+	+

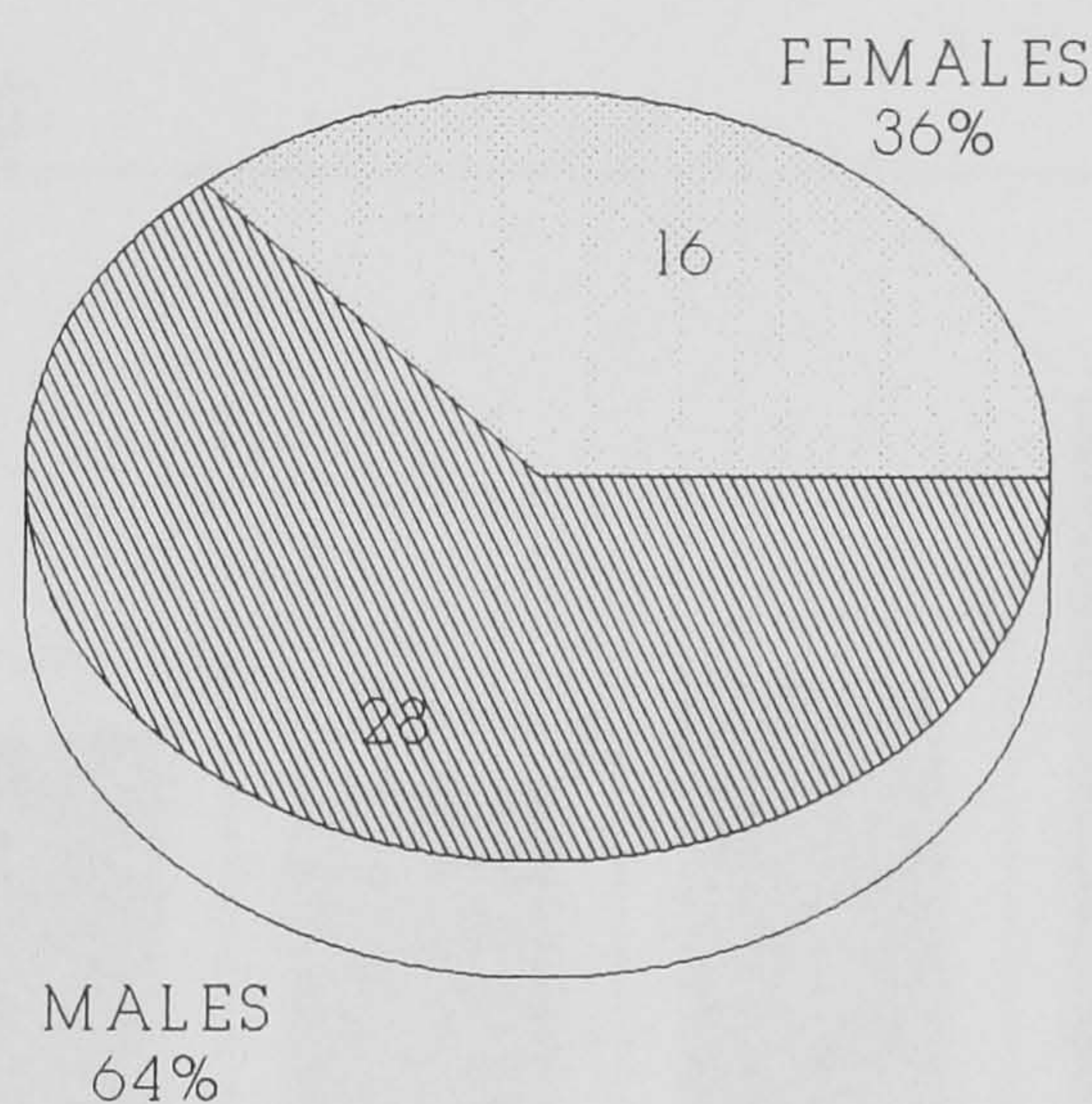
OD: Optical Density

Mean Break Point: 0.262, Calculated as the average of the cut off points of nine plates

Cut off Values: Mean of pooled negative sera in each plate + 2 Standard Deviations (SD)

Positive serology was found in 15% (16/109) of the females and in 19% (28/145) of the males in the population studied, not significant difference (Chi square 0.03, $p=0.864$). However, the sex distribution of these 44 positive individuals is 36% females to 64% males (Figure 3.14).

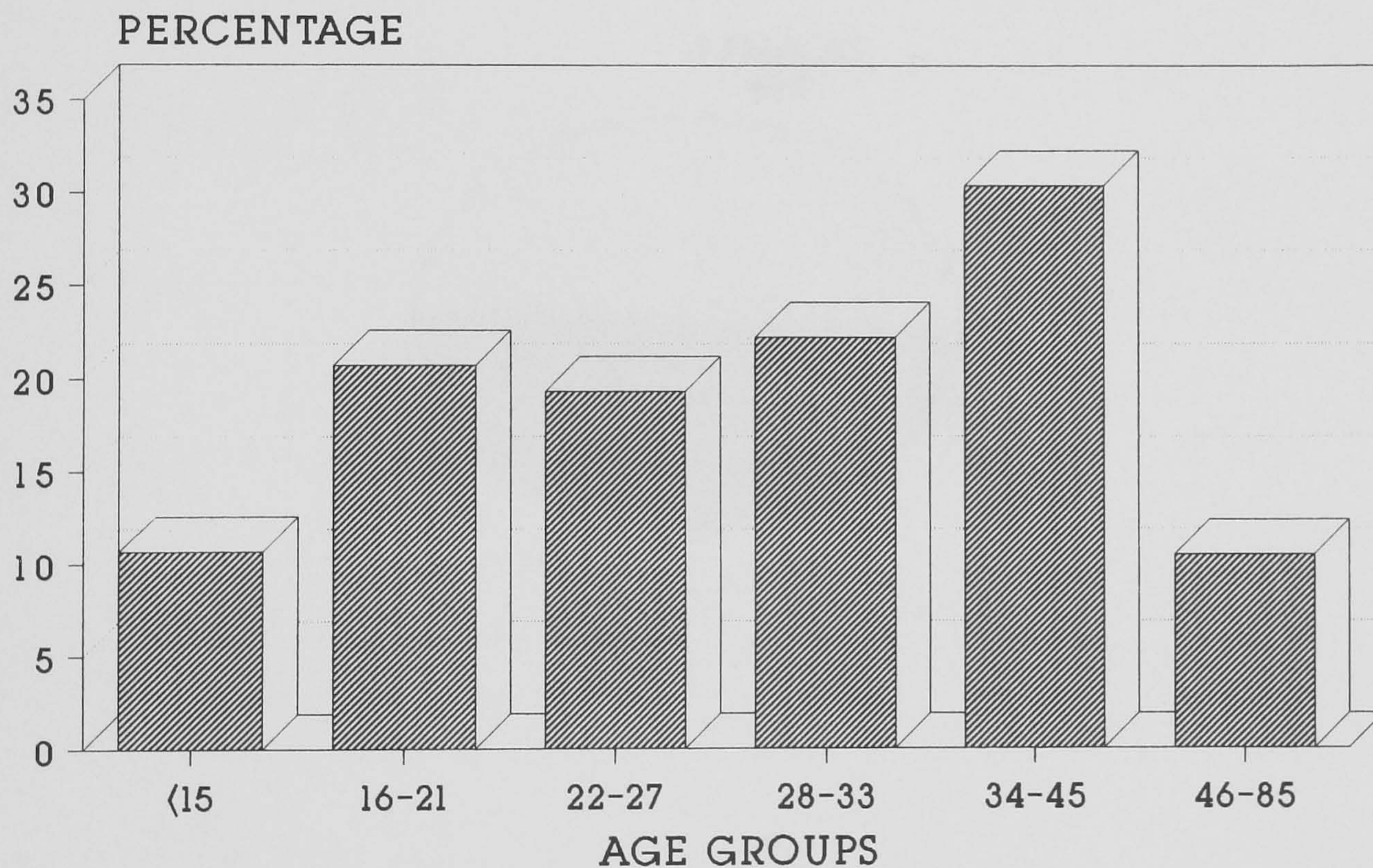
FIGURE 3.14 GENDER DISTRIBUTION AMONG SEROLOGY POSITIVE INDIVIDUALS IBAGUE SAMPLE



When analysing the presence of a positive serological response to *E. histolytica* antigen according to age group, 9 individuals out of 84 (10.7%) were positive in the group of 5-15 years old, 11 of 53 (20.7%) in the group of 16-21 years old, 11 out of 57 (19.2%) in the group of 22-27 years old, 4 out of 18 (22.2%) in the group of 28-33 years old and 2 out of 19 (10.5%) in the group of 46-85 years old (Figure 3.15). Although the highest percentage of positives (30%) was found in the group of 34-45 year olds (7/23), no significant association between age and presence of antibodies was found (Chi square for trend 1.675, $p=0.1956$).

Individuals with other parasites but no *E. histolytica* in their faeces and a positive serology by ELISA 26/44 (60.5%) and those with no parasites 4/44 but with positive serology (9.3%) possibly suffered past invasive intestinal or extraintestinal infections since serology remains positive for up to 5 years (Krupp and Powell, 1971). Also, it is possible that light infections with the parasite were not detected in the stools of those individuals. A specific detection system is necessary to discriminate *E. histolytica* from *E. dispar* in stool samples.

FIGURE 3.15 *E. histolytica* SEROLOGY
POSITIVE SUBJECTS ACCORDING TO AGE GROUP
IBAGUE SAMPLE

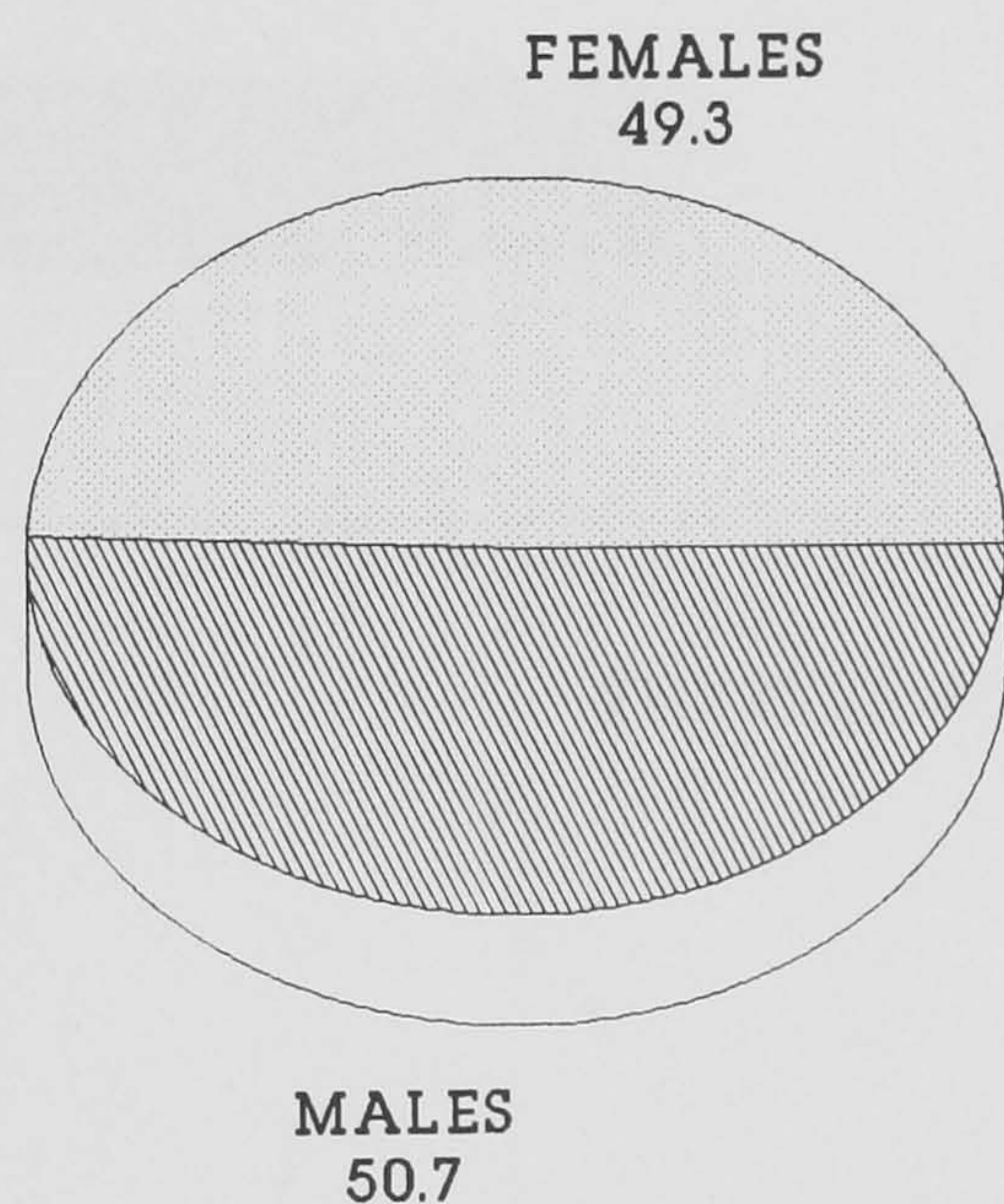


3.3.2 Field Work at La Mesa, Department of Cundinamarca

After the field trip to Ibagué, it was decided that new material was to be collected in a new location with similar population and geographical conditions. From the pilot study it was evident that not many invasive strains were circulating among healthy carriers and consequently, the probability of finding invasive strains would increase by testing material

from symptomatic patients at hospitals in addition to the carriers in the general population. A second field trip to La Mesa involved testing 138 subjects, 50.7% (70) of which were males and 49.3% (68) females from the orphanage, the secondary school and the hospital (Figure 3.16).

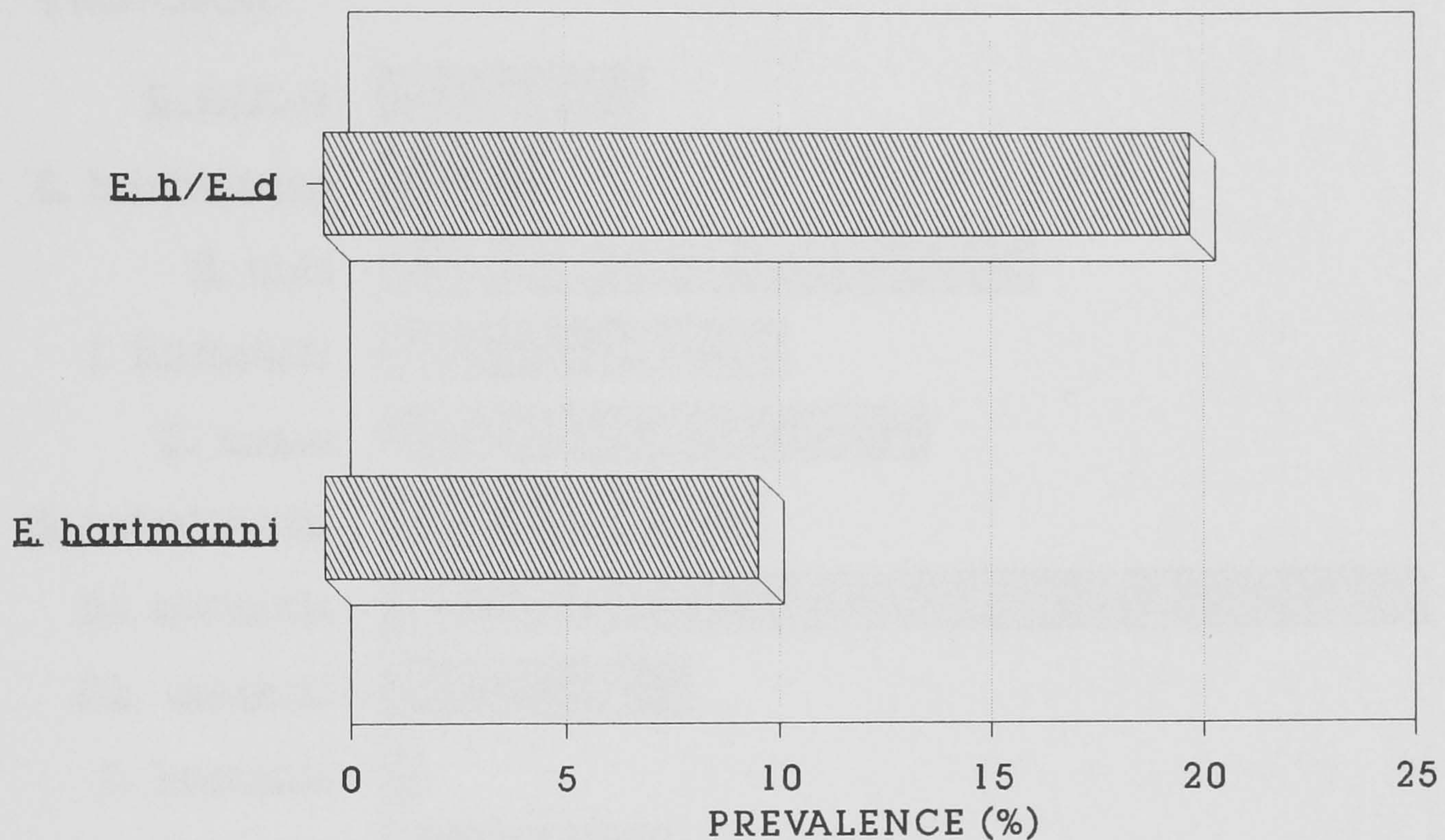
FIGURE 3.16 GENDER RATIO(%)
LA MESA SAMPLE



3.3.2.1 Microscopy

The microscopy on the samples read at La Mesa resulted in high prevalences for amoebic infections such as 20.3% (28/138) of samples positive for *E. histolytica/dispar*, 10.1% with *E. hartmanni* (Figure 3.17), and in 5.8% (14/138) of cases both parasites were present.

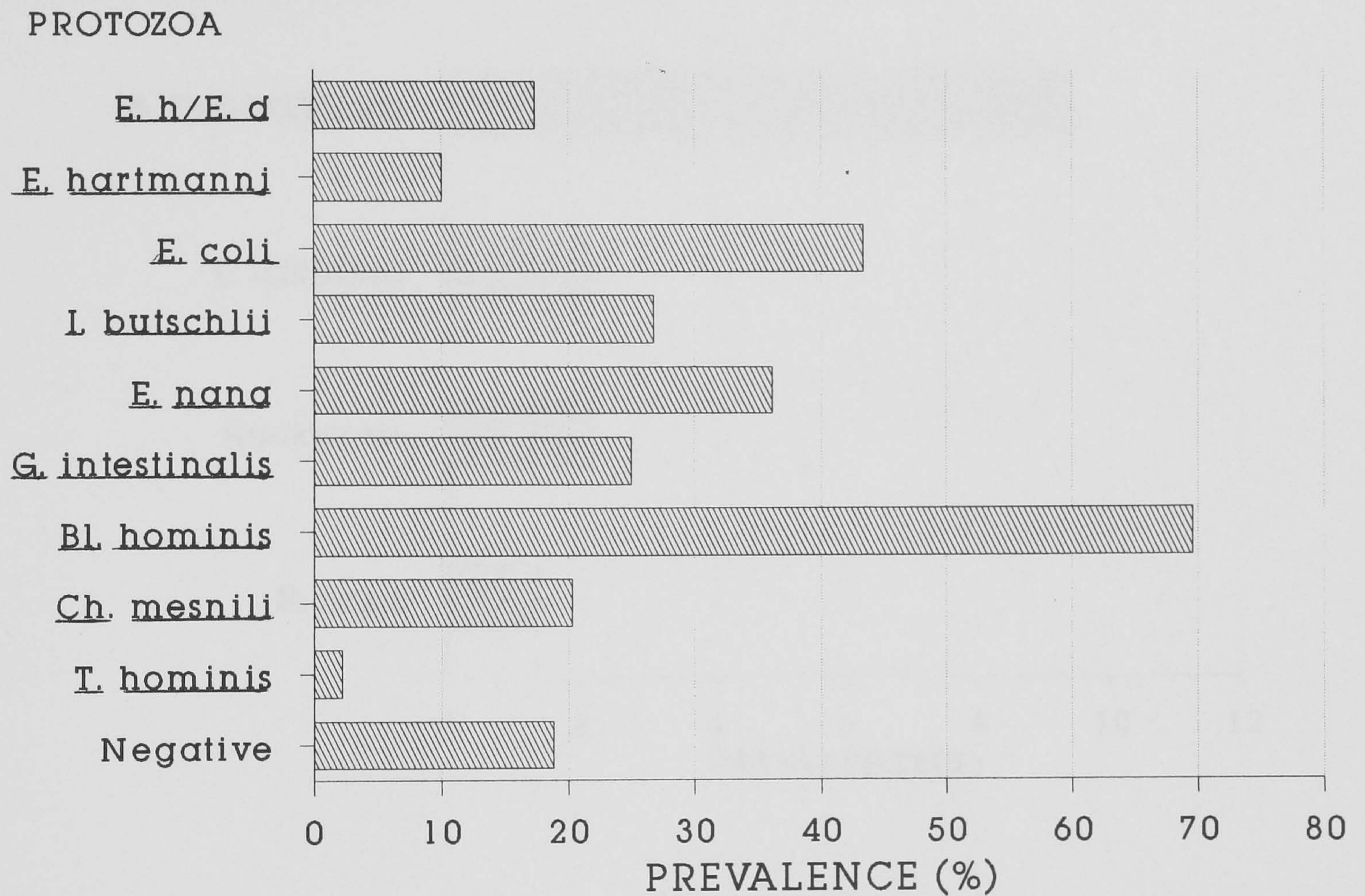
FIGURE 3.17 PREVALENCE OF *E. hartmanni* & *E. histolytica/E. dispar* INFECTIONS AT LA MESA



E. h: *E. histolytica*
E. d: *E. dispar*

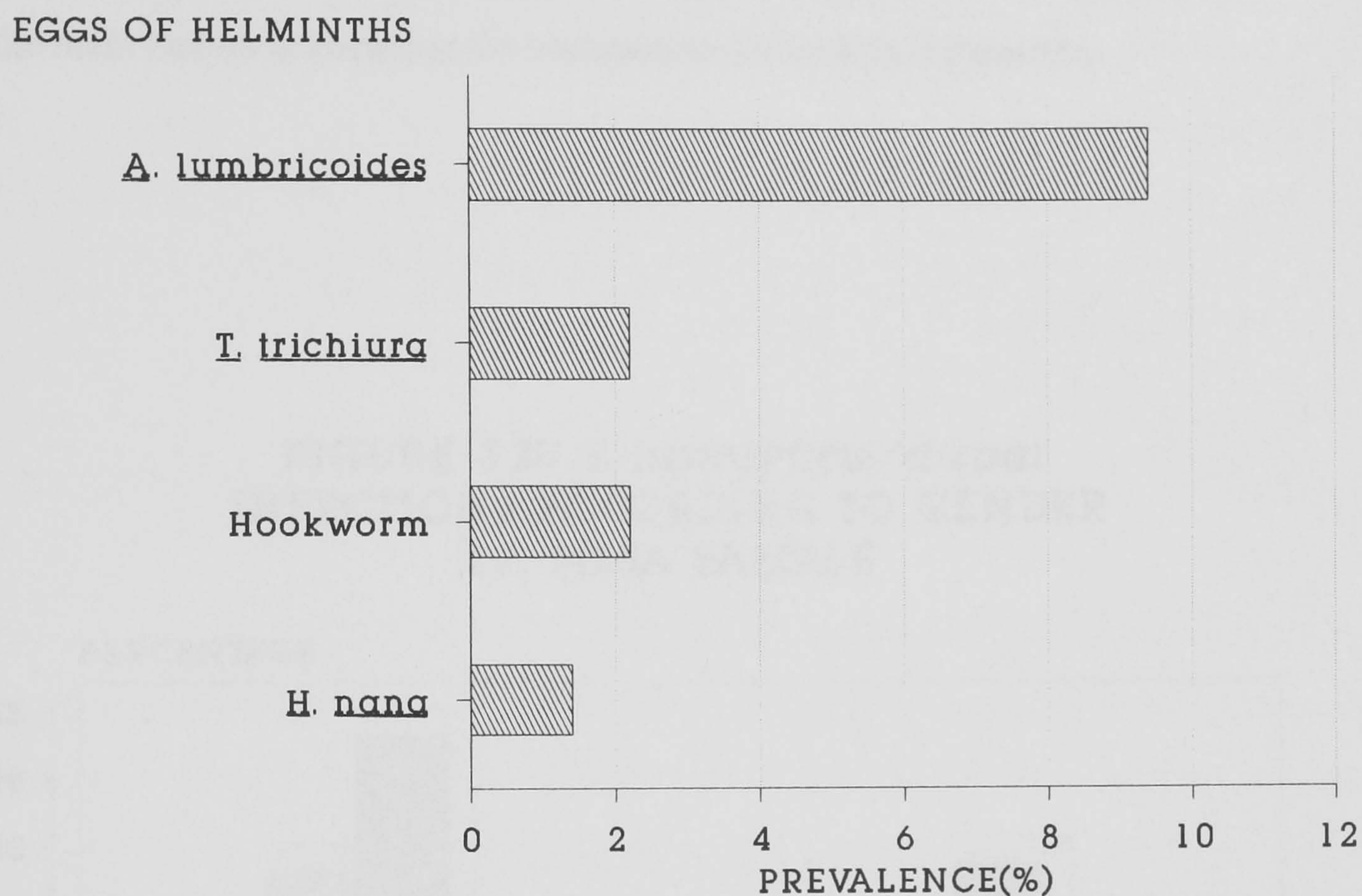
E. nana was found in 36.2% (50/138) of the samples, and *I. butschlii* was present in 26.8% (37/138). The most prevalent parasites were *E. coli* in 43.5% (60/138), and *Bl. hominis* with a prevalence of 69.6% (96/138). Other protozoa found were *G. intestinalis* in 16.6% (23/138) of the population tested, *Ch. mesnili*, 20.3% (28/138) and in a lower proportion, *T. hominis* in 2.2% (3/138) of samples; and at La Mesa, 18.8% (26/138) of the 138 subjects tested were negative for intestinal parasites (Figure 3.18).

FIGURE 3.18 MICROSCOPY OF STOOLS
LA MESA SAMPLE



Among the infections with helminths, the most prevalent was *A. lumbricoides*, found in 9.4% (13/138) of subjects, 2.2% were carrying *T. trichiura* (3/138), or hookworm; 2 (1.4%) were found infected with *H. nana* (Figure 3.19). The low number of infections found may be due to the concentration method used (flotation), and the results might not reflect the real prevalences of helminth infections at La Mesa.

FIGURE 3.19 PREVALENCE (%) OF HELMINTH INFECTIONS AT LA MESA



Prevalence of infection with *E. histolytica*/*E. dispar* was not significantly different (Chi square Yates corrected 0.70, $p=0.401$) between the samples taken from the symptomatic patients from the hospital (8/29) than in the samples collected at the orphanage and at the school (12/56 and 8/53 respectively) at La Mesa. However, for future screenings, close contacts of individuals found carrying *E. histolytica* will be included in the screenings. This might reveal the subjects source of infection in the households, thus their diagnosis and treatment could help to reduce transmission.

When analysing the prevalence of infections according to sex, 17 females out of a total of 68 were found infected with *E. histolytica*/*E. dispar* as compared to only 11 from a total of 70 males screened at La Mesa (Figure 3.20). The difference is not statistically significant (Chi square 1.310, $p=0.2525$), but as discussed previously, it is important because mainly females are in charge of food handling in their households, which is one of the main factors influencing the transmission of intestinal parasites.

FIGURE 3.20 *E. histolytica*/*dispar*
INFECTIONS ACCORDING TO GENDER
LA MESA SAMPLE



ASYMPTOMATIC: Cyst passers at the orphanage and school

3.3.2.2 Cultures

Fourteen cultures were successful after samples of 28 *E. histolytica/dispar* microscopically positive individuals were cultured in Robinson's medium. The microscopy-positive individuals according to location were distributed as follows: 8 came from the school, 12 from the orphanage and 8 from the hospital. Hexokinase characterisation showed slow non-invasive pattern of migration in starch gels, and once again no invasive strains were isolated.

3.4 DISCUSSION

Socio-economical conditions in Colombia have improved steadily during the last 40 years, however, poverty remains the most crucial factor responsible for the regional disparities in the quality of life. According to the Departamento Administrativo Nacional de Estadística (DANE), the national percentage of poor people fell from 45.6% in 1985 to 37.2% in 1993. From the 33.1 million people censused in 1993, almost 20% (6 million) had an income below the subsistence level (less than 1USD/day) and of these, 4.5 million lived in rural areas. Malnutrition affects 10% of children under five, with an infant mortality rate of 37 per 1000 for 1992 (PAHO, 1995). About 3 million (10%) of the population in the country lack access to safe water (World Bank, 1995), accounting for 51,024 hospital cases of enteritis and other diarrhoeal infections in 1994 (DANE, 1996). During the same year, parasitic intestinal infections were the second cause of morbidity in the department of Cundinamarca, with 34,482 cases from a total of 1,046,497 morbidity cases registered by the Servicio Seccional de Salud de Cundinamarca.

The introduction of over the counter antiparasitic treatments in Colombia during the seventies is believed to have dramatically influenced the prevalences of intestinal parasites, with a marked reduction in morbidity and mortality caused by these entities (Robles, 1995),

although transmission rates (reflected in reinfections and prevalences of polyparasitism) continue to be high (Agudelo *et al.*, 1993; Duque *et al.*, 1994; Carvajal *et al.*, 1995). Also, the slight improvement in socioeconomical and sanitary conditions of the population may explain the difference in prevalences for *E. histolytica/E. dispar* among recent and earlier studies, although the high prevalences of other intestinal parasites found still indicates the need for educational campaigns and a greater effort on the development of economical but improved alternatives for sanitation in the country.

The prevalence of *Bl. hominis* brings to attention the ongoing discussion about its pathogenicity. The problem has been actively investigated during the last fifteen years because large numbers of pleomorphic dividing trophozoites have been found in patients suffering from diarrhoea (Zierdt, 1991; Zaman, 1996), however other studies have concluded it is a commensal organism. The results of the present study show no association between *Bl. hominis* and diarrhoeic faeces, although the characteristic polymorphism was present, and large spherical trophozoites (20-40 μm) were frequently observed in Robinson's cultures. Trophozoites of *Bl. hominis* have a higher multiplication rate than *E. histolytica* and competition interfered with the amoebic growth causing the death of many cultures. Contamination was eliminated by two washings of the cultured amoebas in distilled water for 10 minutes and transfer of the pellets to fresh culture medium.

The controversy surrounding *Bl. hominis* shares many features in common with the *E. histolytica/E. dispar* case. The asymptomatic cyst carrier and several symptomatic states have been identified, consisting of self-limited gastroenteritis in some cases, and chronic gastroenteritis in others, where metronidazole is the drug of choice for treatment (Levy *et al.*, 1996), however, in resistant cases emetine, trimethoprim-sulfamethoxazole and pentamidine are claimed to be useful (Zierdt, 1991; Zaman and Zaki, 1996). A link between *Bl. hominis* infection and autoimmune-related disorders such as irritable bowel syndrome (Nagler *et al.*, 1993; Hussain *et al.*, 1997), arthritis (Kruger *et al.*, 1994) and urticaria (Armentia *et al.*, 1993) has been reported.

The presence of "pathogenic" strains with variable virulence, and "non-pathogenic" strains is supported by studies on animal models (Zierdt, 1991; Moe *et al.*, 1996), variation in polypeptide, isoenzyme patterns (Mansour *et al.*, 1995), and serological characterisation (Muller, 1994). Consequently, variation among human isolates has been shown by immunological and DNA hybridization tests, suggesting that different species may infect the same host. Sequencing of the small subunit of ribosomal DNA has provided evidence that *Bl. hominis* is an unusual Stramenopile, which comprises a complex of mitochondria containing organisms including unicellular and multicellular protists (Silberman *et al.*, 1996). As a result, the laboratory diagnosis of *Blastocystis hominis* represents a new challenge for the design of "pathogenic"-specific diagnostic methods.

The need for improved diagnostic techniques, incorporating differential diagnosis between entities such as *E. histolytica* and *E. hartmanni* on one hand and between *E. histolytica* and *E. dispar* on the other is evident. In a recent study on the quality of diagnosis for amoebiasis carried out in 58 laboratories in different areas of Bogota, Guhl *et al.* (1996) found that only one laboratory used a micrometer eye piece, 95% of the laboratories failed in recognising cysts and/or trophozoites of *E. histolytica/dispar* and 81% of the total failed to diagnose *E. coli* cysts in spiked faeces.

The accurate diagnosis of amoebiasis relies on the improvement of the methodology for the analysis of microscopically positive samples with new diagnostic targets, where detection is based not only in sensitive and specific procedures, but also on targets stable enough to permit samples collected in the field to reach reference laboratories. Ideally, such procedures should be applied on the faecal sample directly, avoiding the problems of selection during culture. As an example, although all microscopically positive samples from Ibaguè and La Mesa were cultured in Robinson's medium, very few strains were isolated because of the difficulty of maintaining and transporting cultures in the field. At Ibaguè, the high number of positive samples required high volumes of materials and culture media. Also, the procedure of examining each of them for positivity was time consuming and most of the strains did not survive long term culture. A further disadvantage of

cultured material is the possibility of selection of populations, which for example influences the results on true prevalences of mixed infections. In terms of the lysates for isoenzyme analysis, protein extracts are not stable except under cryopreservation, and the intensity of the banding pattern varies depending on the media components, influencing interpretation of results.

Antigen and nucleic acid detection systems seem to be the most appropriate candidates for differential diagnosis in the future. The field work in Colombia was a valuable source of information and material. Some of the cultures collected and characterised during the field work were used to standardise and evaluate procedures involving antigen detection with monoclonal antibody 20/7D in Chapter 4 and DNA amplification using Polymerase Chain Reaction (PCR) and ELISA in Chapter 5.

CHAPTER 4
DETECTION OF *ENTAMOEB*A HISTOLYTICA USING MONOCLONAL
ANTIBODY 20/7D

4.1 INTRODUCTION

Producing an assay which can distinguish between *E. histolytica* and *E. dispar* species has been one of the main lines for amoebiasis research. Monoclonal antibodies (MAbs) have been the first choice as diagnostic tools when incorporated in IFA or ELISA tests. For this purpose, a series of MAbs have been generated against *E. histolytica* (Table 4.1). Among the most interesting assay formats proposed, Bhaskar *et al.*, (1996) used a rapid, single step immunochromatographic test in which MAb AC55 was conjugated to colloidal gold to detect the presence of *E. histolytica*/*E. dispar* antigen in faeces as a dot in nitrocellulose strips, with a sensitivity of 97.6% and a specificity of 92.6%. Application of this format to specific detection of *E. histolytica* would be valuable.

TABLE 4.1 *ENTAMOEB*A ANTIGEN DETECTION WITH MONOCLONAL ANTIBODIES

MAb ID	ANTIGEN	<i>Eh</i>	<i>Ed</i>	LOCATION	REFERENCE
116	Lipophosphoglycan(LPG)	+	-	S	Mirelman <i>et al.</i> , 1997
EH3023	150 kDa, Gal/GalNac-Inhibitable lectin	+	-	S, I	Tachibana <i>et al.</i> , 1997
AC55	14 kDa, 21 kDa	+	+	I	Bhaskar <i>et al.</i> , 1996
NICED 11	22 kDa, 29 kDa	+	+	ND	Segupta <i>et al.</i> , 1993
Eh208C2-2	122 kDa, 115 kDa, 111 kDa, 65 kDa	+	+	S, I	Wonsit <i>et al.</i> , 1992
4G6	30 kDa, Peroxidoxin	+	-	I	Tachibana <i>et al.</i> , 1990
7F4, 8C12	170 kDa	+	-	S	Petri <i>et al.</i> , 1990a
NA	Serine Rich Antigen	+	+	S	Stanley <i>et al.</i> , 1990
22.5	ND	+	-	I	Strachan <i>et al.</i> , 1988
EH403	96 kDa	ND	ND	S	Torian <i>et al.</i> , 1987
NA	50 kDa, 25 kDa	ND	ND	S	Ortiz <i>et al.</i> , 1986

Eh: *E. histolytica*. *Ed*: *E. dispar*

S: Surface. I: Intracellular

NA: Not Available

ND: Not Determined

Also among those commercially available, the surface serine-rich antigen-specific MAb (Stanley *et al.*, 1990) has been used for clinical diagnosis (Optimum S kit, Merlin Diagnostica, USA). However, since most of the epitopes on this particular and other antigens are shared by *E. histolytica* and *E. dispar*, this test is therefore not specific. The most advanced kits currently available employ a mixture of MAbs specific for the surface adhesin of the parasites and are distributed by either TechLab or Cellabs as the *Entamoeba* test and the *E. histolytica* specific test (Haque *et al.*, 1995). Since the latter test is able to distinguish *Entamoeba histolytica sensu stricto* infections but the presence of *E. dispar* can only be determined if the *Entamoeba* test is positive with a negative result in the *E. histolytica* specific test, each sample should ideally be typed with both kits.

According to the manufacturers of the Techlab *E. histolytica* kit, the sensitivity and specificity of the test are 92.6% and 96.7%, respectively when evaluated against cultures. Two further evaluations using clinical samples from Bangladesh have, however revealed sensitivities of 85% and 86% respectively for antigen detection when compared with microscopy of faecal samples (Haque *et al.*, 1998; Haque *et al.*, 1997). The literature included with the test reports a sensitivity of 100% and a correlation of 84% when the *Entamoeba* test is compared to culture and/or PCR. These two tests are presently the best available for the fast confirmation of *E. histolytica sensu stricto* infection. However, since *E. dispar* is diagnosed only by deduction, and most infected people carry *E. dispar*, deduction is a problem because the *E. histolytica* test is of low sensitivity, thus this limits the use of the kits for epidemiological studies. Furthermore, an independent evaluation determined that the limit of detection for the *E. histolytica* test was 1000 trophozoites/well, which is approximately 100 times less sensitive than PCR when compared to the specific amplification of the small subunit of rDNA (Mirelman *et al.*, 1997).

MAb 20/7D which was able to distinguish invasive from non-invasive strains in clinical isolates was described in 1992 (Figures 4.1 and 4.2).

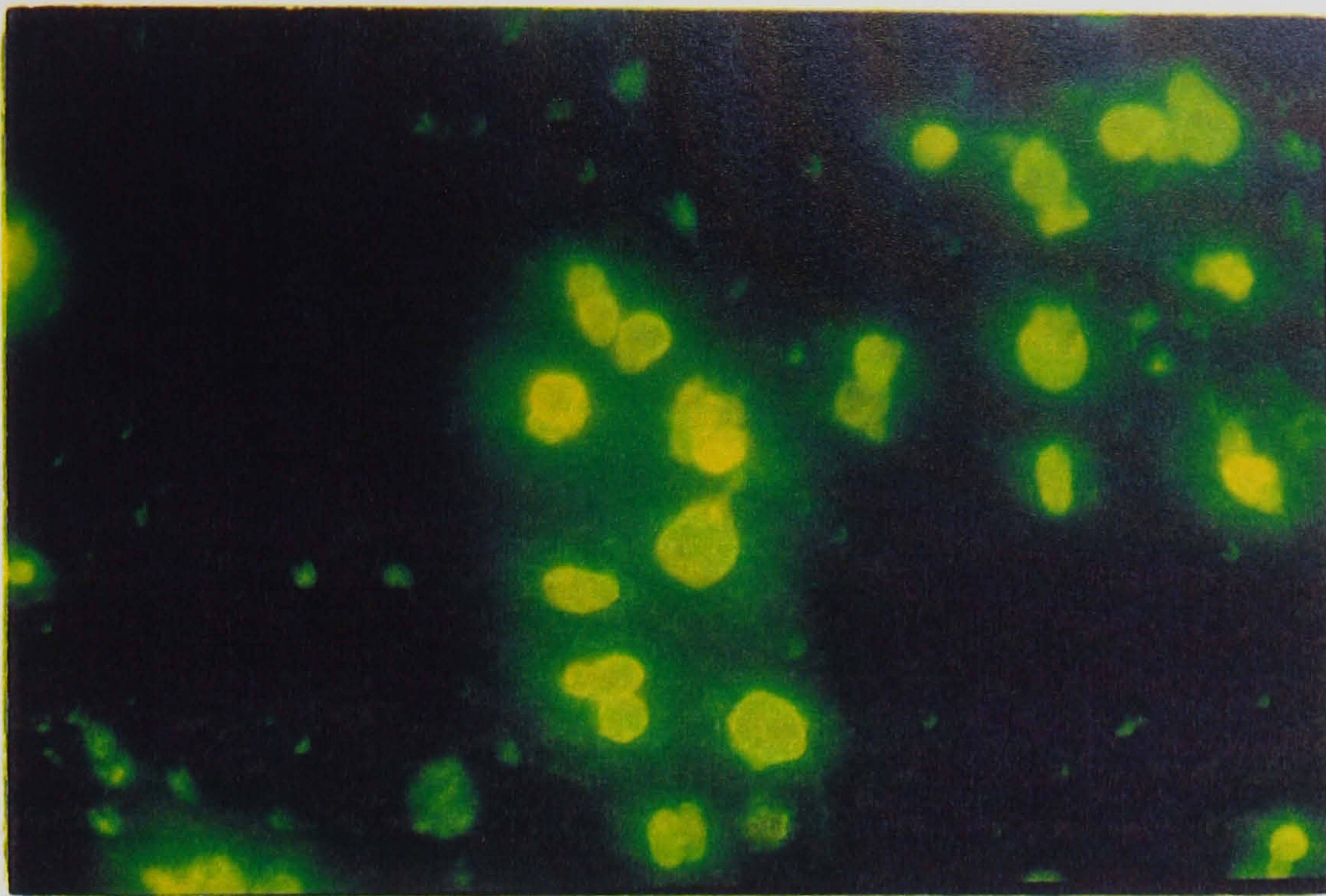


FIGURE 4.1 DETECTION OF *E. histolytica* BY IFA WITH MONOCLONAL ANTIBODY 20/7D. POSITIVE REACTION

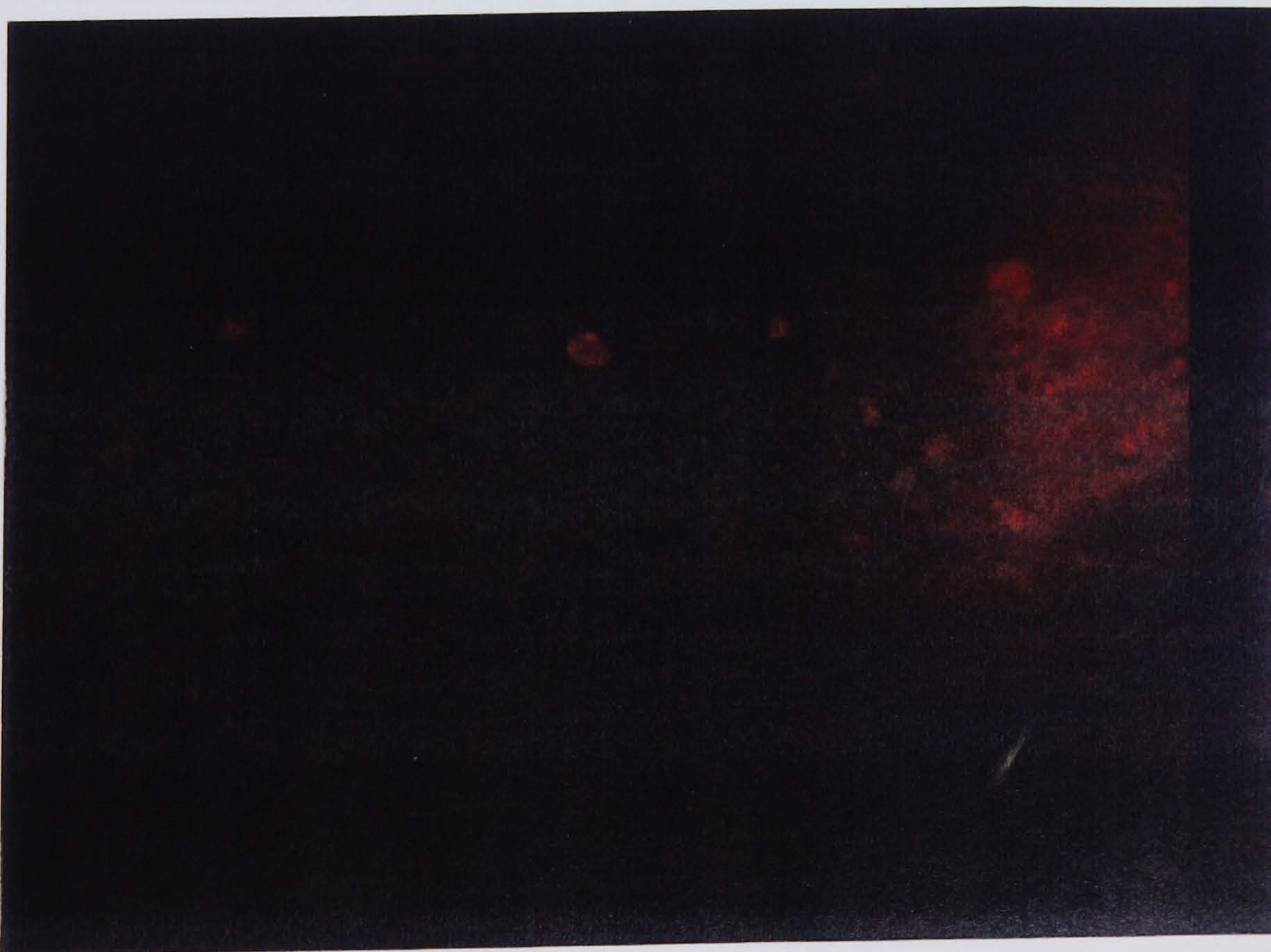


FIGURE 4.2 DETECTION OF *E. dispar* BY IFA WITH MONOCLONAL ANTIBODY 20/7D. NEGATIVE REACTION

The characterisation of MAb 20/7D was performed on lysates of axenic *E. histolytica* reference strains and on invasive clinical isolates from Colombia and it was shown to recognise a protein in the range of 81 and 84 kDa in western blot assays from axenic or polyxenic cultures, respectively (Gonzalez-Ruiz *et al.*, 1992a). Although MAb 20/7D also reacted faintly with a homologous protein in lysates of *E. dispar*, the results of the IFAT testing on a further 107 cultured isolates allowed a 100% correlation with the zymodeme analysis of strains, which corresponded to isolates from different geographical areas in Bangladesh, Mexico and Colombia (Gonzalez-Ruiz *et al.*, 1992c). The ability of MAb 20/7D to recognise an analogous antigen in lysates of *E. dispar* suggested that a common antigen was present in both species, at a lower level in *E. dispar*. However, MAb 20/7D was then tested for its ability as an antigen detection system applied directly in faecal samples for diagnosis of amoebic dysentery. Thus, the initial evaluation of an antigen detection ELISA system (Faecal Antigen Capture ELISA, or FAC-ELISA) using affinity purified rabbit polyclonal antibodies (capture step) and MAb 20/7D-containing ascitic fluid for the detection step was performed with 108 stool samples from Bangladesh (Gonzalez-Ruiz *et al.*, 1994b). The FAC-ELISA system detected 13 out of 15 *E. histolytica* infections. There were no false positives in the remaining 93 samples composed of 32 *E. dispar* carriers, 33 samples from patients with other parasites different from *E. histolytica* or *E. dispar* and 28 true negative samples (i.e. no parasites found). The corresponding sensitivity and specificity values were 87% and 100% respectively. In order to compensate for high background signals, this test employed a control of commercially available non-immune rabbit IgG. The optical densities (ODs) of these controls were then subtracted from the OD readings obtained from the test samples. The cut off value was calculated by the average of these controls plus three standard deviations. Optical densities >0.035 were considered positive.

The encouraging results described above suggested the possibility of further developing the test and it was the aim of the work described in this chapter. One of the modifications proposed was to substitute the use of ascites by the purified MAb in the FAC-ELISA in order to improve the sensitivity of the test. Also, to evaluate further the

dynamics of the individual components of the FAC-ELISA system for the detection of *E. histolytica* infections and their differential diagnosis from *E. dispar*. All the work on the evaluation of MAb 20/7D was performed under the advice and supervision of Dr Andrew Falconar, who carried out all animal procedures.

4.2 MATERIALS AND METHODS

MAb 20/7D cell line was first grown and re-cloned followed by the production of ascitic fluid. This IgG1 MAb was then purified using a Protein-G Sepharose column (Sigma, UK). Then, the immunoaffinity column was prepared using purified MAb 20/7D and this column was used for immunoaffinity purification of cell lysates of *E. histolytica* and *E. dispar* trophozoites. The positive fractions were identified by protein assay, and the further identification of the target proteins obtained from lysates of parasites was carried out in dot-blot assays using either rabbit polyclonal antibody or the purified MAb 20/7D. The selected fractions were pooled and the *E. histolytica* and *E. dispar* proteins were then compared by SDS-PAGE under non-reducing conditions with subsequent western blot analysis.

The proteins were also tested by ELISA using *E. histolytica*-immune rabbit serum and MAb 20/7D. Capture-ELISA for the detection of the purified *E. histolytica* and *E. dispar* proteins was then performed using affinity purified rabbit IgG specific for *E. histolytica* antibody in the capture step, and MAb 20/7D as secondary antibody. The results were then evaluated to assess the feasibility of the assay for use in differential diagnosis.

4.2.1 Hybridoma Culture

One cryopreserved vial of hybridoma 20/7D containing 1×10^6 cells/ml was removed from liquid nitrogen, thawed in a 37°C water bath, wiped in 70% (v/v) ethanol and transferred to a category II laminar flow cabinet. The cells were slowly diluted in 10 ml of

serum-free RPMI (Roswell Park Memorial Institute) 1640 medium (Appendix 1), washed by centrifugation at 700 g for 10 minutes, resuspended in 10 ml of growth medium and transferred to a 25cm² culture flask (1-63371 Nunc, DK). This flask was incubated at 37°C in air containing 5% CO₂ until the cells were confluent. Subcultures were established by gently resuspending the cells in fresh complete RPMI medium (Appendix 1) and transferred to new culture flasks. Once the new cultures were 70% confluent, the cells were cloned.

4.2.2 Cloning of Hybridoma Cells

For cloning, mouse peritoneal mononuclear cell feeder layers (provided by Dr Falconar) at 1×10^4 cells/100 μ l of RPMI complete medium (Appendix 1) were added to each well of 96 well tissue culture plates. These plates were then incubated at 37°C overnight. MAb 20/7D hybridomas were then gently removed from the flasks, counted using a Neubauer haemocytometer and diluted in growth medium to obtain 160 hybridoma cells/ml. Then, 0.9 ml of the cell suspension were added to 0.9 ml of growth medium, to give a final concentration of eight hybridoma cells/200 μ l. 100 μ l of serial two fold dilutions were transferred into columns of the previously prepared 96 well microtitre plate which contained the cell-feeder layers to give 8 cells/ well in column 1, 4 cells/well in column 2, 2 cells/well in column 3, and 1 cell/well in columns 4 and 5.

After incubation for 7 days at 37°C with 5% CO₂, 100 μ l of fresh medium was added to each well and the wells were examined for hybridoma growth. Wells in columns 4 and 5, which contained single colonies were marked, and when they reached 50% confluency, these supernatants were assayed for MAb by IFA. Wells which secreted antibodies were transferred to 24 well plates and when these were 50-80% confluent, these clones were subcloned as described above. The MAb producing clones were then expanded into 80 cm² flasks for freezing and ascitic fluid production.

4.2.3 Screening of 20/7D Subclones by Immunofluorescent Assay (IFA)

12-well immunofluorescence slides (polytetrafluorethene-coated slides, Hendley, PH-001) which contained methanol-fixed trophozoites of polyxenic *E. dispar* SAW 1734 and axenically cultured *E. histolytica* HMI-IMSS or NIH-200 were used to test the MAb 20/7D cell-culture supernatants for *E. histolytica* specificity by IFA. Supernatants from the MAb non-producing myeloma cell line Sp2/O-Ag14 (Shulman *et al.*, 1978) (kindly provided by Dr A. Gonzalez), were used as negative controls for IFA. For the test, 10 µl of two fold dilutions (1/2 to 1/64) from each cell-culture supernatant was added to each well and slides were incubated in a humid chamber for 30 minutes. After washing for five minutes in PBS pH 7.2, 10 µl of a 1/500 dilution of fluorescein-conjugated goat anti-mouse IgG(H+L) (115-016-062, Jackson ImmunoResearch Laboratories, USA) diluted in 1/10,000 dilution of Evans blue were added to each well. After incubation for 30 minutes in humid chamber, and washing in PBS pH 7.2 for five minutes, the slides were allowed to dry and were mounted in glycerol buffer (1000-4 Sigma, UK) under a cover slip. Positive specimens were identified using an immunofluorescence microscope (Diaplan Leitz, Germany).

4.2.4 Screening of 20/7D Subclones by ELISA

MAb 20/7D-secreting cell-culture supernatants which were positive in the IFA were tested by ELISA using a crude lysate of *E. histolytica* HM1-IMSS trophozoites. For the ELISA, 96-well ELISA plates (Immulon 2, Dynatech, UK) were coated with 100 µl of 1.2 µg/ml of protein in sodium carbonate-bicarbonate buffer pH 9.6 (coating buffer, Appendix 1), previously found by titration to be the optimal antigen concentration. The plates were incubated overnight at 4°C in a humid container. Unbound antigen was removed by washing with PBS which contained 0.05% v/v Tween 20 (PBS/T, Appendix 1). The plates were then dried by blotting on to paper towels and stored at -20°C with desiccant for up to one month.

Before use, these plates were then blocked using 100µl/well of 1% gelatin/PBS and incubated for 1 hour at 37°C in a humid container. After washing 3 times with PBS/T, three-fold serial dilutions of each MAb cell-culture supernatant were prepared in PBS/T containing 0.25% gelatin (PBS/T/G) and these plates were then incubated for 1 hour at 37°C. After six washes with PBS/T, 100 µl of a 1/1500 dilution of horse-radish peroxidase conjugated goat anti-mouse IgG antibody (A-2304, Sigma, UK) diluted in PBS/T/G were added to each well and these plates were incubated for 1 hour at 37°C in a humid chamber. After washing, 100 µl of OPD-H₂O₂ substrate prepared in citrate/phosphate buffer pH 5.0 (Appendix 1) was added to each well. The plates were then incubated at room temperature for 20 minutes, before the reactions were then stopped by addition of 50µl/well of 2.5M H₂SO₄, and the absorbance was read at 492 nm (MR700, Dynatech microplate reader, UK).

4.2.5 Cryopreservation of Myeloma Cells, and Hybridomas

Approximately 2x10⁶ cells of each MAb 20/7D hybridoma cell-line were resuspended in 1.8 ml of ice-cold foetal calf serum which contained 10% v/v dimethyl sulphoxide (DMSO) (D-4540, Sigma UK). Two 1 ml aliquots were distributed in cryovials (Nunc, DK) and slowly the vials were frozen to -70°C (-1°C/min) before being transferred to liquid nitrogen.

4.2.6 Production of 20/7D Ascitic Fluid

Groups of 20 eight week old female BALB/c mice were injected intra-peritoneally (i.p.) with 0.5 ml of pristane (2,6,10,14-tetramethyl pentadecane) (Sigma, UK) followed by the i.p. injection of 2x10⁶ hybridoma cells contained in 0.5 ml of PBS (pH 7.2) 10-14 days later. After 14-28 days, ascitic fluid was collected from these animals using a 18 gauge hypodermic needle, clarified by centrifugation for 10 minutes at 700 g and the supernatants were then stored at -40°C.

4.2.7 Production of Immunoaffinity Columns

4.2.7.1 MAb 20/7D Purification

MAb 20/7D was purified from ascitic fluid using a 5ml Protein G-Sepharose Cl 4B fast flow column (P-3296, Sigma, UK). For this, 20 ml of ascitic fluid were diluted to 50 ml in TNE buffer (loading buffer, Appendix 1) and allowed to bind overnight at 4°C. After washing the column with 500 ml of TNE, bound MAb 20/7D was eluted with 0.1 M glycine/HCl pH 2.5 and 2 ml fractions were immediately neutralised with 250µl of 2M Tris-HCl buffer, pH 8.0 and stored at -20°C.

4.2.7.2 MAb 20/7D Immunoaffinity Column

Purified MAb 20/7D was adjusted to a protein concentration of 5 mg/ml (5 ml) in coupling buffer (Appendix 1) by reduced pressure dialysis against three 1 litre changes of coupling buffer. 1 g of cyanogen bromide-activated Sepharose 4B (Pharmacia, UK) was then washed with 500 ml of 1 mM HCl (pH 3.0) at 4°C. The MAb 20/7D was then added and coupling was allowed to proceed overnight at 4°C in a 5 ml polypropylene tube on a rotating mixer. After draining the coupling buffer, the excess binding sites were blocked using 0.2M glycine in 10mM Na₂HPO₄ pH 8.0 for 2 hours in a rotating mixer at room temperature. The coupled Sepharose was then transferred to a 5 ml polypropylene column and subjected to five high and low pH alternations using 0.1M acetate buffer containing 0.5M NaCl pH 4.0 and coupling buffer (pH 8.5) to remove the excess uncoupled protein. The column was then stored at 4°C with PBS which contained 0.02% w/v sodium azide.

4.2.8 Immunoaffinity Purification of Proteins

1x10⁶ trophozoites of axenic *E. histolytica* and polyxenic *E. dispar* cultures were lysed using 0.5% v/v Nonidet P-40 (NP 40) diluted in TNE which contained a mixture of protease inhibitors (Appendix 1) to minimise protein degradation. After incubation

overnight at 4 °C, these lysates were diluted with 50 ml of loading buffer (TNE, Appendix 1) and passed through the MAb 20/7D immunoaffinity column overnight at 4 °C. After washing with 500 ml of TNE which contained protease inhibitors, the bound antigens were eluted using 0.05M diethylamine/TNE (pH 11.3) and 2 ml fractions were immediately neutralised with 250 µl of 2M Tris-HCl pH 7.0.

4.2.9 Dot-ELISA for Detection of *E. histolytica* and *E. dispar* Proteins in Eluates

Nitrocellulose (NC) membranes were soaked in PBS pH 7.4 and dried at room temperature. From each fraction 2µl were applied as dots on 5mm grids on the nitrocellulose membranes and were dried at room temperature. Each membrane also had antigen (2µl) control dots corresponding to Bovine Serum Albumin (1 mg/ml stock), conjugate diluent (PBS-3% casein), faecal diluent (50% FCS/0.02% NaN₃/0.3% PBST, Appendix 1) and *E. histolytica* and *E. dispar* antigens (1.2 mg/ml each). Non-specific binding sites were blocked by incubation with PBS-casein 3% for 15 minutes. After washing with PBST, either immune rabbit serum 1:100 or purified monoclonal antibody diluted 1:50 in PBS-3% Casein were added to the membranes in plastic bags and incubated for 30 minutes at room temperature. A final wash with PBS-T eliminated unbound antibodies.

The corresponding peroxidase labelled conjugates containing goat anti-rabbit IgG or goat anti-mouse IgG were incubated for 15 minutes at room temperature. After washing with distilled water, the substrate (10 ml of PBS-T containing 10 µl of a 3 mg/ml 4-chloro-1-naphthol stock in methanol and 10 µl of 30% hydrogen peroxide) was incubated with the membranes in a plastic bag for 15 minutes at room temperature. Then the membranes were washed in distilled water to stop the reaction. The results were read positive when a purple dot was seen.

4.2.10 Polyacrylamide Gel Electrophoresis (PAGE)

Discontinuous SDS-PAGE (Laemmli, 1970) was performed using a minigel apparatus (Mini-Protean II, 165-2940, Bio-Rad Laboratories Ltd., UK). An 8% w/v polyacrylamide resolving gel and a 3.4% w/v stacking gel were prepared (Appendix 1). Both gels were allowed to polymerise for 1 hour at room temperature. Purified protein and culture lysate samples (8 μ l) were mixed with 2 μ l of 5X sample buffer (without 2-mercaptoethanol) and bromophenol blue (2 μ l of 0.2% bromophenol blue w/v in 50% glycerol/water, Appendix 1). After heating the samples (100°C for three minutes in a boiling water bath), 10 μ l of each sample was loaded/well. Molecular weight standards of the proteins (MW-SDS-200, Sigma, UK) were added to the outer wells to calculate the relative Mr (Appendix 1).

The proteins were separated using 200 V (constant voltage). The gels were then either stained to reveal all proteins or the proteins were transferred directly onto nitrocellulose membranes for immunoblot analysis (western blot assay, see 4.2.10). For staining, the gels were initially fixed using a mixture of 40% methanol and 14% acetic acid in water (Appendix 1) for half an hour before being stained with the 0.25% w/v Coomassie blue R250 solution (Appendix 1) for 1 hour. Destaining was then performed using 5% methanol and 7.5% acetic acid in water until sufficient contrast was obtained.

4.2.11 Western Blotting

After electrophoresis the gels were immersed in 1X blotting buffer (Appendix 1) together with six (8.5 cm² x 5.5 cm²) Whatmann 3MM filter papers. These papers were used to "sandwich" a sheet of 0.2 μ m pore size nitrocellulose (401-196, Schleicher & Schuell, Germany) next to the gel. Semi-dry electroblotting of the proteins was performed at 160mA/gel (Sartoblot II-S, SM 17556, Sartorius Ltd., UK) for 30 minutes at room temperature. The membranes were then air-dried, blocked overnight with 3% w/v skimmed

milk (Cadbury, Marvel, UK) in PBS that contained 0.02% NaN₃ (PBS/T/M) at room temperature. After washing three times with PBS/T, a 1/200 dilution of purified MAb 20/7D was allowed to react with the membrane in a sealed plastic bag overnight on a horizontal shaker (Mk VII, LH Engineering Co., UK). After washing the membranes three times with PBS/T, a 1/2,000 dilution of goat anti-mouse IgG conjugated with horse-radish peroxidase (A-2304, Sigma, UK) in PBS/T/M was allowed to react for one hour at room temperature. After three washing steps, diaminobenzidine/4-chloro-1-naphthol substrate (Appendix 1) was added and allowed to react for 10 minutes at room temperature. The reaction was stopped by washing the membranes with distilled water and residual enzyme activity was destroyed with 0.01M H₂SO₄ for 10 seconds before the membranes were thoroughly washed with distilled water.

4.2.12 Generation of Rabbit Anti-*E. histolytica* Specific Polyclonal Antibodies

Two male New Zealand White (NZW) rabbits were initially bled from the marginal ear vein and the blood was allowed to clot at room temperature for 20 minutes. The clot was detached and centrifuged at 700 x g for 30 minutes at 4°C and the serum was collected. This pre-immune serum was checked for absence of antibodies against *E. histolytica* and as negative control in IFA and ELISA assays. Each animal was then immunised intramuscularly with 1x10⁶ axenically cultured trophozoites of *E. histolytica* strain HMI-IMSS in a total volume of 0.5 ml of Freund's complete adjuvant (FCA, F-5881 Sigma, UK). These animals were then boosted with the same dose of antigen emulsified in Freund's incomplete adjuvant (FIA, F-5506 Sigma, UK) every two weeks for four months. Blood samples were obtained before each antigen boost and aliquots of each serum sample were mixed with an equal volume of glycerol and stored at -20°C.

4.2.13 Titration of Rabbit Polyclonal Anti-*E. histolytica* Specific Antibodies

ELISAs were performed using *E. histolytica* lysate as described for MAb 20/7D (see 4.2.4), except that the bound rabbit IgG was identified using a 1/5,000 dilution of goat anti-rabbit IgG conjugated with horse radish peroxidase (HRP) secondary antibody (111-036-003, Jackson's ImmunoResearch, USA).

4.2.14 Affinity Purification of Rabbit Polyclonal Anti-*E. histolytica* Specific Antibodies

IgG from the rabbit serum samples (see 3.2.10) were precipitated by the addition of an equal volume of ice-cold saturated ammonium sulphate $[(\text{NH}_4)_2\text{SO}_4]$ solution (pH 7.4). After continuous stirring for two hours at 4°C, the mixture was then centrifuged at 5000 g for 10 minutes at room temperature. The pellet was then washed twice with 50% saturated ammonium sulphate solution and then dissolved in half of the original volume of distilled water. These samples were then transferred into dialysis tubing which had a 12.4 kDa cut-off value (D-9777 Sigma, UK) and dialysed overnight against 25 mM Tris/HCl which contained 70 mM NaCl pH 8.8.

Then a column of DEAE/Trisacryl (Reactifs IBF, Villeneuve la Garenne, France) was packed and equilibrated in 25 mM Tris/HCl which contained 35 mM NaCl (TN) pH 8.8. The precipitated rabbit IgG was diluted with an equal volume of 25 mM Tris/HCl pH 8.8 and added to the column at a flow rate of 1 ml/min. This column was washed with TN buffer and elution was then performed using 25 mM Tris/HCl which contained 100 mM NaCl pH 8.8. Twenty, 2 ml fractions were then collected and any remaining proteins were finally eluted with 25 mM Tris/HCl which contained 500 mM NaCl pH 8.8. The fractions that contained the protein were identified using a BCA protein assay (see 2.6), pooled and then dialysed overnight at 4°C against 0.15M NaCl solution and stored at 4°C with 0.02% w/v 3mM NaN_3 . The rabbit IgG was further purified using Protein A coupled Sepharose

6MB (P-6649 Sigma, UK) column. After loading the IgG in TN buffer pH 7.8 and washing with 500 ml of this buffer, the bound IgG was eluted using 0.2 M glycine/HCl pH 3.0 and one ml fractions were immediately neutralised with 200 μ l of 1M Tris/HCl pH 7.6. The purified IgG was then stored at -20°C.

4.2.15 Capture ELISA (FAC-ELISA) Using *E. histolytica* and *E. dispar* Spiked Stool Samples

4.2.15.1 Preparation of Samples

Stool samples found to be negative for any intestinal parasites by microscopy with concentration were pooled and diluted in PBS which contained 0.02% w/v sodium azide, 0.3% v/v Tween 20 and 50% foetal calf serum (PBS/T/FCS) as described previously (Gonzalez-Ruiz *et al.*, 1994b). 1×10^6 trophozoites (approximately 1.2 mg protein/ml) of freshly harvested *E. histolytica* (HMI-IMSS) and *E. dispar* (SAW 1734) reference strains were lysed by three freeze/thaw cycles using liquid nitrogen. These suspensions were then centrifuged at 3500 g for 10 minutes at 4°C, and the supernatants were diluted to 1/100 (12 μ g protein/ml) and to 1/500 (2.4 μ g protein/ml) PBS/T/FCS with or without the negative faeces.

4.2.15.2 Test Procedure

ELISA plates (Immulon 4, Dynatec, UK) were coated (50 μ l/well) with a 10 μ g/ml solution of purified rabbit anti-*E. histolytica* IgG (see 4.2.13) in carbonate-bicarbonate buffer pH 9.6 (coating buffer, Appendix 1) and incubated overnight at 4°C. After three washings with PBS/T, the plates were blocked for 1 hour at 37°C using 200 μ l/well of 1% w/v gelatin/PBS. After three washings, 100 μ l of the *Entamoeba* spiked faecal samples were added to duplicate wells and the plates were incubated for 2 hours at room

temperature. After washing 5 times with PBS/T, 100 µl/well of purified MAb 20/7D diluted to 1/3000 in PBS/T which contained 0.25% w/v gelatin and 50% FCS (PBS/T/G/FCS) were added and the plates were incubated for 2 hours at room temperature. After washing with PBS/T, 100 µl/well of goat anti-mouse conjugated with peroxidase (A-2304 Sigma, UK) diluted 1/1000 in PBST/G/FCS were added and the plates were incubated for 1 hour at 37°C. After washing the plates, bound antibodies were identified by the addition of 100 µl/well of o-phenylene diamine substrate (OPD) in citrate/phosphate buffer pH 5.0 (Appendix 1) and the plates were incubated for 30 minutes in the dark. The reactions were then stopped by the addition of 50 µl/well of 2.5M HCl and the absorbance was determined at 492 nm (MR700, Dynatec, UK).

4.3 RESULTS

4.3.1 Selection of Monoclonal Antibody 20/7D Clones

Four subclones of MAb 20/7D (4A9, 3C6, 1E2, and 1H2) were positive by both IFAT against methanol-fixed *E. histolytica* and *E. dispar* trophozoites (see 4.2.3), and ELISA against crude lysates of *E. histolytica* HM1-IMSS (Table 4.2). Each of these produced higher MAb titres than the original uncloned parent cell-line. Each of these subclones was then further subcloned to obtain 3 to 7 stable hybridoma cell-lines, for a total of 18 antibody-producing hybridomas (Lot 1). The logarithm of the 50% end point titre of antibody from each cell-line is registered in Table 4.2.

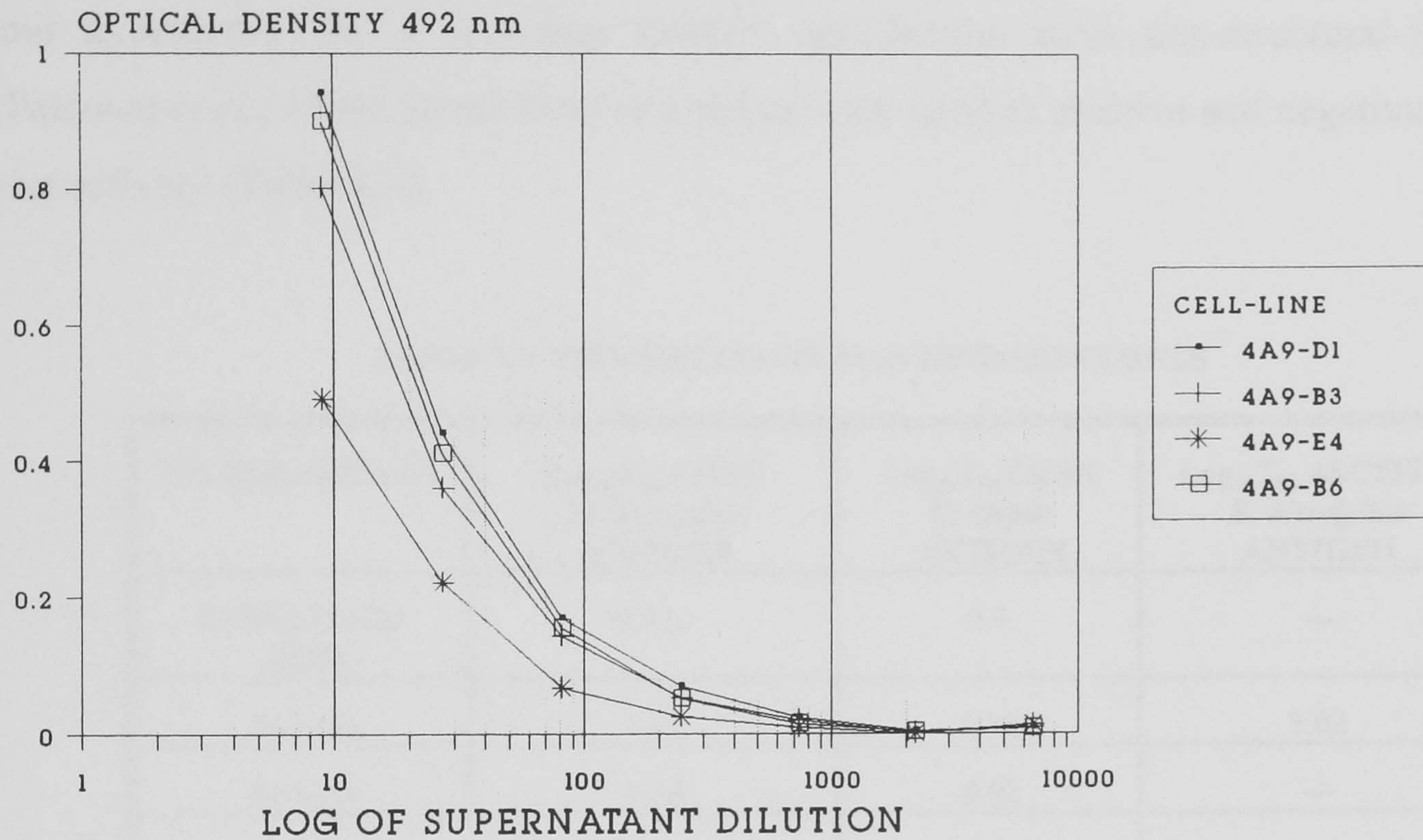
Nine of the hybridomas (Lot 2, Table 4.2) were selected for subculture on the basis of their growth rate, then their antibody titres were confirmed by ELISA using crude lysates of *E. histolytica* antigen as above (Figures 4.3 A and B).

TABLE 4.2 SELECTION OF MAb 20/7D SUBCLONES USING AN *E. histolytica* LYSATE

1st SUBCLONE	2nd SUBCLONE	Log ₁₀ T ₅₀ CCSN (Lot 1)	Log ₁₀ T ₅₀ CCSN (Lot 2)
4A9	B3	1.67	1.41
	B6	1.71	1.41
	D1	1.62	1.42
	E4	1.5	1.43
	H4	1.44	---
3C6	D9	1.44	---
	G10	1.42	---
	F4	1.59	1.44
1E2	A1	1.36	---
	B7	1.42	---
1E2	C5	1.86	1.78
	H2	1.4	---
1H2	B4	1.5	---
	C9	1.67	---
	D1	1.62	---
	D4	1.6	1.76
	E1	1.6	1.72
	E8	1.57	---
	F4	1.6	1.78
	MAb 20/7D	PRECLONED	0.916

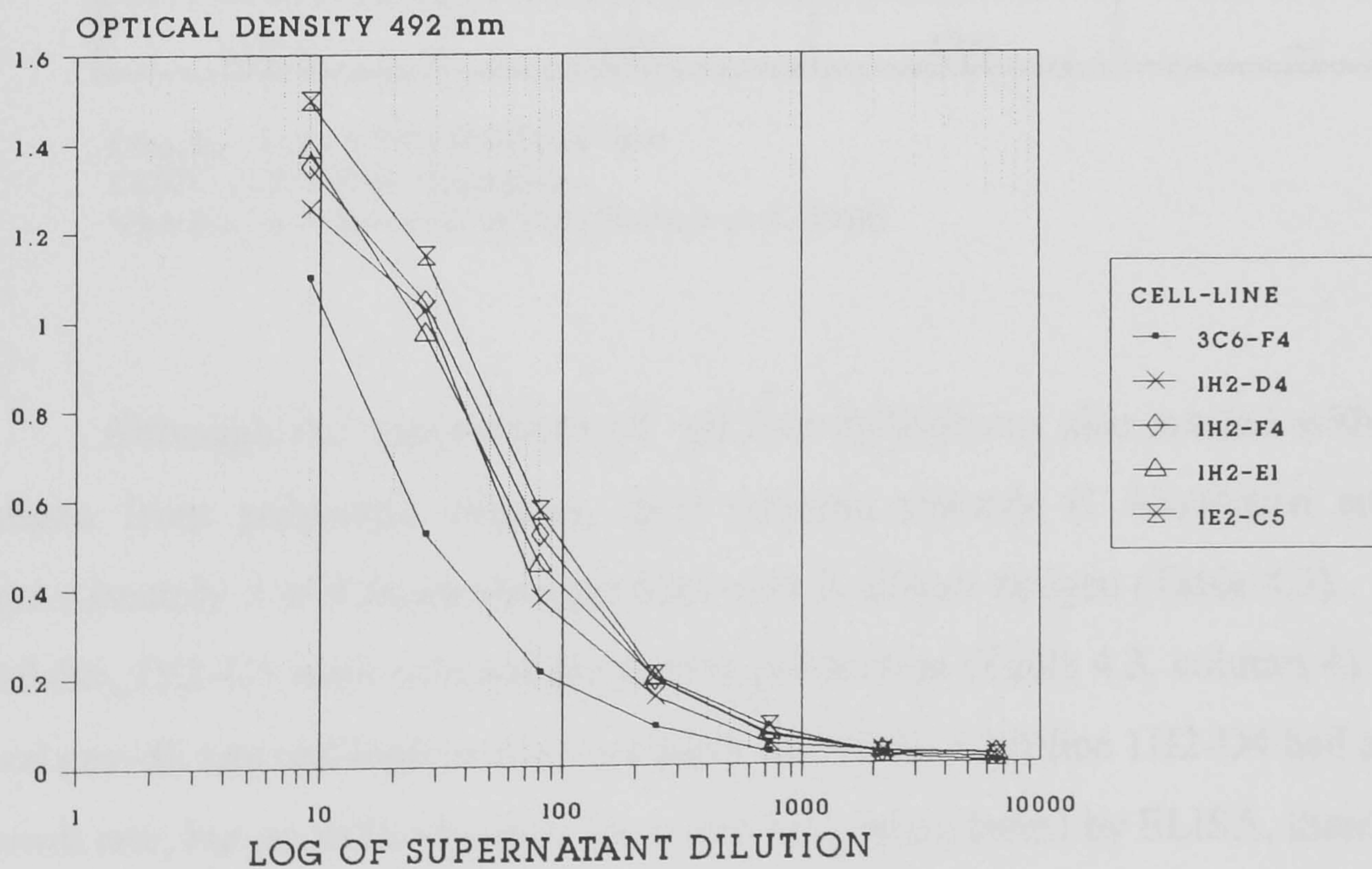
CCSN: Cell Culture Supernatant.
 Log₁₀T₅₀: Log₁₀ 50% End-Point Titre

Figure 4.3A CLONED LINES SUPERNATANT TITRATION FOR MAb 20/7D PRODUCTION WITH *E. histolytica* ANTIGEN BY ELISA



Three fold supernatant dilutions from 1/9

Figure 4.3B



Three fold supernatant dilutions from 1/9

The specificity of supernatants of hybridomas 4A9-B6, 4A92-D1, 1E2-C5, 1H2-D4, and 1H2-F4 was examined by comparison of their antibody titres with *E. histolytica* and *E. dispar* crude antigens by ELISA (see 4.2.4). Supernatants from uncloned 20/7D cell-line and hybridoma 1H7.4 secreting specific anti-dengue virus non-structural-1 protein (Falconar *et al.*, 1994) monoclonal antibodies were used as positive and negative controls respectively (Table 4.3).

TABLE 4.3 SPECIFICITY OF MAb 20/7D SUBCLONES

HYBRIDOMA ID	Log ₁₀ T ₅₀ CCSN <i>E. histolytica</i> ANTIGEN	Log ₁₀ T ₅₀ CCSN <i>E. dispar</i> ANTIGEN	Log ₁₀ T ₅₀ ASCITES <i>E. histolytica</i> ANTIGEN
PRECLONED 20/7D	0.916	0.8	---
4A9-B6	1.42	0.92	3.02
4A9-D1	1.36	0.92	---
1E2-C5	1.58	0.954	3.5
1H2-D4	1.4	0.92	3.36
1H2-F4	1.46	0.89	---
22.5*	NT	NT	4.5
1H7-4	NEG.	NEG.	---

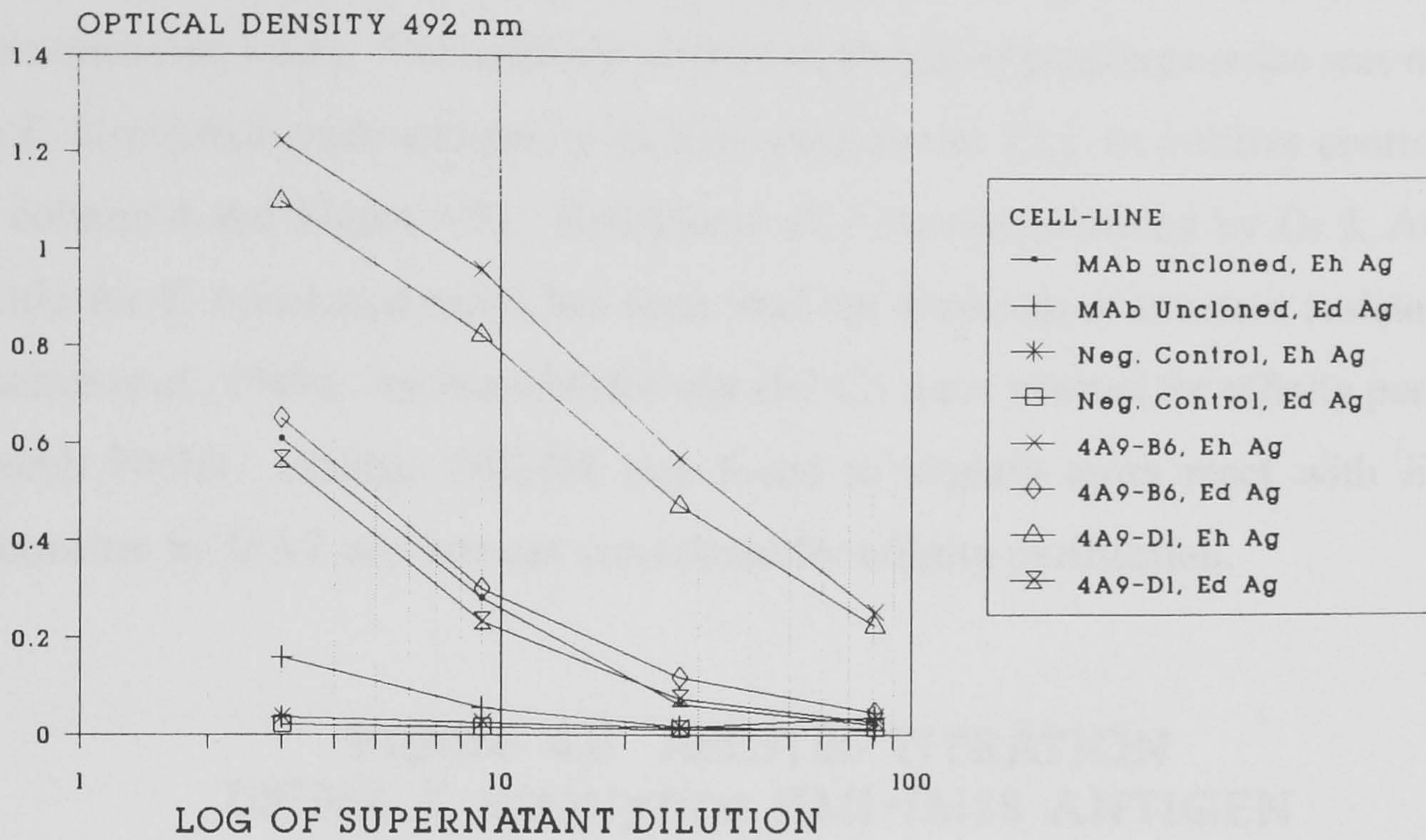
Log₁₀T₅₀: Log₁₀ of 50% End Point Titre

CCSN: Cell Culture Supernatant

*Specific for *E. histolytica* in IFA (Strachan *et al.*, 1988)

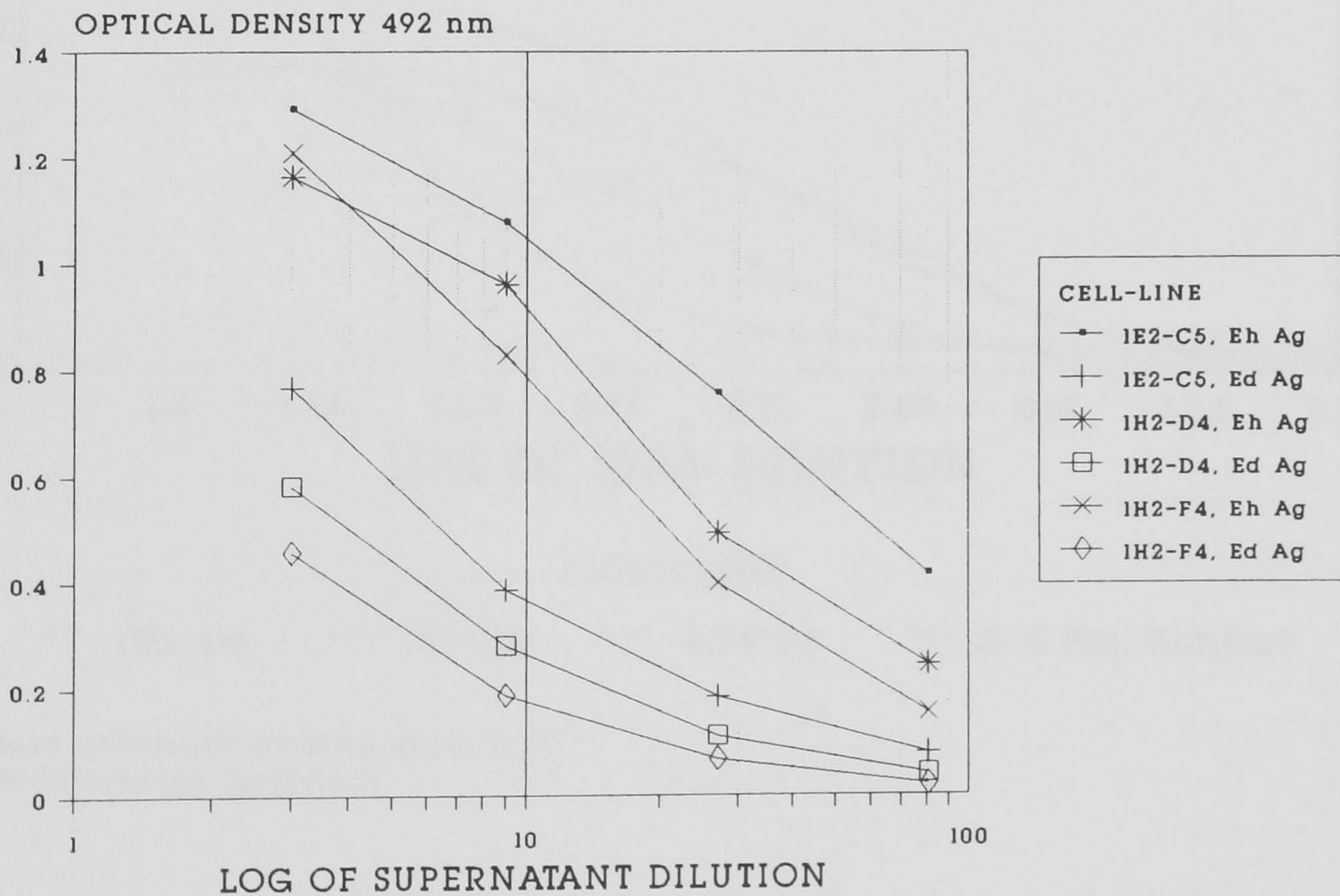
Although the supernatants of all five hybridomas also reacted with *E. dispar* antigen from polyxenic cultures, their reaction towards *E. histolytica* antigen was approximately 3 to 4 times stronger than with *E. dispar* antigen (Table 4.3). Subclones 4A9-B6, 1E2-C5 were selected for ascites production (Table 4.3, column 4) because of good growth rate and high level of antibody secretion. Cell-line 1H2-D4 had a very slow growth rate, but its antibody titres were also high when tested by ELISA, therefore it was also selected for ascites production (Figures 4.4 A and B).

Figure 4.4A TITRATION OF MAb 20/7D
SUPERNATANTS OF CLONES WITH
E. histolytica and *E. dispar* ANTIGENS



Eh Ag: *Entamoeba histolytica* Antigen
Ed Ag: *Entamoeba dispar* Antigen

Figure 4.4B

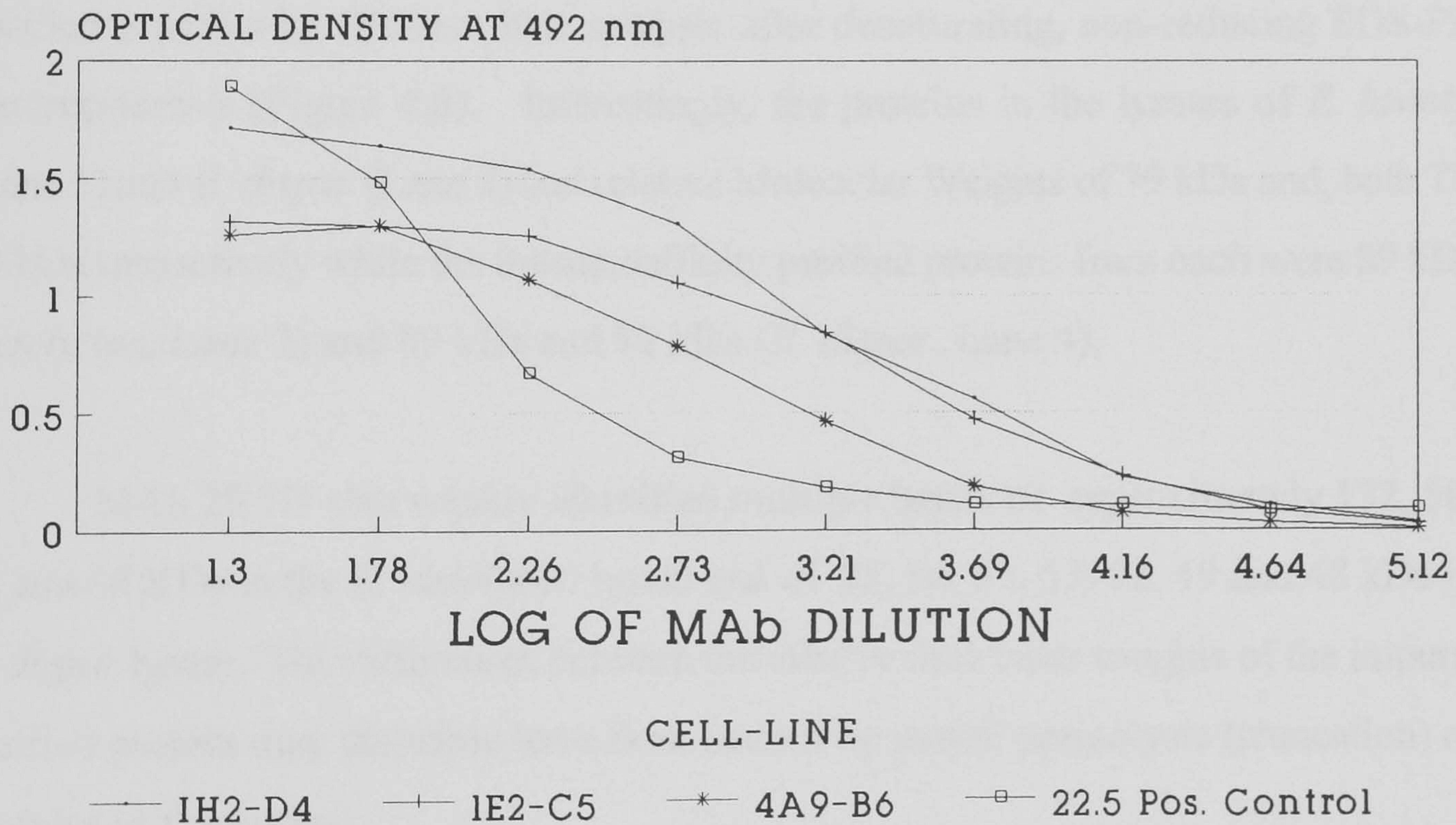


Eh Ag: *Entamoeba histolytica* Antigen
Ed Ag: *Entamoeba dispar* Antigen

4.3.2 Purification of Monoclonal Antibody 20/7D

Hybridomas 4A9-B6, 1E2-C5 and 1H2-D4 were injected into pristane-primed Balb/c mice (see 4.2.6). The antibody activity of the corresponding ascites was measured with *E. histolytica* crude antigen by ELISA using ascites 22.5, as positive control (Table 4.3, column 4 and Figure 4.5). Hybridoma 22.5 (kindly provided by Dr J. Ackers) is specific for *E. histolytica* and it has been used for diagnosis of invasive isolates in IFA (Strachan *et al.*, 1988). Ascites 4A9-B6 and 1E2-C5 were selected for affinity purification of MAb 20/7D. Ascites 1H2-D4 was found to slightly cross react with *E. dispar* trophozoites by IFAT and was not considered for affinity purification.

Figure 4.5 ASCITES TITRATION
USING *E. histolytica* HMI-IMSS ANTIGEN
IN ELISA



Three fold dilutions of MAb from 1/20
MAb: Monoclonal Antibody

4.3.3 *E. histolytica* and *E. dispar* Protein Purification

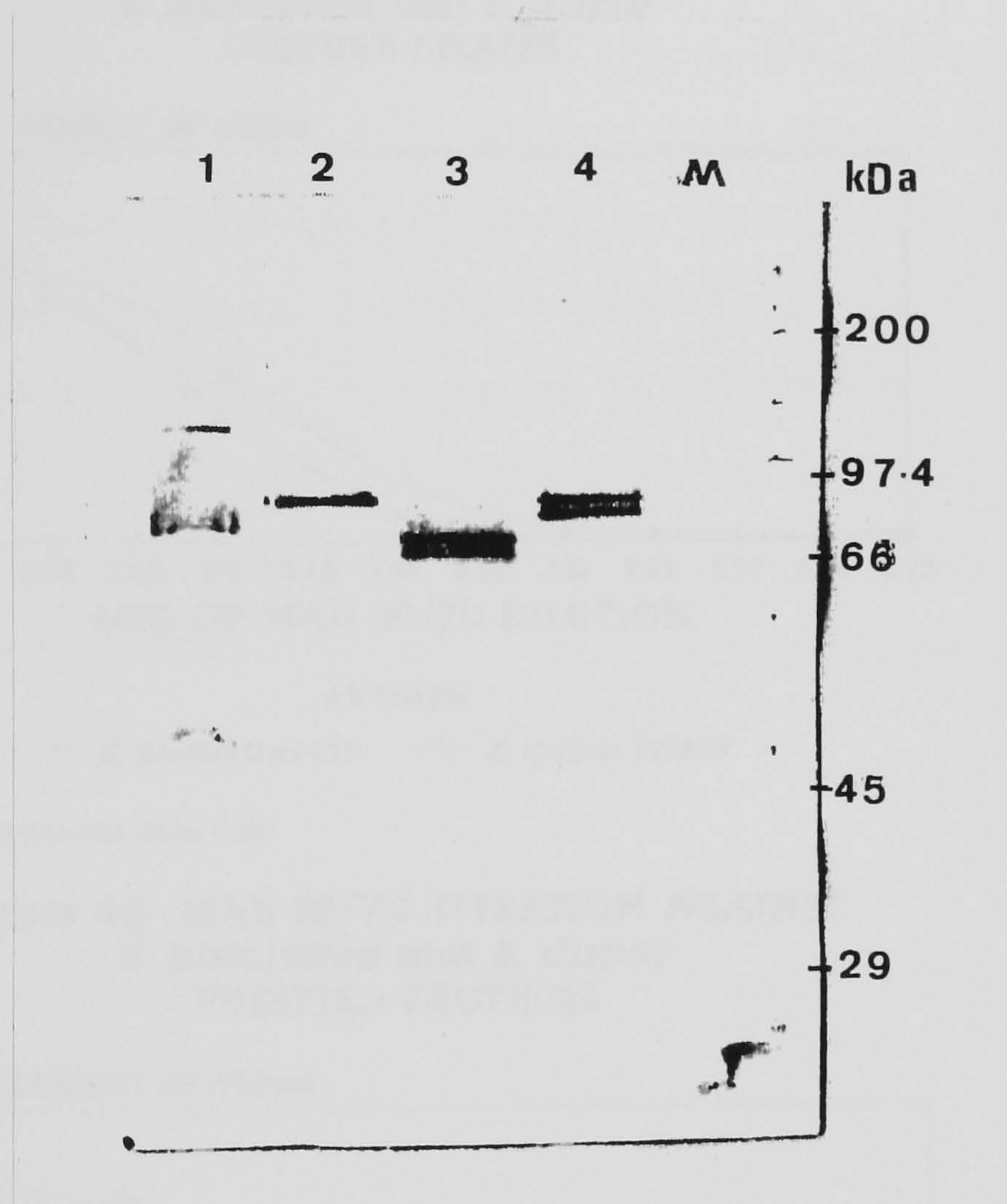
The protein identified by monoclonal antibody 20/7D was obtained from both *E. histolytica* and *E. dispar* immunoaffinity columns. The 20 fractions collected in each case were analysed by Dot-ELISA using the MAb 20/7D and rabbit anti-*E. histolytica* IgG for protein detection. The fractions containing the peak of protein were each pooled before being incorporated in further assays. Both the purified MAb 20/7D and the purified rabbit IgG antibody reacted with *E. histolytica* and *E. dispar* lysates. This was later confirmed when the isolated proteins were used as antigens to coat microtiter plates for ELISA (4.3.5).

4.3.4 SDS-PAGE and Western Blotting of Specific Proteins

Lysates of *E. histolytica* and *E. dispar* were compared with the corresponding purified proteins by Western blot analysis after denaturing, non-reducing SDS-PAGE electrophoresis (Figure 4.6). Interestingly, the proteins in the lysates of *E. histolytica* (Lane 1) and *E. dispar* (Lane 3) had relative Molecular Weights of 79 kDa and, both 77 and 70 kDa respectively while the immunoaffinity purified proteins from each were 89 kDa (*E. histolytica*, Lane 2) and 89 kDa and 82 kDa (*E. dispar*, Lane 4).

MAb 20/7D also weakly identified multiple bands of approximately 132, 58, 54, 52 and 50 kDa in the *E. histolytica* lysate and of 98, 56, 55, 53, 52, 49 and 48 kDa in the *E. dispar* lysate. The differences between the relative molecular weights of the impure and purified protein may therefore have been caused by partial proteolysis (truncation) of the proteins in the lysates.

FIGURE 4.6 Western Blot Analysis of *E. histolytica* and *E. dispar* Lysates and Immunoaffinity Purified Proteins Using MAb 20/7D.

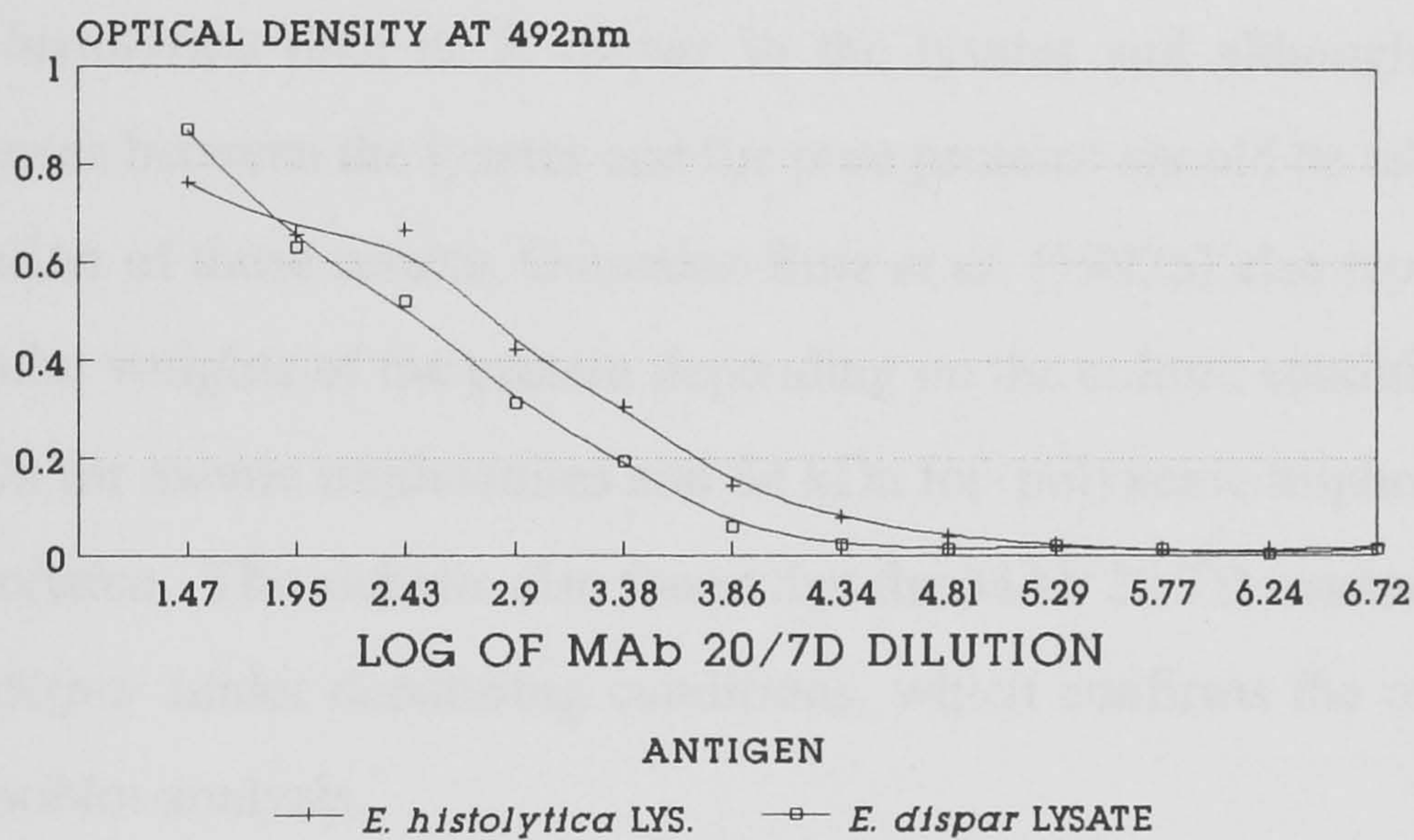


Lane 1: *E. histolytica* Lysate (Axenic), where a protein of approximately 79 kDa reacted with MAb 20/7D. **Lane 2:** *E. histolytica* Pure Protein, where a protein of 89 kDa reacted with MAb 20/7D. **Lane 3:** *E. dispar* Lysate (Polyxenic) showing the reaction of MAb 20/7D with bands at 77kDa and 70 kDa. **Lane 4:** *E. dispar* Pure Protein reacted with bands at 89 kDa and 82 kDa.

4.3.5 Titration of *E. histolytica* and *E. dispar* Lysates and Immunoaffinity Purified Proteins Using MAb 20/7D

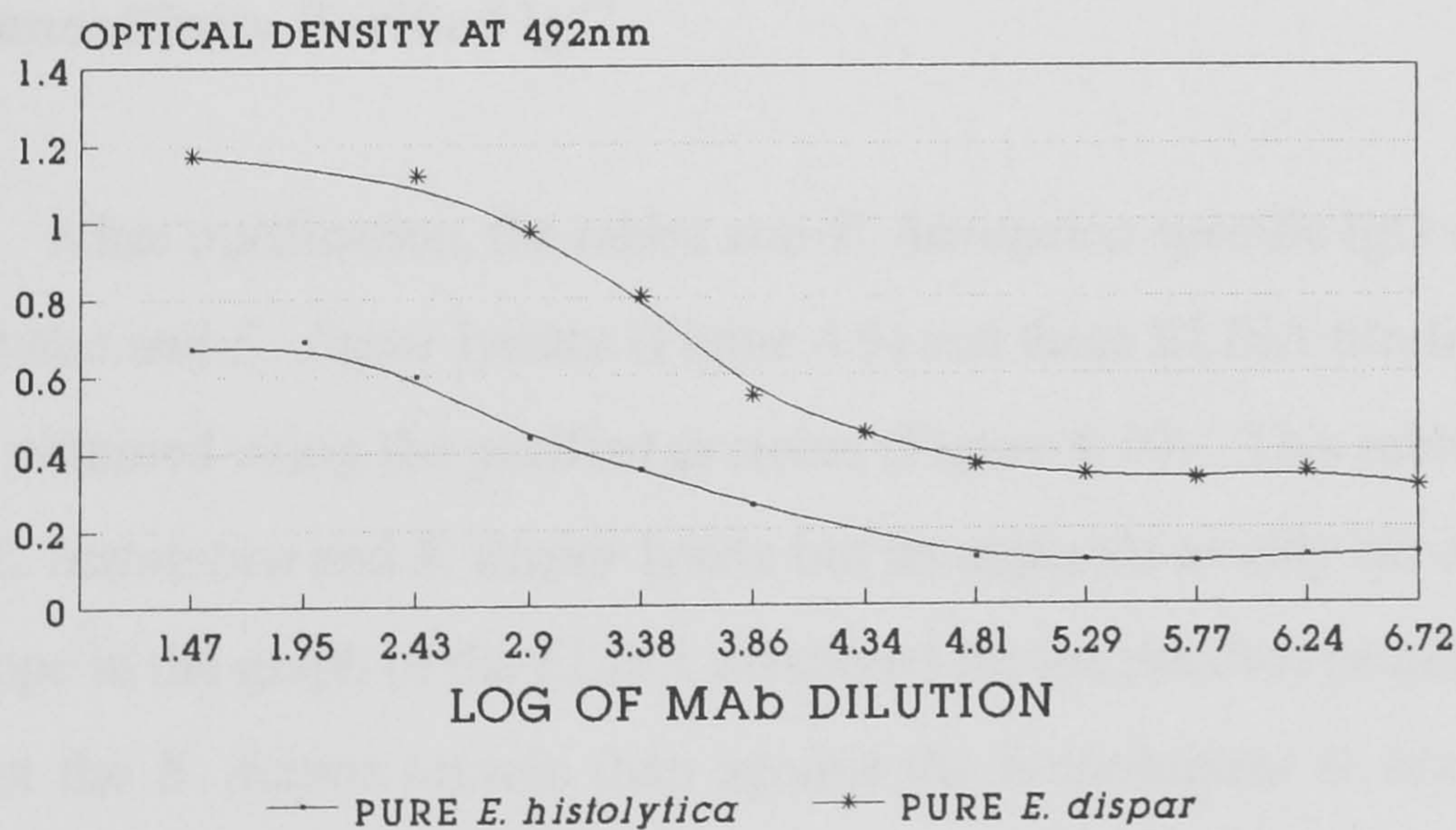
Three-fold dilutions of purified MAb 20/7D starting from 1/30 were titrated with the *E. histolytica* and *E. dispar* culture lysates (Figure 4.7) and purified proteins (Figure 4.8) used as the antigens in ELISA.

Figure 4.7 20/7D MAb TITRATION AGAINST *E. histolytica* and *E. dispar* CULTURE LYSATES



Three fold MAb dilutions from 1/30

Figure 4.8 MAb 20/7D TITRATION AGAINST *E. histolytica* and *E. dispar* PURIFIED PROTEINS



Three fold dilutions of MAb from 1/30
Antigen Protein Concentration 10µg/ml

The purified MAb 20/7D reacted with both lysates, but its titre ($\log_{10} t_{50}$) against the *E. histolytica* lysate was approximately three times higher than that against the *E. dispar* lysate (Figure 4.7). In contrast, when ELISA plates were coated with the same concentration (10 μ g/ml) of each of the purified proteins, MAb 20/7D showed a higher titre and higher maximum absorbance values against the *E. dispar* antigen ($\log_{10} t_{50}$:3.7) than the *E. histolytica* antigen ($\log_{10} t_{50}$:3.4) (Figure 4.8).

These results may indicate that there is between three and ten times more protein in *E. histolytica* than in *E. dispar* in the lysates and although the molecular weight differences between the lysates and the pure proteins should be taken into account for the evaluation of these results, Gonzalez-Ruiz *et al.* (1992a) also reported differences in the molecular weights of the protein depending on the culture conditions of the trophozoites (81 kDa for axenic trophozoites and 84 kDa for polyxenic trophozoites) in the lysates of *E. histolytica*. The authors also found that the MAb 20/7D reacted with a similar epitope in *E. dispar* under denaturing conditions, which confirms the results found during the immunoblot analysis.

4.3.6 Titration of *E. histolytica* and *E. dispar* Lysates and Proteins Using Rabbit Immunoaffinity Purified IgG

After purification, the rabbit anti-*E. histolytica* specific IgG was assayed against *E. histolytica* and *E. dispar* lysates (Figure 4.9) and these ELISA titrations were compared to those obtained using the purified proteins (Figure 4.10). This rabbit serum reacted with both *E. histolytica* and *E. dispar* lysate but its apparent avidity (as indirectly measured by the slope in the graph of the ELISA titrations) for the purified proteins was slightly higher against the *E. dispar* protein than against the homologous *E. histolytica* protein. This implied that this serum would, therefore, capture these proteins from both *E. histolytica* and *E. dispar* during the screening of faecal samples.

FIGURE 4.9 RABBIT SPECIFIC IgG VS
E. histolytica AND *E. dispar*
CULTURE LYSATES

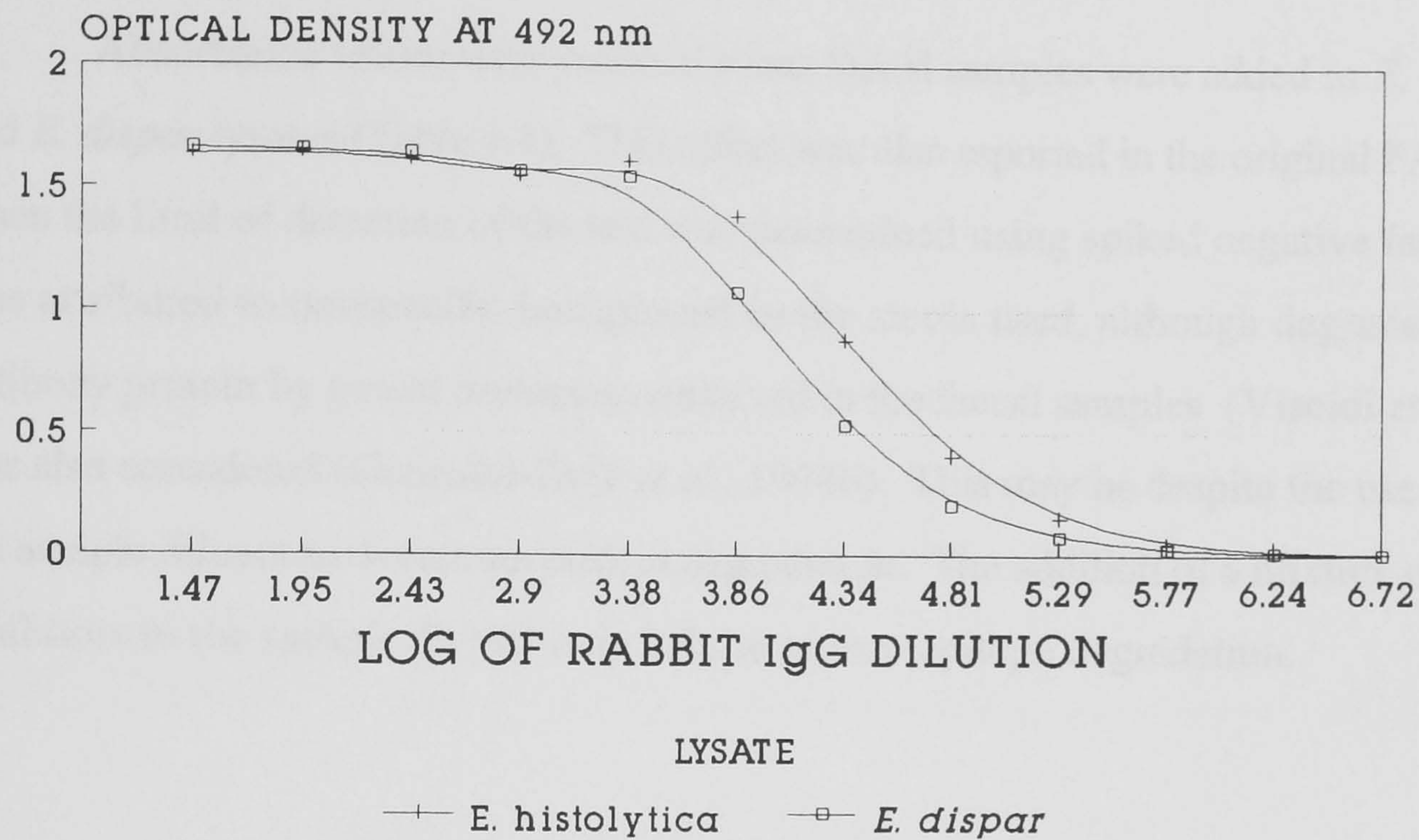
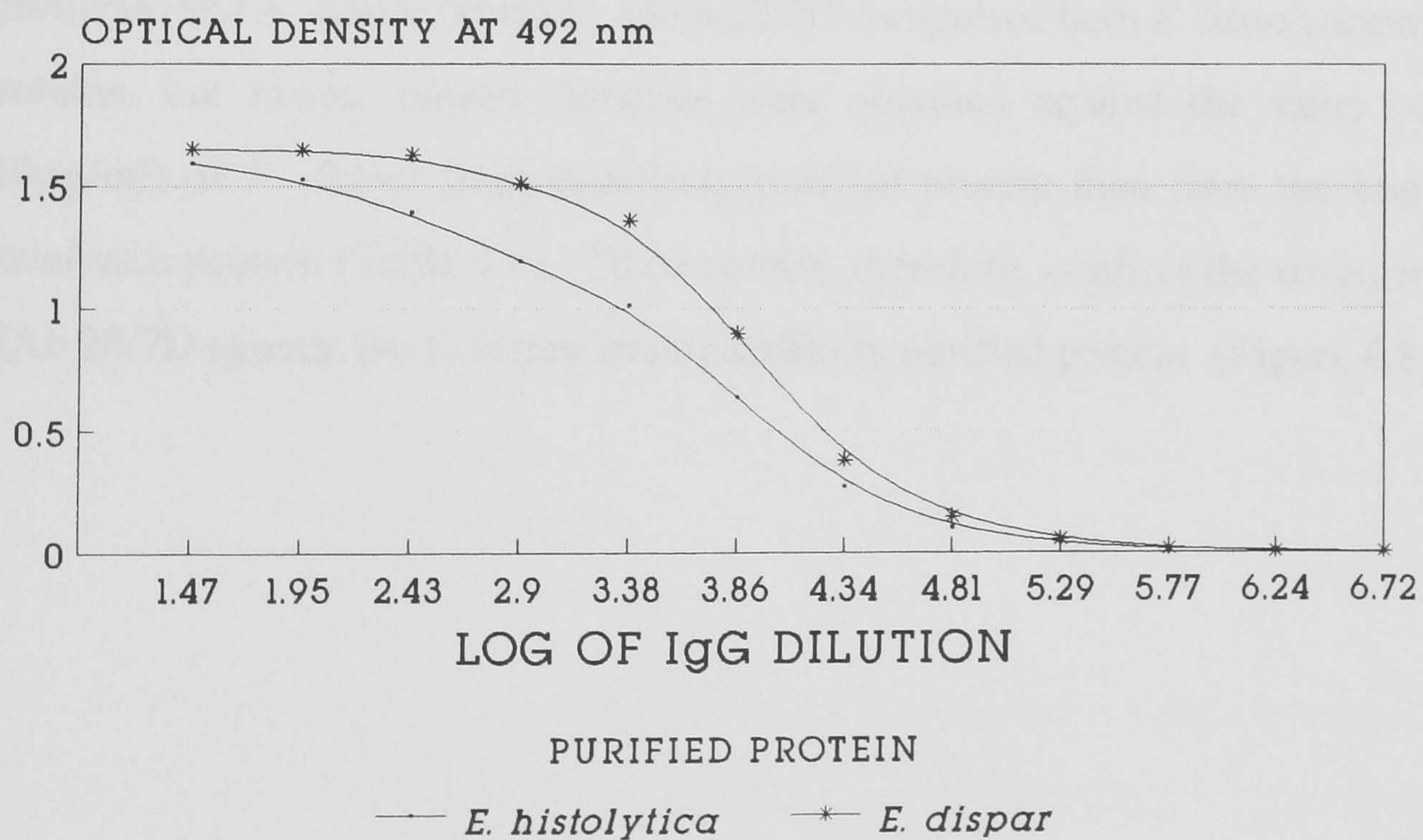


FIGURE 4.10 RABBIT SPECIFIC IgG VS
E. histolytica and *E. dispar*
PURIFIED PROTEINS



Purified Protein Concentration 10µg/ml

4.3.7 FAC-ELISA Using Affinity Purified MAb 20/7D Against *E. histolytica* and *E. dispar* Lysates

Absorbance values were reduced when faecal samples were added to *E. histolytica* and *E. dispar* lysates (Table 4.4). This effect was also reported in the original FAC-ELISA when the limit of detection of the test was determined using spiked negative faeces and it was attributed to nonspecific background in the stools used, although degradation of the antibody protein by potent proteases contained in the faecal samples (Viscidi *et al.*, 1984) was also considered (Gonzalez-Ruiz *et al.*, 1994b). This may be despite the use of FCS in the sample diluent to overcome protein degradation. The addition of a mixture of protease inhibitors to the sample diluent may help to reduce epitope degradation.

4.3.8 FAC-ELISA Using Immunoaffinity Purified MAb 20/7D to Detect *E. histolytica* and *E. dispar* Immunoaffinity Purified Proteins

The capture in this assay was also tested using immunoaffinity purified *E. histolytica* and *E. dispar* proteins. MAb 20/7D recognised both *E. histolytica* and *E. dispar* proteins, but higher optical densities were obtained against the same concentration (10µg/ml) of *E. dispar* immunoaffinity purified protein than from the homologous *E. histolytica* protein (Table 4.4). These results, therefore, confirm the stronger reaction of MAb 20/7D against the *E. dispar* immunoaffinity purified protein (Figure 4.8, page 134).

TABLE 4.4 COMPARISON OF ABSORBANCES IN ANTIGEN CAPTURE ELISA USING LYSATES AND PURIFIED PROTEIN OF *E. histolytica* and *E. dispar* IN THE ABSENCE OR PRESENCE OF FAECES

ANTIGEN	PROTEIN µg/ml	ABSORBANCE WITHOUT FAECES**		ABSORBANCE WITH FAECES**	
		OD	SD	OD	SD
<i>E. histolytica</i> Lysate*	12.0	0.643 ± 0.034		0.538 ± 0.014	
<i>E. histolytica</i> Lysate*	2.4	0.537 ± 0.027		0.485 ± 0.012	
<i>E. dispar</i> Lysate*	12.0	0.485 ± 0.032		0.439 ± 0.020	
<i>E. dispar</i> Lysate*	2.4	0.421 ± 0.028		0.422 ± 0.007	
<i>E. histolytica</i> Protein ^a	10.0	0.516 ± 0.028		NT	
<i>E. dispar</i> Protein ^a	10.0	0.886 ± 0.026		NT	
Blank ^b (No Antigen)	0.0	0.320 ± 0.014		0.308 ± 0.011	
Cut Off Value (X Blank OD+3SD)	---	0.362		0.341	

** : PBS/T/FCS used as diluent

* : Average OD of four Observations.

Protein equivalences: 1×10^4 Trophozoites=approx. 12 µg/ml and 2×10^3 Trophozoites= approx. 2.4 µg/ml of protein

^a : Average OD of 16 Observations,

^b : Average OD of 8 Observations

4.4 DISCUSSION

Subcloning of MAbs has been routinely used to eliminate non-producing hybridoma cells which eventually outnumber the MAb secreting cells (Tijssen, 1985). This strategy was successfully used to produce stable cell-lines of MAb 20/7D which, therefore, had higher titres. While the subclones also showed a greater specificity for *E. histolytica* rather than *E. dispar* in the IFAT as described previously (Gonzalez-Ruiz *et al.*, 1992a), they all reacted with a protein present in both parasites in immunoblot assay.

Purification of the components to be used in the antigen capture ELISA was performed in an attempt to improve the sensitivity of the original test (Gonzalez-Ruiz *et al.*, 1994b). The substitution of the impure MAb 20/7D (ascites) and of the rabbit anti-*E. histolytica* antisera by affinity purified MAb and IgG for the capture step did not affect the capture of antigen, but the assay could not discriminate between *E. histolytica* and *E.*

dispar and MAb 20/7D was therefore not *E. histolytica* specific. While this may have resulted from the different concentration of the antigens in *E. histolytica* and *E. dispar* lysates, this was confirmed by the comparison of the reaction of the same quantity (10 µg/ml) of the purified proteins attached to the wells in ELISA, where the purified rabbit IgG and the MAb both reacted better with *E. dispar*. Similar results were found for the antigen capture ELISA, when the purified rabbit IgG was attached to the wells to capture the purified proteins and the MAb was used for detection (Table 4.4). This is not surprising, since it has been established that *E. histolytica* and *E. dispar* have homologous antigens (e.g. superoxide dismutase, hexokinase, phosphoglucomutase, cysteine proteinases, and pore-forming peptides) with only slight differences in either their protein concentration or epitopes in these proteins (Tannich *et al.*, 1991; Ortner *et al.*, 1997; Horstmann *et al.*, 1992; Petri *et al.*, 1990b; Leippe, 1992).

The successful use of MAb 20/7D for detection of this antigen in faecal lysates from cases of amoebic dysentery may be the result of the higher output of the protein per cell and of the overall larger number of trophozoites present in infections by *E. histolytica*. Thus, differences between detection of the two proteins in *E. histolytica* and *E. dispar* may relate to the differences in concentration of both proteins in the lysates, as it is the case for the galactose-specific adherence lectin, which is present in both *E. histolytica* and *E. dispar* but in significantly different concentration (Petri *et al.*, 1990a).

The presence of potent bacterial proteases in polyxenic cultures could have been responsible for differences in the protein mobility noted, in particular for the double band of the protein in *E. dispar*. A possible alternative explanation for the stronger reaction observed using MAb 20/7D against the purified *E. dispar* protein could have been mechanically or chemically-induced degradation of the *E. histolytica* protein during the process of purification, which was reflected in lower ODs when the MAb 20/7D reacted with the *E. histolytica* purified protein in ordinary ELISAs (Figure 4.8, page 134) and in the antigen capture assay (Table 4.4, page 138). This could also explain the fact that the rabbit antibody reacted slightly better with the pure protein from *E. dispar*. Indeed, MAb 20/7D

detected further bands in both lysates by western blot (Figure 4.6), which could be truncated products. Immunofluorescence assays gave evidence that the epitope detected by the MAb 20/7D is located internally, mainly in the cytoplasm (Gonzalez-Ruiz *et al.*, 1992a) and by ELISA titration it has been indirectly revealed that there is approximately a three to ten fold difference in concentration of the protein between *E. histolytica* and *E. dispar* lysates. This difference in antigen concentration is likely to be responsible for its detection in *E. histolytica* but not in *E. dispar* isolates by IFAT, as it was shown for MAb 4G6 (Tachibana *et al.*, 1990), which was reported to react specifically with *E. histolytica* but not with *E. dispar* by IFAT. However, this MAb also recognised a homologous antigen in lysates of *E. dispar* trophozoites by western blot analysis (Tachibana *et al.*, 1990). Another more recent example is the report by Walderich *et al.*, (1998) of the production of three MAbs that reacted with cysts of *E. histolytica* in faecal smears. One of these, MAb 1.11D10, strongly reacted with a protein of 80 kDa in cysts and trophozoites of *E. histolytica*, but also reacted (although faintly) with trophozoites of *E. dispar* by IFAT. Further investigations could clarify the dynamics of expression and function of both proteins recognised by MAb 20/7D in *E. histolytica* and *E. dispar*, and the first step was taken when the monoclonal antibody was used to isolate a cDNA clone from a expression library from *E. histolytica* (Rehman, 1992). The recombinant protein exhibited promising results for its use in serological test as a substitute for culture-derived antigen (Gonzalez-Ruiz *et al.*, 1992d). Although MAb 20/7D represents an advance in the detection of invasive *E. histolytica* infections in dysenteric faecal samples, and the use of MAb 20/7D will be pursued for IFAT from cultured isolates (see Table 5.9, page 178), the modifications to the original FAC-ELISA format were abandoned for the purpose of fulfilling the aims proposed in this thesis and a change in strategy was decided in order to produce a test that would be able to specifically detect *E. histolytica*, to differentiate it from *E. dispar* and *vice-versa*. For this, taking advantage of the fact that gene sequences in *E. histolytica* and *E. dispar* differ to a greater or lesser extent (Tannich *et al.*, 1991; Ortner *et al.*, 1997; Que and Reed, 1997; Petri *et al.*, 1990b; Leippe, 1992; Spice and Ackers, 1992; Clark and Diamond, 1993), the design, standardisation and evaluation of a new differential test based on known genomic sequences was adopted and is discussed in the following chapter.

CHAPTER 5

THE POLYMERASE CHAIN REACTION-SOLUTION HYBRIDIZATION ENZYME-LINKED IMMUNO-ASSAY (PCR-SHELA) FOR THE DIFFERENTIAL DIAGNOSIS OF *ENTAMOEBIA HISTOLYTICA* AND *ENTAMOEBIA DISPAR*

5.1 INTRODUCTION

Until recently, duality in amoebiasis was a matter of discussion (Sargeant, 1987, Mirelman, 1987). The question whether *E. histolytica sensu lato* was able to establish a commensal relationship with its host without causing any harm, unless certain environmental unknown conditions turned it into an invasive pathogen, or whether two morphologically identical species with different pathogenic potential existed. Vigorous research on this area by several groups demonstrated numerous differences at the nucleic acid level and among them, the sequences of genes encoding for ribosomal RNA (Bhattacharya *et al.*, 1988, Bhattacharya *et al.*, 1989; Huber *et al.*, 1989), which were found to diverge by 2.2% and it was concluded that sequences for ribosomal DNA were divergent enough to separate *E. histolytica sensu lato* into two species, namely *E. histolytica sensu stricto* for the invasive forms and *E. dispar* for the non-invasive forms (Clark and Diamond, 1991; Diamond and Clark, 1993).

Because one of the priorities for research is to produce tools able to identify potentially invasive populations of what by microscopy has been identified as *E. histolytica sensu lato* (WHO/PAHO, 1992), the exploration of *E. histolytica* and *E. dispar* genomic libraries in search of clones with inserts able to differentiate between invasive and non-invasive forms resulted in the production of the first DNA probes by two different teams, Garfinkel and colleagues and Samuelson *et al* in 1989. Both groups reported probes derived from the specific and abundant repetitive sequences found in the 24.5 kb episome of the

organism, such as the *PvuI* 140 bp and the *BamHI* 133 bp fragments in *E. histolytica* and *E. dispar* respectively (Figure 5.1).

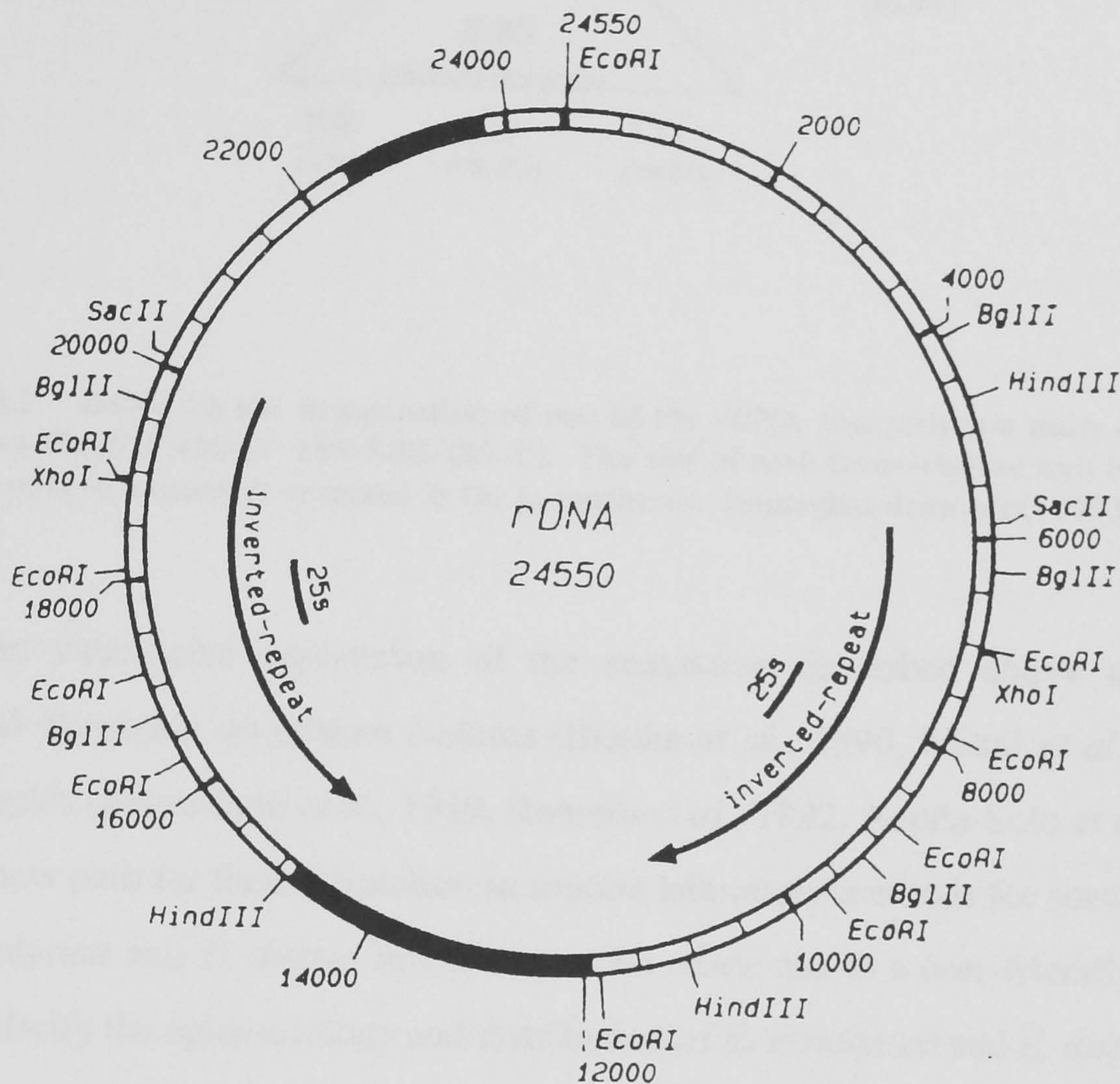


FIGURE 5.1 Circular map of the 24.5 kb episomal molecule containing two copies of rDNA genes in *E. histolytica* HMI-IMSS according to Huber *et al.*, 1989 and showing the 15 *PvuI* 140 bp repeats located between positions 22000 and 24000. Another 4 kb fragment containing *DraI* repeats is located downstream of the two rDNA genes around the position 14000.

This extra chromosomal circular molecule also contains one or two copies of the rDNA in invasive and non-invasive forms (Figure 5.2, Huber *et al.*, 1989; Bhattacharya *et al.*, 1989). Located downstream of those sequences, a *Scal* repeat with 90-95% sequence homology to the *PvuI* repeat was also found in *E. histolytica* (Mittal *et al.*, 1992a, Mittal *et al.*, 1992b)

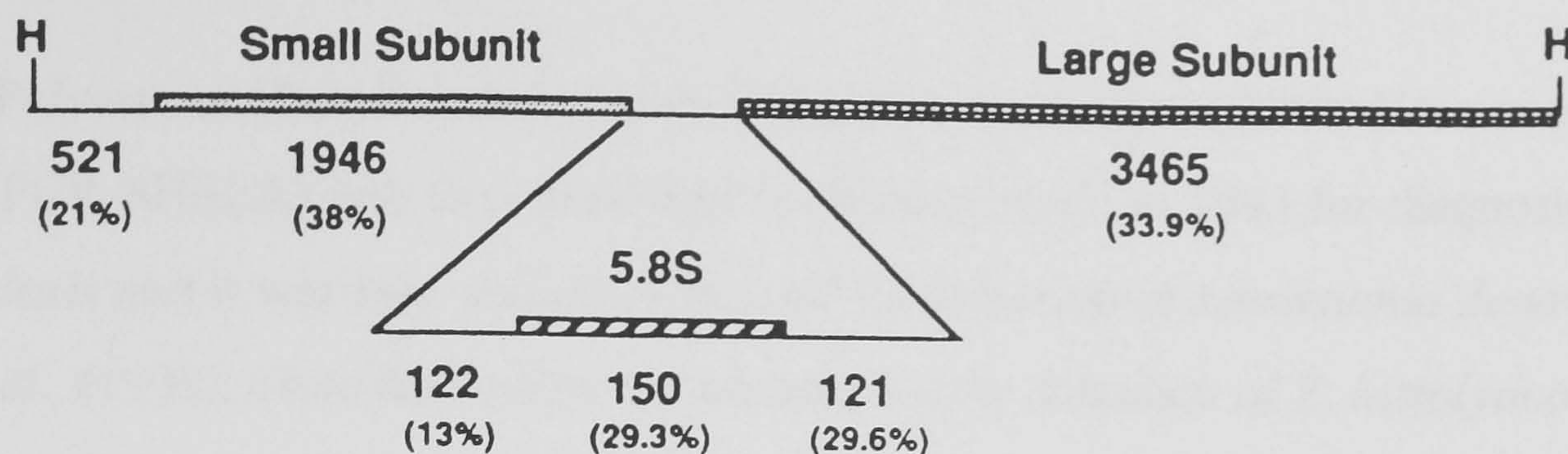


FIGURE 5.2 Detail on the organisation of one of the rRNA transcription units of *E. histolytica* between two *HindIII* sites (5'-18S-5.8S-28S-3'). The size of each transcription unit is given, and the percentage of G+C content is enclosed in the parentheses. Ramachandran *et al.*, 1993, Bhattacharya *et al.*, 1998.

The successful application of the sequences described above as probes for differential diagnosis on culture isolates (Bracha *et al.*, 1990, Mittal *et al.*, 1992b) and faecal samples (Samuelson *et al.*, 1989, Romero *et al.*, 1992, Acuña-Soto *et al.*, 1993) has opened a new path for their adaptation as routine laboratory methods for specific diagnosis of *E. histolytica* and *E. dispar* infections. Also, their use in a user-friendly format will permit to clarify the epidemiology and distribution of *E. histolytica* and *E. dispar* infections in endemic and non-endemic areas with particular emphasis for their application in the developing world (WHO/PAHO/UNESCO, 1997).

The main aim of the work described in this chapter was to produce a diagnostic tool with improved sensitivity, and specificity based on gene sequence information already available. With the use of such a tool, the specific objectives were to reduce the time required to produce a confirmatory result for species distinction and to allow for a high number of samples to be processed at once, which is necessary for future epidemiological studies.

5.2 MATERIALS AND METHODS

Polymerase Chain Reaction-Solution Hybridization Enzyme-Linked Immunosorbent Assay (PCR-SHELA) was first described by Wilson *et al.*, in 1993 for diagnosis of *M. tuberculosis* and it was later successfully used for detection of *Leishmania donovani* by Qiao *et al.*, (1995). Here the system was adapted for the detection of *E. histolytica* and *E. dispar* in cultures, stools and body fluids. PCR-SHELA involves the use of a digoxigenin labelled primer in each specific pair, which once incorporated into a PCR product, is able to hybridise to a biotin labelled specific probe. ELISA is then used for detection of the hybrids which bear biotin and digoxigenin ends. Briefly, the double marked products are captured in an avidin coated plate and detected by an anti-digoxigenin antibody conjugated to alkaline phosphatase (AP). The reaction is developed by addition of the specific substrate (p-Nitrophenyl phosphate, pNPP) using $MgCl_2$ as the cofactor for the enzymatic reaction (Appendix 1, Figure 5.3 A and B).

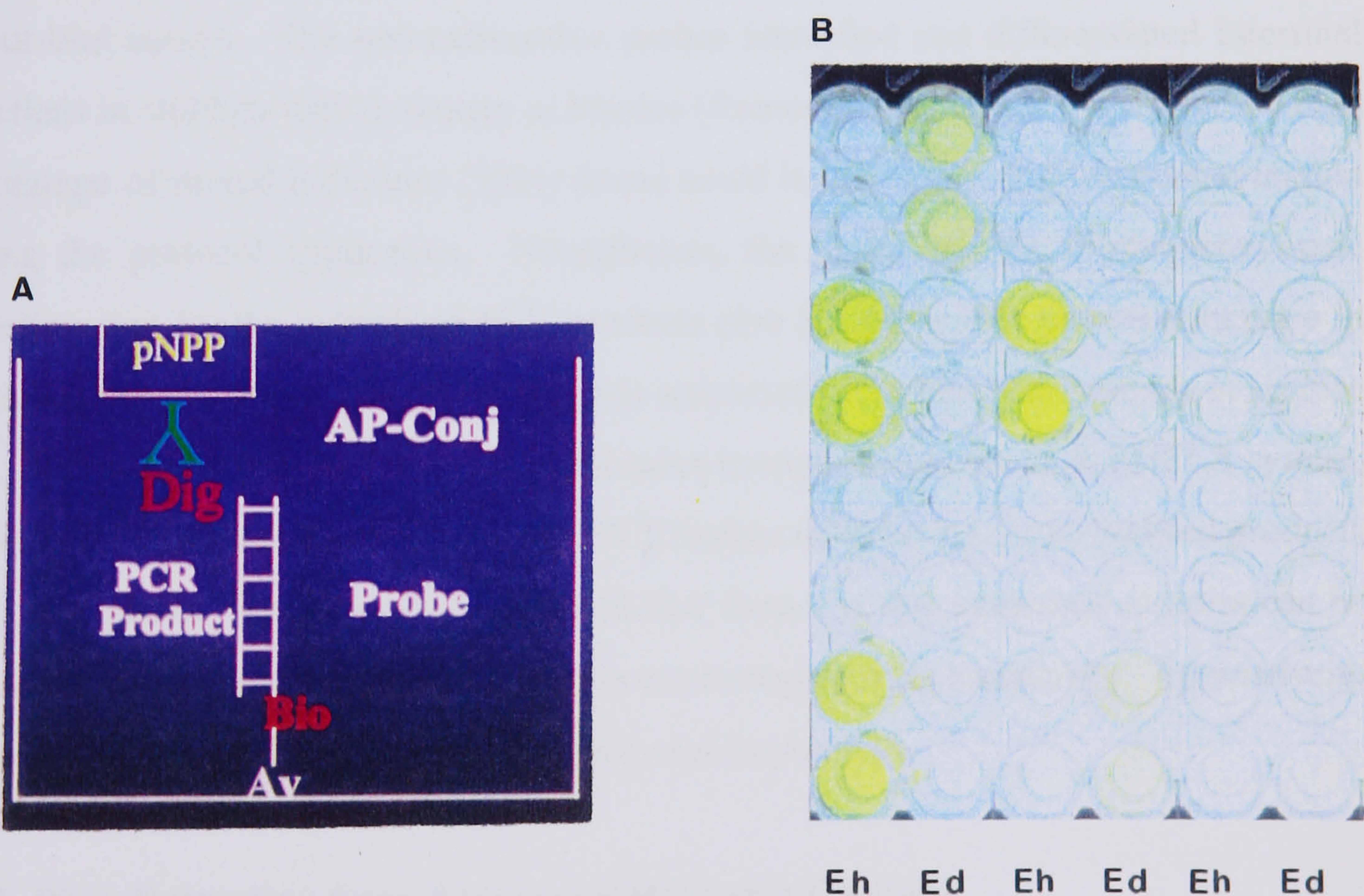


FIGURE 5.3 A. PCR-SHELA FORMAT. AV: Avidin. Bio: Biotin labelled probe. Dig: Digoxigenin labelled PCR product. AP-Conj: Alkaline phosphatase-anti-digoxigenin labelled antibody. pNPP: p-Nitrophenyl phosphate, the substrate. **B. ELISA, Eh:** Products of amplification with *E. histolytica* specific primers and probe. **Ed:** Products of amplification with *E. dispar* specific primers and probe. Samples in duplicate wells, side by side.

PCR-SHELA for *E. histolytica* and *E. dispar* is based on the amplification of a 125 bp *PvuI* tandemly repeated region on the episomal DNA of *E. histolytica* and a 133 bp *BamHI* region in *E. dispar* episomal DNA with specific primers. The sequences are located between the two copies of the rDNA. It has been calculated that there are approximately 200 episomes per trophozoite, each containing about 14-15 copies in tandem repeats (Bhattacharya *et al.*, 1988), which are equivalent to 50-100 fg of target DNA per trophozoite of *E. histolytica sensu stricto* (Samuelson *et al.*, 1989).

Among the advantages of using this PCR with hybridisation is the fact that the abundance of the region to be amplified provides a high probability for amplification in samples with low numbers of parasites, and the specificity of the system is guaranteed by the hybridisation step with the probe located within the sequence amplified in each species. Horse-radish peroxidase labelled probes to the 145 bp and 133 bp sequences on the episome of *E. histolytica* and *E. dispar* were successfully used to hybridise PCR products in dot-blot assays. The non-radioactive probes identified and differentiated intestinal infections in children with dysentery in Mexico (Romero *et al.*, 1992). However, the high percentage of mixed infections (93%) found could be the result of cross contamination during the protocol application. Nevertheless, the probe and the immunoenzymatic detection step for the hybridised PCR products give an appreciable potential increase in sensitivity when compared to electrophoresis and detection by ethidium bromide in agarose gels. The same probes, labelled with biotin were incorporated in the PCR-SHELA system, where hybridisation was carried out after PCR before transferring the hybridised products to ELISA plates. The advantage of the ELISA format is that numerous samples can be processed at the same time and absorbances can be registered automatically. Alternatively, colorimetric qualitative reading of results by eye is possible.

5.2.1 DNA Extraction from Axenic and Polyxenic Cultures

DNA from axenic cultures was extracted after confluent monolayers were observed in the culture flasks. Trophozoites were made to detach from the flasks walls by immersion

in an ice water bath for 20 minutes. The culture medium containing the trophozoites was then transferred to sterile 50 ml conical tubes (Sterilin, UK) and pelleted at 700 g for 10 minutes. The pellets were pooled and resuspended in 1 ml of PBS pH 7.2 and counted in a haemocytometer. Lysis buffer (Appendix 1) containing 100 μ g/ml of proteinase K was added in a 1:1 ratio (v/v) and after an overnight incubation at 37°C, the DNA was extracted using standard phenol-chloroform method with ethanol precipitation (Romero *et al.*, 1992).

For extraction of polyxenic Robinson's cultures, the content of 1 to 5 bijoux bottles was pooled together, and the trophozoites were pelleted by spinning at 700 g for 5-10 minutes. The supernatant was discarded and the pellet was transferred to a 1.5 ml screw capped centrifuge tube and lysed and extracted as described above.

5.2.2 DNA Extraction from Faecal Samples and Other Clinical Specimens

The protocol used for DNA extraction was successfully used for processing faecal samples from Mexican patients during the evaluation of the 145bp probe (Samuelson *et al.*, 1989). For our study, approximately 1g of each faecal sample was resuspended in 3 ml of PBS, passed through gauze to eliminate particles and frozen/thawed three times in a mixture of dry ice-ethanol and thawed in a water bath. The suspension was then micro-centrifuged for one minute at 6500 rpm and supernatants were diluted 1:1 in lysis buffer (Appendix 1), containing 100 μ g/ml of proteinase K, incubated overnight at 37°C and extracted the next morning using the phenol-chloroform method followed by ethanol precipitation as before. The phenol-chloroform purification was introduced in order to reduce the amount of possible PCR inhibitors from the faeces and the lysis buffer, such as bilirubin-derived pigments and EDTA. The DNA was diluted in 50 μ l of sterile distilled water and if the resulting solution was coloured, it was diluted 1/100 prior to PCR. DNA was also extracted from pus after liver puncture, for which 500 μ l of lysis buffer were added to 500 μ l of pus, containing 100 μ g/ml of proteinase K. After incubating overnight, the suspension was treated as described above.

5.2.3 Estimation of DNA Concentrations

Once the DNA was resuspended in 50 μ l of sterile distilled water, 10 μ l were run in a 0.8% agarose mini-gel for 1 hour at 75 V in order to check integrity. Concentration and purity were assayed by absorbance measurement at 260 and 280 nm using a 1/100 dilution of each DNA. For calculation of the DNA concentration of each sample, it was assumed that an absorbance of 1 at A_{260} was equivalent to 50 μ g/ml of double stranded DNA. A 260/280 ratio of 1.8 is indication of high purity. Ratios of less than 1.8 indicate contamination by proteins or phenol.

5.2.4 *E. histolytica* and *E. dispar* Target Sequences

DNA from reference strains *E. histolytica* HMI-IMSS and *E. dispar* SAW 1734 was amplified with two different pairs of primers for each species (5.2.4). The *E. histolytica* "P145" target sequence was cloned from the axenic strain HM-1:IMSS cl6, GeneBank accession number A01325 (Samuelson *et al.*, 1989; Garfinkel *et al.*, 1989):

```

          +10      +20      +30      +40      +50
1  GATCGATTTT CCCAGTTAGA AATTATTGTA CTTTGTACTT TGTATTTAGT
   -----
   CTAGCTAAAA GGGTCAATCT TTAATAACAT GAAACATGAA ACATAAATCA

          +10      +20      +30      +40      +50
51 ACTTTTCATT AAAAATGGTG AGGTTCTTAG GAAATCCGAA AATCAACGGT
   -----
   TGAAAAGTAA TTTTACCAC TCCAAGAATC CTTTAGGCTT TTAGTTGCCA

          +10      +20      +30      +40      +50
101 GTAAATTGGT CAAAAAATAT TTTGCCTAGA CGACGACCAT TTTGAATAAT
   -----
   CATTTAACCA GTTTTTTATA AAACGGATCT GCTGCTGGTA AAAC TTATTA

151 TCGATC
   -----
   AGCTAG

```

The *E. histolytica* PCR System incorporates the following pair of primers derived from the sequence described above:

Forward Primer (positions 13-37 of P145):

5'...CAG TTA GAA ATT ATT GTA CTT TGT A...3'

T_m= 62°C

Reverse Primer (positions 145-124 of P145):

5'...DIG-TCA AAA TGG TCG TCG TCT AGG C...3'

T_m= 66°C

The *E. dispar* "B133" target sequence was cloned from polyxenic strain SAW 1734R clAR, Genbank accession number A01326 (Garfinkel *et al.*, 1989):

```

          +10      +20      +30      +40      +50
1  GGATCCTCCA AAAAATAAAG TTTTATCAGA AATGGTGAGG TTGTAGCAGA
   -----
   CCTAGGAGGT TTTTATTTC AAAATAGTCT TTACCACTCC AACATCGTCT

          +10      +20      +30      +40      +50
51 GATATTAATT CAACAATTTT ATTGAAAAAA GGTGCACTTT TTTGATTTTA
   -----
   CTATAATTAA GTTGTTAAAA TAACTTTTTT CCACGTGAAA AACTAAAAT

          +10      +20      +30
101 ATATAAAGTA CTAGGTATCC AATATCGTTC TGTGGATCC
     -----
     TATATTCAT GATCCATAGG TTATAGCAAG ACACCTAGG

```

Forward Primer (positions 1-23 of B133):

5'...GGA TCC TCC AAA AAA TAA AGT TT...3'

T_m= 60°C

Reverse Primer (Positions 133-109):

5'...DIG-ACA GAA CGA TAT TGG ATA CCT AGT A...3'

T_m=68°C

The primers were prepared by automated oligonucleotide synthesis (British Biotechnology Products Ltd, UK) on a scale of 0.2 μmol, with one of each pair digoxigenin-labelled at the 5' end. For the calculation of molar primer concentrations the following

formulas were used:

Molecular Weight (MW) of Primer: Average MW of a base (=333) x No. of bases

Concentration of the primer ($\mu\text{g/ml}$): $A_{260} \times 33$

Molar concentration of the primer: Concentration of Primer in g/l divided by the MW of the Primer

Where,

A_{260} : Absorbance at 260 nm in a 1 cm path length cell.

5.2.5 Optimisation of PCR Reactions

Standard reaction mixtures were prepared in Promega Taq buffer A (M-1861) containing 500 mM KCl, 100 mM Tris-HCl, 1% Triton X-100, 4mM MgCl₂, and 0.2 mM of each deoxynucleoside triphosphate per 100 μl of mixture. Two concentrations of Promega Taq polymerase (5U and 2.5U per 100 μl of reaction mixture) were tested in 40 μl PCR reactions containing DNA of *E. histolytica* HMI-IMMS and *E. dispar* SAW 1734. The "hot start" PCR protocol (Wilson *et al.*, 1993) was adopted to avoid the contact of the primers with the Taq before denaturation begins. The hot start requires a physical barrier provided by a pellet of wax with melting temperature between 57°C and 58°C, thus avoiding extension of any primer dimers formed at room temperature. For this purpose, the 50 pmol of primers in each PCR reaction were contained in a fixed volume of 18 μl to which two wax pellets were added, melted at 58°C and allowed to solidify. Over this layer, 20 μl of reaction mix were added and finally, 2 μl of DNA template were added last. When the PCR cycle was initiated at 94°C the two layers mixed for the first time.

For standardisation purposes, the amplification conditions were set as described by Acuña-Soto *et al.* (1989): a cycle of denaturation at 94°C for 2 minutes was followed by 30 cycles of:

Denaturation	94°C	1 minute
Annealing	55°C	2 minutes
Polymerisation	72°C	2 minutes

and one final cycle of extension at 72°C for 7 minutes.

5.2.6 Titration of *E. histolytica* and *E. dispar* DNA

DNA obtained from axenic cultures of *E. histolytica* HM1-IMSS and polyxenic Robinson's cultures of *E. dispar* SAW 1734 as described in 5.2.1 was diluted and titrated after quantitation according to the method in 5.2.3. The quantities tested ranged from 10 pg to 10 ng for *E. histolytica* DNA and from 10 pg to 100 ng for *E. dispar* DNA. The concentrations in the stocks were adjusted by diluting the DNA with distilled water to a suitable volume.

5.2.7 Polymerase Chain Reaction (PCR) on *E. histolytica* and *E. dispar* Cultures

PCR amplification of 2 to 4µl of undiluted DNA extracted from axenic (TYI-S-33, Appendix 1) and polyxenic (Robinson's medium, Appendix 1) cultures was done using the conditions described in 5.3. For clinical diagnosis DNA obtained from cultures derived from microscopy positive samples was tested with both sets of primers. Controls containing water as sample replacement and positive reactions containing reference DNA from *E. histolytica* and *E. dispar* were included for each batch of amplifications. PCR products were initially detected by electrophoresis in 2% agarose gels using ethidium bromide. A product of 125 bp was expected for *E. histolytica* positive controls and a band of 133 bp was expected for *E. dispar* positive controls. Band migration was compared against a 123bp DNA marker ladder (Gibco BRL, Life Technologies, UK).

5.2.8 Southern Blot Analysis of *E. histolytica* and *E. dispar* PCR Products

Due to a sequence homology of 43.6% between the *E. histolytica* and *E. dispar* targets (Garfinkel *et al.*, 1989) and because of reports by both Samuelson *et al.* (1989) and Mirelman *et al.* (1990) on cross reactivity of the *E. histolytica*-derived P145 sequence with *E. dispar*, the products of amplification of reference strains of both species were purified from agarose gels according to the Magic PCR prepsTM DNA Purification System protocol (Promega, UK) and further isotope-labelled and used as probes against each other. After PCR, electrophoretic separation of the PCR products was carried out in a 2% agarose gel for 1h at 75 V. After staining with ethidium bromide, the gel was photographed followed by immediate denaturation of the double-stranded DNA in the gel with 250 ml of denaturing solution (Appendix 1). A change to 250 ml of fresh denaturing solution was made after 15 minutes, with incubation for further 30 minutes. The gel was then transferred to neutral pH first by rinsing with distilled water for 5 minutes and then by addition of 500 ml of neutralising solution (Appendix 1) for 45 minutes.

Meanwhile, 3 sheets of Whatman 3MM paper were cut to the exact size of the gel, with a further sheet cut to the width of the gel but longer to allow contact between the gel and the buffer (20X SSC) in the plastic tray. A piece of nylon membrane (Hybond-N, Amersham) was cut to the size of the gel and soaked with 2X SSC. The gel was then transferred facing down onto the 3 MM paper wick in contact with the buffer in the tray taking care to remove trapped bubbles by rolling a plastic pipette over the surface. The nylon membrane cut to the size of the gel was then placed on the top of the gel, again removing air bubbles and also marking the orientation of the gel (well positions and sample positions). Then, three sheets of soaked Whatman 3 MM paper (removing air bubbles) and a stack of adsorbent paper towels were placed on the top of the membrane. Finally, a glass plate and a heavy weight were applied and the transference was carried out overnight. Next day, the weight, the glass and the paper towels were removed and the membrane was soaked in 2X SSC for 5 minutes and then dried facing up on a dry sheet of 3MM paper.

After covering with cling film, the DNA was covalently bound to the membrane by exposure to UV light on an automatic illuminator, Stratalinker (Stratagene, UK).

5.2.9 Cross Hybridisation Assay With *E. histolytica* and *E. dispar* PCR Products as Probes in Southern Blot Analysis

A prehybridisation step was carried out overnight at 42°C in a solution containing 50% formamide, 5X SSC(1X SSC= 0.15 M NaCl-0.015 M sodium citrate), 5X Denhardt's solution and 100 µg/ml denatured salmon sperm DNA to block non-specific binding sites. Membranes were hybridised overnight in the same solution with addition of 0.1% sodium dodecyl sulphate (SDS) and the corresponding labelled-oligo DNA PCR product (125 bp or 133 bp respectively). Probes were labelled with $\alpha^{32}\text{P}$ -dCTP (Amersham, UK) using a random primer DNA labelling kit (rediPrime, Amersham, UK) according to the protocol provided by the manufacturer. The membrane was washed for 15 min in 2x SSC-0.1% SDS at room temperature and twice for 15 min in 0.2x SSC-0.1% SDS at 60°C. The membrane was exposed to X-ray film (Kodak XAR-5 film) at -70°C.

5.2.10 Optimisation of Hybridisation of Amplicons With Specific Digoxigenin Labelled Probes and Detection of Hybrids by ELISA (SHELA)

After successful amplification of DNA from cultured reference strains and isolates from clinical samples, the next step was to establish the optimal conditions for hybridisation of PCR products and their detection by ELISA. The use of probes was introduced to increase the specificity of the test. The sequence of each biotinylated probe is complementary to the corresponding strand primed by the digoxigenin labelled primer and this facilitates detection after the capture of the hybrids on avidin coated ELISA plates.

alkaline phosphatase-labelled (Fab fragment, Boehringer Mannheim, Germany) antibody diluted 1/5000 was followed by three washings with TBS-T and a final wash with TBS.

The ELISA was developed by addition of 100 µl/well of carbonate buffer (coating buffer) containing 1 mg/ml of p-nitrophenyl phosphate (pNPP) and 1 mM of MgCl₂. After 1 hour incubation at room temperature, absorbance was read at 405 nm.

5.2.11 Detection Limit of *E. histolytica* and *E. dispar* Isolates from Polyxenic Cultures by PCR-SHELA

Diagnosis of amoebiasis based on polyxenic cultures or faeces implies that the sample used for PCR contains DNA from several species, mainly from bacterial flora. In order to establish the limit of detection of PCR-SHELA in polyxenic cultures, trophozoites of *E. histolytica* strain 882 and of *E. dispar* 533 strain were used for the test. Both strains had been isolated from faecal samples and were maintained in Robinson's culture at the Hospital Clinic i Provincial in Barcelona and were brought to the LSHTM where hexokinase characterisation and PCR were carried out.

For the assay, the trophozoites were washed in PBS pH 7.2, counted in a Neubauer chamber and diluted to a starting stock of 1×10^6 trophozoites per ml with PBS (see 5.2.1, page 145). From this stock, dilutions were made to final concentrations of 1×10^5 trophozoites, 1×10^4 trophozoites, 1×10^2 trophozoites and 1 trophozoite in a volume of 0.5 ml and to each of them 0.5 ml of lysis buffer were added for DNA extraction. The final volume extracted was 1 ml and after extraction, the DNA was diluted in 50 µl of sterile distilled water. Two microlitres were used for PCR reactions, equivalent to 4000 trophozoites per ml, 400 trophozoites per ml, 4 trophozoites per ml and less than 1 trophozoite per ml.

5.2.12 Evaluation of PCR-SHELA With *E. histolytica* and *E. dispar* Polyxenic Clinical Isolates from Endemic Areas

A collection of frozen pellets of 60 strains cultured in Robinson's medium from Bangladesh and Colombia were tested by PCR-SHELA. Pellets of trophozoites from 28 strains isolated at the International Centre for the Control of Diarrhoeal Diseases (ICDDR), in Dhaka, Bangladesh were transported frozen to the LSHTM. Similarly, 32 pellets obtained from cultures at the Centro de Investigaciones en Microbiología y Parasitología Tropical, in Bogota, Colombia were also brought to the LSHTM. DNA was extracted as described in 5.2.1 and 2 µl of each DNA were used for the PCR reactions.

5.2.13 PCR Amplification of the Small Ribosomal Subunit (rSSU) DNA from *E. histolytica* and *E. dispar*

Amplification of the SSU rDNA in cultures of *E. histolytica* and *E. dispar* with species specific primers was used by Clark and Diamond to test the two species hypothesis between *E. histolytica* and *E. dispar* (Clark and Diamond, 1991). The advantage of using ribosomal DNA is the abundance of those genes, which are also very conserved within the same species but different between closely related but different species.

Because of the background amplification found on the cultures from Bangladesh and Colombia by PCR-SHELA, the rSSU PCR was used as a complementary tool for confirmation of results since it does amplify *E. histolytica* DNA even in the presence of very small amounts of homologous *E. dispar* DNA and viceversa (Clark, 1997).

The amplification of the diagnostic fragment of 876 bp is based on the 1932 bp *E. histolytica*-specific sequence of rSSU submitted by Que and Reed in 1991, GeneBank accession number X61116, from which bases 1-1091 are shown:

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1 CCACCGTCCG CAGTATTATA TGCTGATGTT AAAGATTAAG CCATGCATGA

GGTGGCAGGC GTCATAATAT ACGACTACAA TTTCTAATTC GGTACGTA
51 GTAAGTATAA AGACCAAGTA GGATGAACT GCGGACGGCT CATTATAACA

CATTCAATATT TCTGGTTCAT CCTACTTTGA CGCCTGCCGA GTAATATTGT
101 GTAATAGTTT CTTTGGTTAG TAAAATACAA GGATAGCTTT GTGAATGATA

CATTATCAAA GAAACCAATC AATCATGTTT CCTATCGAAA CACTTACTAT
151 AAGATAATAC TTGAGACGAT CCAGTTTGTA TTAGTACAAA ATGGCCAATT

TTCTATTATG AACTCTGCTA GGTCAAACAT AATCATGTTT TACCGGTAA
201 **CATTCAATGA** **ATTGAGAAAT** GACATTCTAA GTGAGTTAGG ATGCCACGAC

GTAAGTTACT TAACTCTTTA CTGTAAGATT CACTCAATCC TACGGTGCTG
251 AATGTAGACA ACACAGTGTT TAACAAGTAA CCATGAGAAT TTCTGATCTA

TTACATCTGT TGTGTCACAA ATTGTTTATT GGTACTCTTA AAGACTAGAT
301 TCAATCAGTT GGATAGTATCG AGGACTACCA AGTTATAACG GATAACGAGG

AGTTAGTCAA CCATCATAGC TCCTGATGGT TCAATATTGC CTATTGCTCC
351 AATTGGCGTT CGACATCGGA GAGGGAGCTT TACAGATCGG ATTACCACTT

TTAACCGCAA GCTGTAGCCT CTCCTCGAA ATGTCTAGCC TAATGGTGAA
401 CTAAGGAAGG CAGCAGGCGC GTAAATTACC CACTTTCGAA TTGAAGAGGT

GATTCCTTCC GTCGTCCGCG CATTTAATGG GTGAAAGCTT AACTTCTCCA
451 AGTGACGACA CATAACTCTA GAGTTGAGTA AAATCAATTC TTGAAGGAAT

TCACTGCTGT GTATTGAGAT CTCAACTCAT TTTAGTTAAG AACTTCCTTA
501 GAGTAGGAGG TAAATTCTCC TACGAAATCA ATTGGAGGGC AAGTCTGGTG

CTCATCCTCC ATTTAAGAGG ATGCTTTAGT TAACCACCCG TTCAGACCAC
551 CCAGCAGCCG CGGTAATTCC AGCTCCAATA GTGTATATTA AAGTTGCTGT

GGTCGTCCGC GCCATTAAGG TCGAGGTTAT CACATATAAT TTCAACGACA
601 GATTAAAACG CTCGTAGTTG AATTAAAATG TGGTTTTATA CTTTTGAAG

CTAATTTTGC GAGCATCAAC TTAATTTTAC ACCAAAATAT GTAAAACCTC
651 ACTTTATGTA AGTAAAGTTT CTAGAAATGT TAAATTAAAA TCAAAGAAGG

TGAAATACAT TCATTTCAAA GATCTTTACA ATTTAATTTT AGTTTCTTCC
701 AAACAATTCA AGTAATTGAG TTGTTATTAC TTTGAATAAA ATAAGGTGTT

TTTGTTAAGT TCATTAACTC AACAATAATG AACTTATTT TATTCCACAA

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```
751 TAAAGCAAAA CATTATGTTA ATGAATATTC AAGCATGGGA CAATGCTGAG
-----
ATTTCGTTTT GTAATACAAT TACTTATAAG TTCGTACCCT GTTACGACTC
801 GGGATGTCAA TAAGACATTT CGAGAGAAGG ATTAAAAGGA ACAATTGGGG
-----
CCCTACAGTT ATTCTGTAAA GCTCTCTTCC TAATTTTCCT TGTTAACCCC
851 TGATTCAGAA AATAACGGGA GAGGTGAAAA TCCATGATCG CTATAAGATG
-----
ACTAAGTCTT TTATTGCCCT CTCCACTTTT AGGTACTAGC GATATTCTAC
901 CAGCGAGAGC GAAAGCAGAT TTCACTCAAC TGTGTCCATT AATCAAGAAC
-----
GTCGCTCTCG CTTTCGTCTA AAGTGAGTTG ACACAGGTAA TTAGTTCTTG
951 GAAAGTTAGG GGATCGAAGA CGATCAGATA CCGTCGTAGT CCTAACTATA
-----
CTTCAATCC CCTAGCTTCT GCTAGTCTAT GGCAGCATCA GGATTGATAT
1001 AACGATGTCA ACCAAGGATT GGATGAAATT CAGATGTACA AAGATAGAGA
-----
TTGCTACAGT TGGTTCCTAA CCTACTTTAA GTCTACATGT TTCTATCTCT
1051 AGCATTGTTT CATGATCTGA GTATATCAAT ATTACCTTGT TCAGAACTTA
-----
TCGTAACAAA GTACTAGACT CATATAGTTA TAATGGAACA AGTCTTGAAT
```

The sequences of the primers (highlighted in bold from the sequence above) used for *E. histolytica* rDNA amplification are:

Forward Primer (Positions 193 to 216)

Psp5: 5'...GGC CAA TTC ATT CAA TGA ATT GAG...3'

T_m= 66°C

Reverse Primer (Positions 1071-1047)

Psp3: 5'...CTC AGA TCT AGA AAC AAT GCT TCT C...3'

T_m= 70°C

The sequence of the rSSU in *E. dispar* has a 2.2% divergence from the sequence described above, represented by 17 different point mutations (Clark and Diamond, 1991) in the positions shown below marked as *E. dispar*:

5'...				3'...	
101	GTAATAGTTT	CTTTGGTTAG	TAAAATACAA		<i>E. histolytica</i>
			G		<i>E. dispar</i>
170	CCAGTTTGTA				<i>E. histolytica</i>
	A				<i>E. dispar</i>
191	ATGGCCAATT	CATTCAATGA	A TTGAGAAAT		<i>E. histolytica</i>
	G	T GT GT A			<i>E. dispar</i>
931	TGTGTCCATT				<i>E. histolytica</i>
	G				<i>E. dispar</i>
1041	AAGATAGAGA				<i>E. histolytica</i>
	GA				<i>E. dispar</i>
1051	AGCATTGTTT	CATGATCTGA			<i>E. histolytica</i>
	A	A CA			<i>E. dispar</i>
1081	ATTACCTTGT				<i>E. histolytica</i>
	C				<i>E. dispar</i>

The sequences of the specific primers used for *E. dispar* rDNA amplification are:

Forward primer:

Npsp5: 5'...GGC CAA TTT ATG TAA GTA ATT TGA G...3'

T_m= 66

Reverse Primer:

Npsp3: 5'...CTT GGA TTT AGA AAC AAT GTT TCT TC...3'

T_m= 68

The conditions for 35 cycles of amplification were set as follows:

Denaturation	94°C	1 minute
Annealing	55°C	1.5 minutes
Elongation	72°C	2 minutes

The two pairs of primers used for specific amplification differ in 5 and 6 bases respectively between *E. histolytica* and *E. dispar*, and the expected result is the presence of a band of 876 bp (Clark and Diamond 1992). DNA from reference axenic and polyxenic strains of *E. histolytica* and *E. dispar* respectively was amplified during standardisation, and later these DNA stocks were used as controls (Table 5.1).

TABLE 5.1 REFERENCE STRAINS USED AS CONTROLS FOR DNA TESTS

STRAIN	ZYMODEME	ORIGIN	ISOLATED BY
<i>E. histolytica</i> HM1-IMSS	II	Amoebic Dysentery	B. Sepulveda, M. de la Torre
<i>E. histolytica</i> NIH:200	II	Amoebic Dysentery	J. E. Tobie
<i>E. histolytica</i> NIH:H-303	II	Amoebic Dysentery	L. S. Diamond
<i>E. histolytica</i> SD157	NA	NA	G. Clark
<i>E. histolytica</i> SD184	NA	NA	G. Clark
<i>E. histolytica</i> ST 138	II	Amoebic Dysentery and Amoeboma	J. Osisañya
<i>E. histolytica</i> ST 140	II	Amoebic Colitis	J. Osisañya
<i>E. dispar</i> SAW 1734	III	Cyst Carrier	P. G. Sargeant and J. Williams
<i>E. dispar</i> SAW 760	NA	NA	P. G. Sargeant and J. Williams
<i>E. dispar</i> ST 119	I	Cyst Carrier	J. Osisañya
<i>E. dispar</i> ST Bean	NA	NA	NA
<i>E. dispar</i> ST 145	I	Asymptomatic Carrier	J. Osisañya

NA: Data Not Available

5.2.14 Detection of *E. histolytica* and *E. dispar* in Faecal Samples and Other Body Fluids by PCR-SHELA

PCR-SHELA was first successfully used for direct clinical diagnosis in 7 faecal samples (Table 5.2) from an endemic area for intestinal parasites at La Mesa, Cundinamarca (Colombia). The samples were provided by individuals with intestinal complaints suggestive of amoebiasis attending the outpatients clinic at the Hospital Pedro Leon Alvarez. The samples were examined by microscopy at the parasitology section in

the clinical laboratory at the hospital and immediately frozen and transported to the LSHTM where DNA was extracted following the methodology described in 5.2.2.

TABLE 5.2 MICROSCOPY ON FAECES FROM LA MESA

SAMPLE ID	PATIENT ID	MICROSCOPY
F1	LAC	<i>E. histolytica</i> Haematophagous trophozites
F2	JA	<i>Entamoeba</i> sp. trophozoites
F3	RM	<i>Entamoeba</i> sp. trophozoites, <i>Bl. hominis</i>
F4	MS	<i>E. coli</i> cysts
F5	FC	<i>E. histolytica</i> haematophagous trophozoites
F6	DG	<i>E. histolytica</i> / <i>E. dispar</i> cysts
F7	PC	Hookworm eggs

Also, as part of initial evaluation of PCR-SHELA with clinical samples, a small series of faecal samples and pus drained from liver abscesses from patients attending the Hospital for Tropical Diseases (HTD) in London were processed according to the methodology described in 5.2.2, although the overnight incubation with lysis buffer at 37°C was substituted by a two hours incubation at 55°C and screened by PCR-SHELA (Table 5.3).

TABLE 5.3 HTD CLINICAL SPECIMENS FOR DIAGNOSIS BY PCR-SHELA

SAMPLE ID	SPECIMEN	CLINICAL DIAGNOSIS	MICROSCOPY
001472	Faeces	Ulcerative Colitis	Negative
AW	Faeces	Dysentery	Haematophagous Trophozoites
65004534	Faeces	Amoebic Liver Abscess	Negative
65004534	Pus	Amoebic Liver Abscess	Negative
1079	Pus	Amoebic Liver Abscess	Negative
65004534	Blood	Amoebic Liver Abscess	NT
65004534	Urine	Amoebic Liver Abscess	NT
65004534	Serum	Amoebic Liver Abscess	NT

HTD: HOSPITAL FOR TROPICAL DISEASES

NT: Not Tested

For PCR, each 100 µl of reaction mixture contained 4 mM MgCl₂, 5 Units of *Taq* polymerase in Promega *Taq* Buffer system (M-1861) and 0.2 mM of each deoxynucleoside triphosphate. 40 µl PCR reactions were set to contain 25 pmol of each primer separated from the other reaction components by a wax layer, 0.5-1 ng of sample DNA and between 0.5 and 10 ng of reference *E. histolytica* and *E. dispar* DNA for control reactions. Also, negative controls including *Escherichia coli* DNA grown in R medium (Robinson's polyxenic culture medium, Appendix 1), DNA from parasite-free faeces and water as a sample replacement were used.

Modifications to the amplification conditions initially standardised for DNA from cultured material were introduced when testing multiple faecal samples: The first cycle of denaturation was reduced from 2 to 1 minute at 94°C and was followed by an increment in the number of cycles to 35. A reduction in the time for primer annealing and omission of the final 7 minutes of elongation were other modifications introduced according to the protocols published by Acuña-Soto *et al.*, in 1993.

The final modified protocol for the 35 cycles of the PCR was then set as follows:

Denaturation at 94°C for 30 seconds

Annealing at 55°C for 1 minute and,

Elongation at 72°C for 2 minutes,

Modifications were also introduced for the hybridisation step, which was reduced from 90 to 15 minutes. The methodology for the ELISA step was followed as described in 5.2.9.

5.2.15 Application of PCR-SHELA for the Diagnosis of Amoebiasis in a Dutch Family

Faecal samples from 7 members of a Dutch household and their derived polyxenic cultures (Table 5.4) were tested by PCR-SHELA following the methodology described above. The index case was a 5 year old female (Child I) who was diagnosed as suffering from amoebic dysentery although she had never been abroad (Visser *et al.*, 1998). However, the mother had an amoebic liver abscess five year before on return from a trip to India. Because of the risk and the epidemiological implications of leaving asymptomatic cyst carriers untreated, it was decided to screen all the individuals in the household. Faecal samples were referred to the Laboratory of Parasitology of the Faculty of Medicine at the University of Leiden for microscopy, and from there the samples were sent by post to LSHTM where an aliquot was cultured in Robinson's medium by Mr J. Williams at the Malaria Reference Laboratory and a second aliquot was used for DNA extraction. The DNA was then diluted 1/10 and 1/100 and 2 µl of the corresponding dilution were used for the PCR reactions.

Hybridisation and ELISA were performed according to the modifications described in 5.2.12.

TABLE 5.4 MICROSCOPY OF FAECAL SAMPLES FROM HOLLAND

SAMPLE ID	PATIENT ID	MICROSCOPY RESULT
1H(1204)	Nanny	<i>D. fragilis</i> , <i>Bl. hominis</i>
2H(1206)	Nanny's Husband	<i>Bl. hominis</i>
3H(1203)	Mother	<i>D. fragilis</i> , <i>E. histolytica/dispar</i> cysts, <i>E. coli</i> cysts, <i>Bl. hominis</i>
4H(1187)	Child I	<i>E. histolytica</i> haematophagous troph., <i>E. histolytica/dispar</i> cysts, <i>E. coli</i> cysts
5H(1205)	Father	<i>E. histolytica/dispar</i> cysts, <i>E. coli</i> cysts
6H(1095)	Child II	<i>E. histolytica/dispar</i> cysts
7H(1096)	Child III	<i>E. histolytica/dispar</i> cysts

Number in parentheses: Corresponding Culture ID number

Serology data were obtained from the Laboratory of Parasitology at the Faculty of Medicine in Leiden after testing all the samples and cultures blindly by PCR-SHELA.

5.2.16 Evaluation of PCR-SHELA for Detection of *E. histolytica* and *E. dispar* Intestinal Infections in Holland and Colombia

The development of PCR-SHELA as a diagnostic aid for differentiation of amoebic intestinal infections with *E. histolytica* and *E. dispar* required a wider evaluation in two fronts: at the reference laboratory level in non endemic areas such as developed countries where the main affected group are travellers and their close contacts and in tropical endemic areas, where a high percentage of carriers are present.

The results obtained with PCR-SHELA at the time encouraged our collaborators to initiate a further evaluation of both systems. For that purpose, funding was obtained through the EC INCO-DC program for a multicentric collaborative project between the London School of Hygiene and Tropical Medicine, the Laboratory of Parasitology attached to the Faculty of Medicine at the University of Leiden (The Netherlands), the Centro de Investigaciones en Parasitología and Medicina Tropical (CIMPAT) at Los Andes University, Bogota (Colombia), the Laboratorio de Microbiología and the Department of Tropical Medicine at the Hospital Clinic i Provincial of Barcelona (Spain). Training on culture and isoenzyme electrophoresis when required, DNA extraction using the phenol chloroform method with ethanol precipitation and PCR-ELISA was provided to the research teams before the start of the pilot studies for PCR-SHELA evaluation.

The pilot study was carried out in Leiden by Teig *et al.*, (1997), where 31 *E. histolytica/E. dispar* microscopy positive and culture positive stool samples were tested by PCR-SHELA and compared to two other PCR systems, the amplification of the 30 kDa antigen gene (Tachibana *et al.*, 1991) and of the rSSU DNA (Clark and Diamond, 1992).

Although PCR-SHELA was successful for the distinction of amoebic intestinal infections and results were produced the day following receipt of the samples, it was evident that modifications to the whole protocol were necessary in order to produce results within one working day. As part of the modifications, the QIAamp Tissue DNA Extraction Kit, from QIAGEN® (Figure 5.4) was introduced for the evaluation of a total of 369 faecal samples arriving from different laboratories in the Netherlands to the reference laboratory at the Department of Parasitology of the University of Leiden (Polderman and Verweij, 1997; Verweij *et al.*, 1998). All the faecal samples were referred to the reference laboratory because of microscopical or clinical suspicion of *E. histolytica/dispar* infection and were concentrated on arrival and processed by the formol-ether method (Allen and Ridley, 1970).

A second evaluation of PCR-SHELA was carried out with samples from Quibdo, the capital of the department of Choco, on the pacific coast of Colombia. The Department of Choco has a population of 406,199 inhabitants surrounded by a tropical lowland forest. The geographical characteristics of the region have kept it isolated from the rest of the country, which influences its very poor economy and sanitary conditions. For the pilot study, faecal samples were collected from an heterogeneous population of volunteers belonging to all age groups (0-70 years), involving personnel from the police force and from the slums around Quibdo. A total of 427 single stool samples were examined by microscopy with micrometric measurement by Sandra Molina and Patricia Fuya from the CIMPAT (Universidad de los Andes, Bogota). The samples that were positive for *E. histolytica/E. dispar* or *E. hartmanni* by microscopy were transported frozen to Bogota for DNA extraction.

DNA from samples harbouring *E. histolytica/dispar* cysts and *E. hartmanni* cysts was extracted using the QIAGEN® Tissue kit (QIAGEN® Cat. No.29304, Figure 5.4). The DNAs were tested by PCR-SHELA and the results were compared to the results obtained with the amplification of the small ribosomal subunit (rSSU) DNA (5.2.13). Unfortunately, no cultured material was obtained for comparison of the PCR results with zymodeme analysis.

QIAamp spin column procedure



FIGURE 5.4 QIAamp DNA EXTRACTION METHOD WITH THE QIAGEN[®] TISSUE KIT

5.2.17 Characterisation of Intestinal Amoebic Infections in Travellers Returning From the Tropics to Barcelona (Spain)

Diagnosis of amoebiasis in travellers is one of the most important issues for the control of the dispersion of *E. histolytica*. The laboratory of Parasitology at the Microbiology Department Hospital Clinic i Provincial in Barcelona screens the faecal samples from patients with gastrointestinal complaints referred by the hospital's travellers clinic. The microscopy of 57 samples (Table 5.5) and the primary polyxenic cultures were carried out in Barcelona and results were stored in a database in Access for windows. An aliquot of each unpreserved sample was frozen at -70°C until transported to the LSHTM, where DNA extraction and PCR-SHELA were carried out.

The microscopy of the samples collected at Barcelona consisted of 29 *E. histolytica/dispar* single infections and 28 faeces containing *E. histolytica/dispar* and other parasites, from which 19 harboured other amoebas (4 containing cysts of *E. hartmanni*) and 9 contained other parasites as well as *E. histolytica/dispar*, but no other amoebas. The country visited was recorded in most cases, and this collection represents infections acquired from around the world (Table 5.5).

TABLE 5.5 SAMPLES FROM TRAVELLERS RETURNING TO BARCELONA FROM THE TROPICS

SAMPLE ID	COUNTRY VISITED	MICROSCOPY-MICROBIOLOGY
2675	MEXICO	<i>E. histolytica/dispar</i> , <i>E. hartmanni</i> cysts
2436	PERU	<i>E. histolytica/dispar</i> cysts
219	CUBA	<i>E. histolytica/dispar</i> cysts, <i>Bl. hominis</i>
622	ECUADOR	<i>E. histolytica/dispar</i> cysts; Negative coproculture
819 20/3	NA	<i>E. histolytica/dispar</i> , <i>E. coli</i> cysts
2394	MADAGASCAR	<i>E. histolytica/dispar</i> , <i>E. coli</i> cysts, <i>G. intestinalis</i>
423	SENEGAL	<i>E. histolytica/dispar</i> , <i>E. coli</i> cysts
505	MEXICO	<i>E. histolytica/dispar</i> , <i>E. coli</i> cysts
1614	NICARAGUA	<i>E. histolytica/dispar</i> , <i>E. nana</i> cysts, <i>T. trichiura</i> Eggs, <i>Bl. hominis</i>
695	GUATEMALA	<i>E. histolytica/dispar</i> , <i>E. coli</i> cysts

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SAMPLE ID	COUNTRY VISITED	MICROSCOPY-MICROBIOLOGY
696	GUATEMALA	<i>E. histolytica/dispar</i> , <i>E. coli</i> cysts
2635	TOGO	<i>E. histolytica/dispar</i> , <i>E. hartmanni</i> , <i>E. coli</i> , <i>E. nana</i> cysts,
2877	ZAIRE	<i>E. histolytica/dispar</i> , <i>E. coli</i> cysts
3210	INDONESIA	<i>E. histolytica/dispar</i> trophozoites; <i>Escherichia coli</i> NET, NEP
3187	NA	<i>E. histolytica/dispar</i> trophozoites
280	NA	<i>E. histolytica/dispar</i> cysts
910	INDONESIA	<i>E. histolytica/dispar</i> cysts
1060	NA	<i>E. histolytica/dispar</i> , <i>E. nana</i> cysts
2529	MALI, BURKINA FASO	<i>E. histolytica/dispar</i> , <i>E. hartmanni</i> , <i>E. nana</i> cysts
2103	SALVADOR	<i>E. histolytica/dispar</i> cysts, <i>Bl. hominis</i>
88	BENIN	<i>E. histolytica/dispar</i> cysts
112	COLOMBIA	<i>E. histolytica/dispar</i> , <i>E. coli</i> cysts; Negative coproculture
764	NA	<i>E. histolytica/dispar</i> cysts
1628	INDIA	<i>E. histolytica/dispar</i> cysts, <i>T. hominis</i>
2333	SALVADOR	<i>E. histolytica/dispar</i> , <i>E. coli</i> cysts
264	GUINEA EQUATORIAL	<i>E. histolytica/dispar</i> , <i>E. coli</i> , <i>I. butschlii</i> cysts, <i>T. trichiura</i> Eggs
2512	UGANDA, KENYA	<i>E. histolytica/dispar</i> cysts; <i>Escherichia coli</i>
287	NA	<i>E. histolytica/dispar</i> cysts
2407	SENEGAL	<i>E. histolytica/dispar</i> cysts
323	ZAMBIA, TANZANIA	<i>E. histolytica/dispar</i> cysts, <i>Klebsiella pneumonie</i>
2597	UGANDA, KENYA	<i>E. histolytica/dispar</i> cysts
2015	NA	<i>E. histolytica/dispar</i> cysts
2564	INDIA	<i>E. histolytica/dispar</i> , <i>E. nana</i> cysts; Negative coproculture
3201	MOROCCO	<i>E. histolytica/dispar</i> , <i>E. coli</i> cysts
746	BURKINA FASO	<i>E. histolytica/dispar</i> , <i>E. coli</i> , <i>E. nana</i> cysts
963	NA	<i>E. histolytica/dispar</i> cysts
107	MALI	<i>E. histolytica/dispar</i> cysts
617	INDONESIA	<i>E. histolytica/dispar</i> cysts
2552	ETIOPIA	<i>E. histolytica/dispar</i> trophozoites; <i>Escherichia coli</i>
971	MEXICO	<i>E. histolytica/dispar</i> , <i>E. coli</i> , <i>E. nana</i> cysts, <i>Bl. hominis</i> ; <i>Escherichia coli</i>
2828	NA	<i>E. histolytica/dispar</i> , <i>E. nana</i> cysts
1127	ECUADOR	<i>E. histolytica/dispar</i> cysts
277	SALVADOR	<i>E. histolytica/dispar</i> , <i>I. butschlii</i> cysts

SAMPLE ID	COUNTRY VISITED	MICROSCOPY-MICROBIOLOGY
1198	INDIA	<i>E. histolytica/dispar</i> cysts
2399	VIETNAM	<i>E. histolytica/dispar</i> , <i>E. coli</i> cysts, <i>Campylobacter jejunii</i>
2891	BURKINA FASO	<i>E. histolytica/dispar</i> cysts; <i>Campylobacter jejunii</i>
822	VIETNAM	<i>E. histolytica/dispar</i> cysts
563	BURKINA FASO	<i>E. histolytica/dispar</i> cysts
533	BURKINA FASO	<i>E. histolytica/dispar</i> cysts
472	NA	<i>E. histolytica/dispar</i> cysts
466	MOROCCO	<i>E. histolytica/dispar</i> cysts
2024	INDIA	<i>E. histolytica/dispar</i> cysts
675	GUINEA ECUATORIAL	<i>E. histolytica/dispar</i> cysts; <i>Salmonella spp.</i>
315	IVORY COAST	<i>E. histolytica/dispar</i> cysts, <i>G. intestinalis</i> ; Negative coproculture
1136	SPAIN	<i>E. histolytica/dispar</i> , <i>E. coli</i> , <i>E. nana</i> cysts
2409	TCHAD	<i>E. histolytica/dispar</i> , <i>E. hartmanni</i> , <i>I. butschlii</i> cysts, <i>S. mansoni</i> ; <i>Escherichia coli</i> NEP, NET
428	IVORY COAST	<i>E. histolytica/dispar</i> cysts

NA: Data not available

NET: Not Enterotoxigenic *E. coli*

NEP: Not Enteropathogenic *E. coli*

5.2.18 Sensitivity and Specificity of PCR-SHELA

The sensitivity and specificity of PCR-SHELA were calculated according to the following formulas (Altman, 1991):

Sensitivity= $[TP / (TP + FN)] \times 100\%$, defined as the proportion of gold-standard positives correctly identified by the test.

Specificity= $[TN / (TN + FP)] \times 100\%$, defined as the proportion of negatives that are correctly identified by the test.

Positive Predictive Value= $[TP / (TP + FP)] \times 100\%$, defined as the proportion of patients with positive test results who are correctly diagnosed.

Negative Predictive Value= $[\text{TN} / (\text{TN} + \text{FN})] \times 100\%$, defined as the proportion of patients with negative test results who are correctly diagnosed.

Accuracy= $[(\text{TP} + \text{TN}) / (\text{TP} + \text{FN} + \text{TN} + \text{FP})] \times 100\%$, defined as the proportion of gold standard positives and negatives correctly identified by the test.

Where,

TP: True Positives are the individuals identified as positive by the gold standard test and the test under trial.

TN: True Negatives are the individuals identified as negative by the gold standard test and the test under trial.

FP: False Positives, are the individuals with a negative result in the gold standard but positive with the test being evaluated.

FN: False Negatives are the individuals identified as positive by the gold standard but which were negative with the test being evaluated.

5.3 RESULTS

5.3.1 PCR System for Amplification of *E. histolytica* and *E. dispar* Reference DNA

PCR amplification was achieved in reactions containing 2.5U of Taq polymerase, however, 5 Units of enzyme gave optimal results, producing a band corresponding to 125 bp for *E. histolytica* and to 133 bp for *E. dispar*. Specificity of the products was tested by southern blot hybridisation. Each product was used as a probe and made to react under the same conditions of stringency with its homologous product and the product from the other species (Figure 5.5).

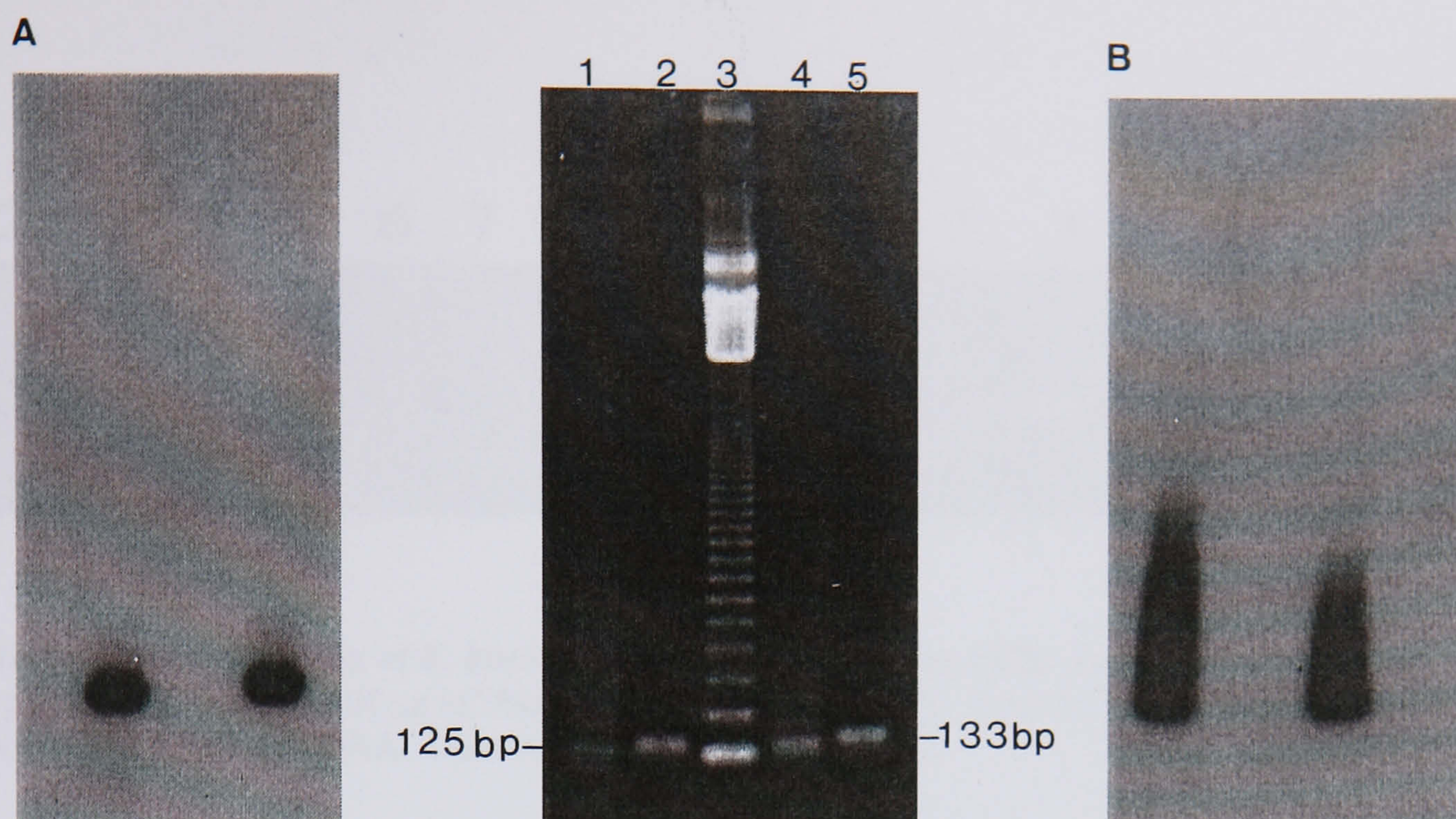


FIGURE 5.5 Southern Blot Analysis of *E. histolytica* (125 bp) and *E. dispar* (133 bp) PCR Products. PCR products in a 2% agarose gel (centre), lanes 1 and 4: *E. histolytica*, lanes 2 and 5: *E. dispar*, lane 3: 123 bp Molecular Weight Marker Ladder. Each homologous radioactive PCR Product was used as the probe. *E. dispar* probe (A), *E. histolytica* probe (B).

No cross reactivity was observed, indicating that the PCR products are species specific. Also, because the sequence amplified is present in multiple copies in both organisms, multiple sites for primer annealing are present and products of amplification of multiple molecular weights are also expected. The presence of bands on a "ladder" pattern depend on the number of repeats amplified so that bands of 125 bp, 250bp, and 375bp correspond to one, two, or three copies of the *E. histolytica* repeat. Similarly, bands of 133bp, 266bp, and 399bp are one to three copies of the corresponding repeat respectively.

5.3.2 Assessment of PCR Sensitivity by DNA Titration

DNA concentrations ranging from 10 pg/2 μ l to 10 ng/2 μ l for *E. histolytica* and from 10 pg/2 μ l to 100 ng/2 μ l for *E. dispar* produced specific bands of the expected molecular weights with their respective PCR systems (Figure 5.6 A and B). The multiple banding pattern of amplification observed correlates with the variation in the concentration of DNA. No amplification was observed in the controls in which water replaced the sample.

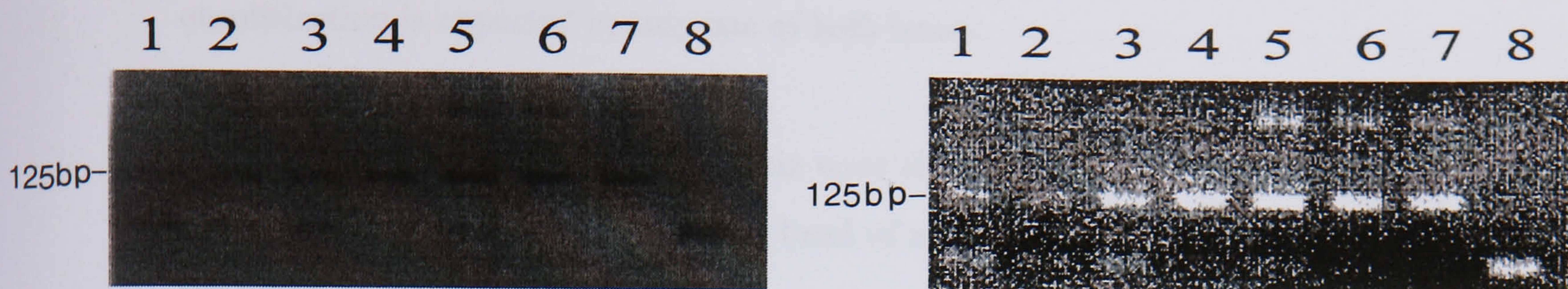


FIGURE 5.6 A Titration of *E. histolytica* HM1-IMSS DNA by PCR. Lane 1: 10 pg of DNA. Lane 2: 50 pg of DNA. Lane 3: 100 pg of DNA. Lane 4: 500 pg of DNA. Lane 5: 1 ng of DNA. Lane 6: 5 ng of DNA. Lane 7 10 ng of DNA. Lane 8: Water control (No DNA).



FIGURE 5.6 B Titration of *E. dispar* SAW 1734 DNA by PCR. Lane 1: 10 pg of DNA. Lane 2: 50 pg of DNA. Lane 3: 100 pg of DNA. Lane 4: 500 pg of DNA. Lane 5: 1 ng of DNA. Lane 6: 5 ng of DNA. Lane 7 10 ng of DNA. Lane 8: 50 ng. Lane 9: 100 ng and Lane 10: Water control (No DNA).

5.3.3 PCR Amplification of DNA From Colombian Isolates in Polyxenic Cultures

In order to test the PCR systems using isolates from the field, DNA from 32 *E. dispar* isolates derived from microscopically positive samples was extracted at the Centro de Investigaciones en Microbiología y Parasitología Tropical, at Los Andes University in Bogota. The DNAs were transported frozen to the LSHTM and the PCR was carried out in London with both sets of primers and the products of amplification were first detected

by agarose gel electrophoresis. The products of each reaction with primers for *E. histolytica* and primers for *E. dispar* were placed side by side in the gel so that amplification is expected in only one of both lanes.

All strains isolated in Colombia were correctly amplified as *E. dispar*, however, some cultures also produced a single band of approximately 375 bp with *E. histolytica* specific primers (Figure 5.7).

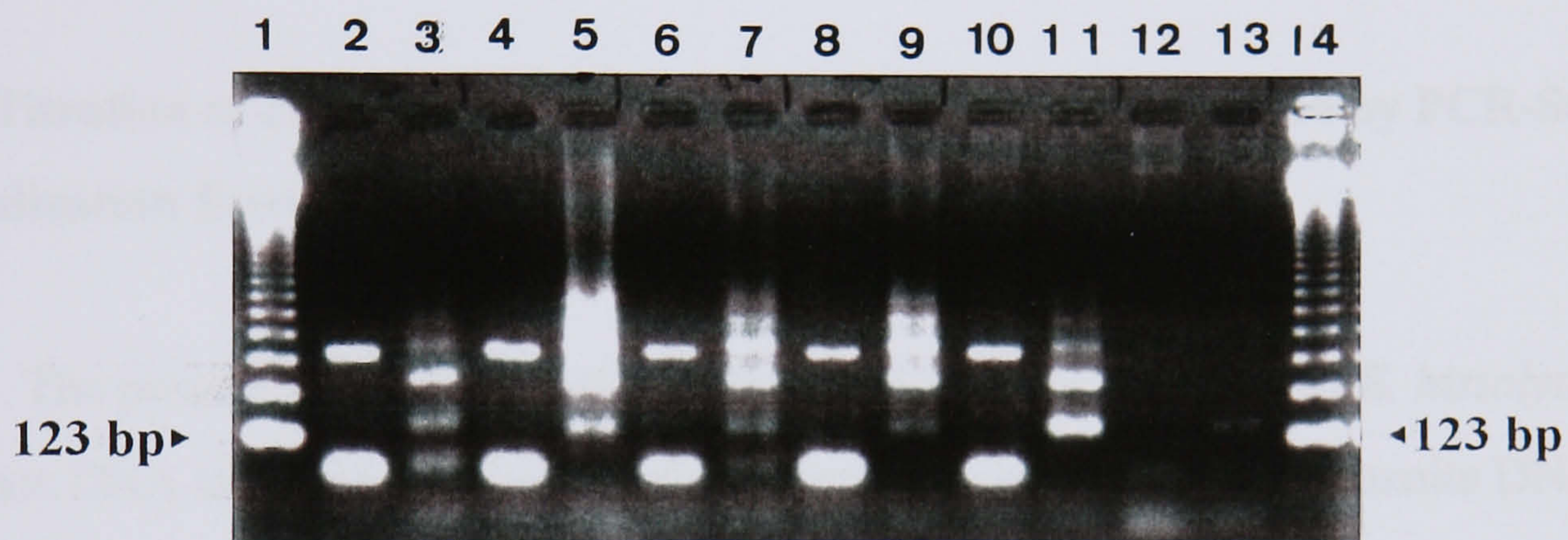


FIGURE 5.7. 2% Agarose Gel Electrophoresis of PCR Products of Cultured Material from Colombia. Lanes 2, 4, 6, 8, 10 and 12 correspond of products of amplification with *E. histolytica* primers. Lanes 3, 5, 7, 9, 11 and 13 correspond to products of amplification with *E. dispar* primers. Lanes 1 and 14: 123 bp Molecular Weight Ladder Marker. Lanes 2 and 3: Jennifer. Lanes 4 and 5: Luisa. Lanes 6 and 7: 217. Lanes 8 and 9: Mauricio. Lanes 10 and 11: Pol 10. Lanes 12 and 13: 86.

Those cultures were retested with similar results. Contamination of the components of the PCR reaction was ruled out when no amplification was present in the water controls. Because no cloning procedure was followed prior to the collection of the trophozoites all those cultures were classified as possibly mixed cultures. However, cross contamination with minimal amounts of DNA can occur on cultured material while subculturing great numbers of isolates or during extraction of DNA.

In order to avoid possible contaminations during the manipulation of cultured material, modifications to the protocol for collection of the culture for DNA extraction from Colombia, an improvement in the drying process of the glassware by increasing the temperature over 170°C was introduced, and also the collection of cultured material in disposable tubes only. In addition, the use of aerosol protected tips and the use of screw capped tubes during extraction and further manipulations of DNA, were adopted. The frequent change of gloves during DNA extractions and for the preparation of the PCR reactions was consistently encouraged until it became part of the routine process.

5.3.4 Titration of *E. histolytica* HMI-IMSS and *E. dispar* SAW 1734 by PCR-Solution Hybridisation Enzyme-Linked Immuno Assay (SHELA)

The process of standardisation of PCR-SHELA for detection of *E. histolytica* and *E. dispar* DNA involved the titration of different concentrations of trophozoite DNA from reference strains HM1-IMSS and SAW 1734 with their corresponding specific primers and probes. After extraction, the DNA was diluted in 50µl of sterile distilled water and its concentration measured as described in 5.2.3 (page 147). For titration, dilutions were prepared to reach concentrations of 10 pg/2µl, 50 pg/2µl, 0.1 ng/2µl, 0.5 ng/2µl, 1 ng/2µl, 5 ng/2µl, 10 ng/2µl, 50 ng/2µl and 100 ng/2µl, and added to the PCR reactions. Hybridisation with the corresponding probes followed PCR, and detection of the hybrids was carried out by ELISA (5.2.10, page 152). ODs are registered in Table 5.6.

For the *E. histolytica* system, positive reactions were observed from the first concentration of HM1-IMSS DNA tested (10 pg/2µl) and it was observed that the ODs increased as the concentration of DNA was increased up to 10 ng/2µl. For the *E. dispar* system, a yellow positive reaction was also readable by eye in all the concentrations tested, however the increase in the DNA concentration was not directly proportional to the optical densities developed.

Because many factors may affect DNA amplification in polyxenic samples, the variation in the behaviour of the optical density trend in regard to concentration may be explained by the variable bacterial content of the polyxenic Robinson's medium, which would account for much of the total DNA estimated after extraction. Also, during the amplification, the number of repeats amplified varies and depends on the accessibility of the multiple annealing points on the target sequences, which also affects the annealing of the probe during the hybridisation step.

TABLE 5.6 *E. histolytica* HM1-IMSS AND *E. dispar* SAW 1734 DNA TITRATION BY PCR-SHELA

DNA CONCENTRATION PER 2µl OF SAMPLE	<i>E. histolytica</i> HM1-IMSS OD	<i>E. dispar</i> SAW 1734 OD
0 (Water Control)	0.037	0.029
10 pg	0.096	0.252
50 pg	0.099	0.262
0.1 ng	0.181	0.152
0.5 ng	0.227	0.114
1 ng	0.309	0.518
5 ng	0.315	0.513
10 ng	0.406	0.174
50 ng	NT	0.130
100 ng	NT	0.240

OD: Optical Density at 410 nm.

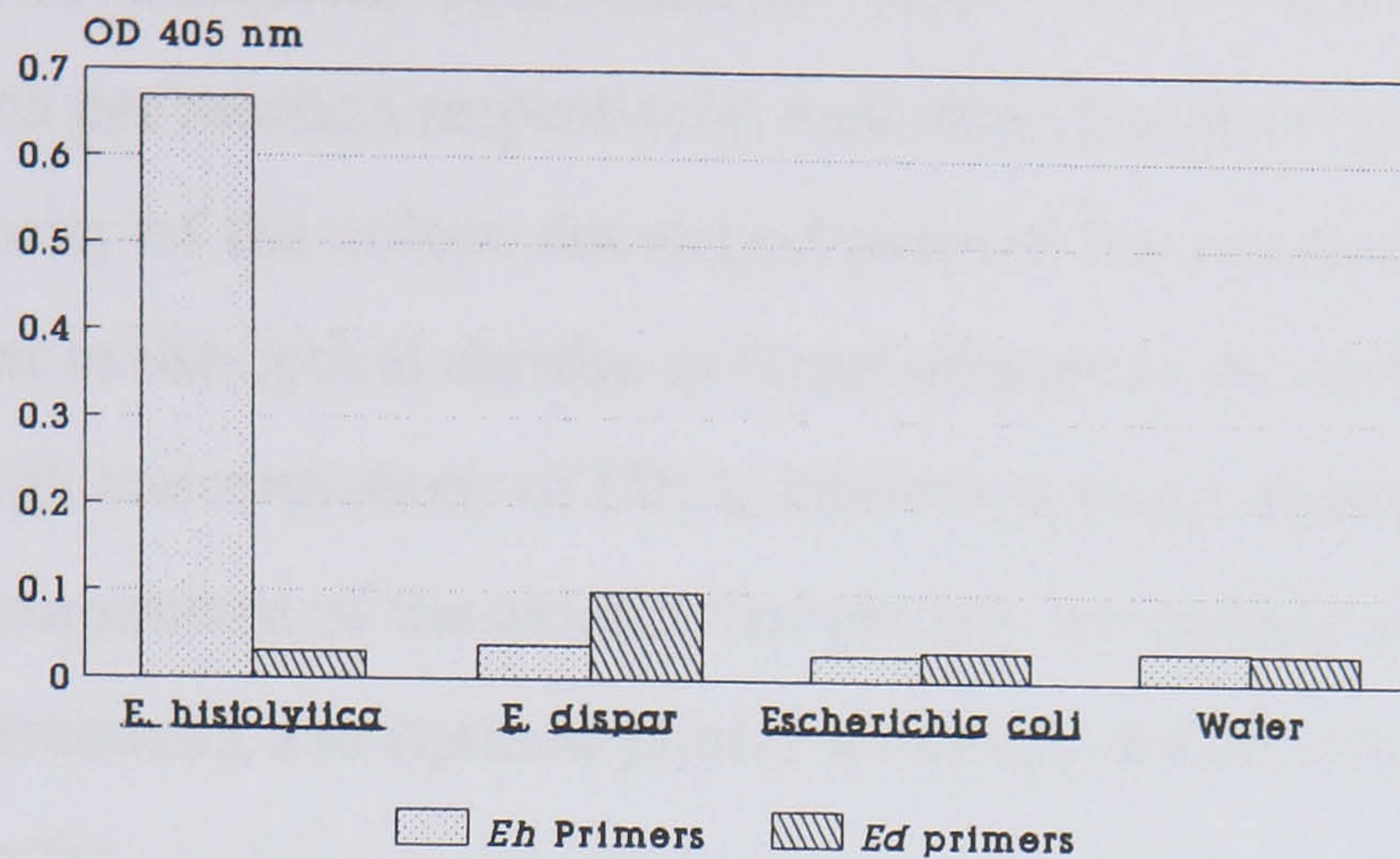
Eh Break Point $\bar{x}+2SD^* = 0.0194+2(0.022) = 0.0639$

Ed BP* = $0.0188+2(0.021) = 0.0630$

*BP calculated with duplicate OD readings of water controls in 15 independent assays.

Also, the isolation of new strains from faeces can only be done in polyxenic culture medium, which contains *Escherichia coli* strain B and some of the original bacterial flora from the faecal sample. DNA from *E. coli* grown in Robinson's medium was amplified with both sets of primers and compared to the same amount (2ng) of *E. histolytica* and *E. dispar* DNA in order to test the possibility of cross reactions (Figure 5.8). No PCR products were obtained with *E. coli* DNA when amplified with the two specific set of primers used in the test and it produced minimal non-specific background in the ELISA.

Figure 5.8 PCR-SHELA AMPLIFICATION OF CONTROL DNA (2 ng)



E. histolytica(Eh) HMI-IMSS DNA
E. dispar(Ed) SAW 1734 DNA
E. coli DNA (Robinson's Medium)

5.3.5 Detection Limit of PCR-SHELA With *E. histolytica* and *E. dispar* Trophozoites From Polyxenic Cultures

The DNA extracted from dilutions of trophozoites derived from polyxenic Robinson's cultures were tested by PCR-SHELA to determine the limit of detection of the tests for material isolated in culture (see 5.2.1, page 146). Numbers were adjusted to 1×10^5 , 1×10^4 , 1×10^2 and 1 trophozoites, in a volume of 0.5 ml of PBS and completed to 1 ml with lysis buffer before extraction with phenol-chloroform and ethanol precipitation (see 2.9, page 70). PCR-SHELA results on *E. histolytica* culture 822 and on *E. dispar* culture 533 are found in table 5.7.

TABLE 5.7 PCR-SHELA TITRATION OF POLYXENIC *E. histolytica* and *E. dispar* TROPHOZOITES

ESTIMATED No. OF TROPHOZOITES PER PCR REACTION	<i>E. histolytica</i> 822 PCR-SHELA OD \pm SD	<i>E. dispar</i> 533 PCR-SHELA OD \pm SD
4×10^3	0.071 ± 0.090	0.037 ± 0.001
4×10^2	0.114 ± 0.014	0.133 ± 0.005
4	0.230 ± 0.021	0.078 ± 0.007
<1	0.281 ± 0.026	0.005 ± 0.0007

OD: Optical Density at 410 nm. SD: Standard Deviation.

Eh Break Point $\bar{x} + 2SD^* = 0.0194 + 2(0.022) = 0.0639$

Ed BP* = $0.0188 + 2(0.021) = 0.0630$

*BP calculated with duplicate OD readings of water controls in 15 independent assays.

Positive reactions were observed using two microlitres from a final 50µl stock of each DNA extracted, equivalent to 4×10^3 , 400, 4 and less than 1 trophozoite of *E. histolytica* per reaction respectively, indicating that the sensitivity of the system is high and the intensity of the colour developed permits the reactions to be read by eye. Also, the increment in the optical density in direct relation to the dilution of the sample may indicate that at high concentrations of DNA, inhibition occurs during PCR amplification, (probably due to components of the extract that are not completely eliminated during the process of DNA extraction), and optimal primer annealing conditions are reached after dilution of the DNA stocks.

The results of PCR-SHELA for DNA extracted from trophozoites of *E. dispar* grown in polyxenic cultures indicated that this system could probably be less sensitive than the assay for detection of DNA of *E. histolytica* trophozoites grown in the same culture medium.

5.3.6 Detection of *E. histolytica* and *E. dispar* Polyxenic Clinical Isolates by PCR-SHELA

Pellets of trophozoites from field isolates made at the ICCDR in Dhaka (Bangladesh) and at CIMPAT in Bogota (Colombia) were tested by PCR-SHELA (Table 5.8). The polyxenic cultures from Bangladesh had been classified according to zymodeme analysis as 23 *E. histolytica* strains, 13 belonging to zymodeme II and 10 to zymodeme XIV. An additional culture (798) had not been characterised by zymodeme analysis. Further 4 isolates were classified as *E. dispar*, zymodeme I, for a total of 28 isolates to be tested by PCR-SHELA. The *E. histolytica* system correctly identified 23 cultures, 15 of which were pure cultures, 6 belonging to zymodeme II and 9 to zymodeme XIV. However, mixture with *E. dispar* was detected in material from 6 cultures with zymodeme II and in one with zymodeme XIV. Culture 798 was identified as *E. histolytica* by PCR-SHELA.

The absorbances obtained with the PCR-SHELA system from the 4 *E. dispar* cultures according to zymodeme analysis at the ICCDR evidenced surprising results: two cultures (1351400 and 616) were positive with the *E. histolytica* system only, the material from one culture (6191) was confirmed as *E. dispar* but also contained some background DNA from *E. histolytica*, and the remaining culture with zymodeme I (1383875) was negative with both systems. Conclusions on the specificity of the test were difficult to reach until a greater number of *E. dispar* cultures from Colombia were tested (Table 5.9).

TABLE 5.8 PCR-SHELA RESULTS OF CULTURES FROM BANGLADESH

SAMPLE ID	ZYMODEME	Eh OD \pm SD	Ed OD \pm SD	PCR-SHELA RESULT
1363050	II	0.525 \pm 0.027	0.003 \pm 0.0007	<i>E. histolytica</i>
2701	II	0.231 \pm 0.004	0.006 \pm 0.0007	<i>E. histolytica</i>
1351400	I	0.520 \pm 0.018	0.009 \pm 0.006	<i>E. histolytica</i>
48286	II	0.378 \pm 0.011	0.013 \pm 0.0007	<i>E. histolytica</i>
44298	XIV	0.386 \pm 0.001	0.009 \pm 0.0007	<i>E. histolytica</i>
3771	II	0.630 \pm 0.021	0.140 \pm 0.002	Mixed Infection
8691	XIV	0.595 \pm 0.002	0.044 \pm 0.002	<i>E. histolytica</i>
4530	XIV	0.465 \pm 0.002	0.005 \pm 0.002	<i>E. histolytica</i>
46385	XIV	0.571 \pm 0.002	0.004 \pm 0.001	<i>E. histolytica</i>
6942	XIV	0.348 \pm 0.002	0.005 \pm 0.0007	<i>E. histolytica</i>
798	ND	0.631 \pm 0.003	0.001 \pm 0.002	<i>E. histolytica</i>
144536	XIV	0.496 \pm 0.002	0.009 \pm 0.000	<i>E. histolytica</i>
1381150	XIV	0.519 \pm 0.001	0.190 \pm 0.002	Mixed Infection
1353600	XIV	0.491 \pm 0.003	0.002 \pm 0.0014	<i>E. histolytica</i>
1375975	II	1.052 \pm 0.066	0.060 \pm 0.019	<i>E. histolytica</i>
1374300	II	0.416 \pm 0.0007	0.169 \pm 0.007	Mixed Infection
1339525	II	0.565 \pm 0.012	0.000 \pm 0.004	<i>E. histolytica</i>
58996	XIV	0.569 \pm 0.004	0.007 \pm 0.000	<i>E. histolytica</i>
1320300	II	0.668 \pm 0.007	0.031 \pm 0.004	<i>E. histolytica</i>
1383875	I	0.009 \pm 0.002	0.017 \pm 0.009	Negative
6191	I	0.128 \pm 0.002	0.458 \pm 0.014	Mixed Infection

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SAMPLE ID	ZYMODEME	Eh OD ± SD	Ed OD ±SD	PCR-SHELA RESULT
1378275	II	0.578 ±0.048	0.007 ±0.0007	<i>E. histolytica</i>
7098	XIV	0.631 ±0.007	0.007 ±0.000	<i>E. histolytica</i>
506	II	0.665 ±0.094	0.608 ±0.113	Mixed Infection
616	I	0.699 ±0.008	0.004 ±0.001	<i>E. histolytica</i>
372	II	0.819 ±0.012	0.581 ±0.019	Mixed Infection
379	II	0.745 ±0.024	0.288 ±0.0007	Mixed Infection
478	II	0.677 ±0.008	0.397 ±0.0007	Mixed Infection

Eh OD: Optical density with *E. histolytica* PCR-SHELA. **Eh SD:** Standard deviation of two readings in the *E. histolytica* system. **Ed OD:** Optical density with *E. dispar* PCR-SHELA. **Ed SD:** Standard deviation of two readings in the *E. dispar* system. **Eh Break Point** $\bar{x}+2SD^* = 0.0194+2(0.022) = 0.0639$. **Ed BP*** $= 0.0188+2(0.021) = 0.0630$
 *BP calculated with duplicate OD readings of water controls in 15 independent assays.

The analysis of PCR-SHELA results on 32 additional colombian isolates (Table 5.9), revealed amplification on DNA of 26 *E. dispar* cultures, however, 8 of those cultures also contained background *E. histolytica* DNA and were classified as mixed infections. The six remaining cultures were negative when tested by both PCR-SHELA systems.

TABLE 5.9 PCR-SHELA RESULTS ON CULTURES FROM COLOMBIA

SAMPLE ID	IFAT MAb 20/7D	HK	<i>E. histolytica</i> OD ± SD	<i>E. dispar</i> OD ± SD	PCR-SHELA RESULT
11 HOSP FUT	Negative	SLOW	0.056 ±0.070	0.191 ±0.013	<i>E. dispar</i>
11 HOSP COL	NT	NT	0.013 ±0.001	0.171 ±0.005	<i>E. dispar</i>
10 COL	Negative	SLOW	0.031 ±0.004	0.085 ±0.004	<i>E. dispar</i>
16 COL	Negative	SLOW	0.008 ±0.003	0.165 ±0.012	<i>E. dispar</i>
34 COL	Negative	SLOW	0.037 ±0.004	0.102 ±0.009	<i>E. dispar</i>
33 COL	NT	NT	0.010 ±0.002	0.042 ±0.002	Negative
41 COL	Negative	SLOW	0.023 ±0.012	0.177 ±0.007	<i>E. dispar</i>
11 FUT	Negative	SLOW	0.186 ±0.001	0.089 ±0.007	Mixed Infection
12 FUT	NT	NT	0.039 ±0.0007	0.120 ±0.003	<i>E. dispar</i>
43 HOSP FUT	NT	NT	0.007 ±0.002	0.148 ±0.008	<i>E. dispar</i>
7 FUT	Negative	SLOW	0.108 ±0.0056	0.122 ±0.000	Mixed Infection
5 FUT	Negative	SLOW	0.014 ±0.0007	0.0007 ±0.002	Negative

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SAMPLE ID	IFAT MAb 20/7D	HK	<i>E. histolytica</i> OD ± SD	<i>E. dispar</i> OD ± SD	PCR-SHELA RESULT
19 FUT	NT	NT	0.130 ±0.002	0.183 ±0.003	Mixed Infection
83 LMP	Negative	SLOW	0.016 ±0.002	0.132 ±0.001	<i>E. dispar</i>
86	NT	NT	0.000 ±0.001	0.126 ±0.004	<i>E. dispar</i>
66	Negative	SLOW	0.111 ±0.0004	0.199 ±0.028	Mixed Infection
217	NT	NT	0.001 ±0.000	0.176 ±0.004	<i>E. dispar</i>
MAURICIO	NT	NT	0.005 ±0.004	0.048 ±0.002	Negative
POL 9	NT	NT	0.352 ±0.028	0.064 ±0.0007	Mixed Infection
POL 10	NT	NT	0.009 ±0.001	0.035 ±0.004	Negative
JULIA	Negative	SLOW	0.108 ±0.001	0.106 ±0.024	Mixed Infection
JENNIFER	NT	NT	0.016 ±0.0007	0.136 ±0.006	<i>E. dispar</i>
RENE	NT	NT	0.001 ±0.004	0.008 ±0.003	Negative
9 LMP	Negative	SLOW	0.092 ±0.016	0.164 ±0.019	Mixed Infection
MIREYA	NT	SLOW	0.006 ±0.004	0.036 ±0.004	Negative
LUISA	NT	NT	0.004 ±0.002	0.127 ±0.006	<i>E. dispar</i>
MARCOS	NT	NT	0.046 ±0.006	0.131 ±0.0007	<i>E. dispar</i>
64 LMP	NT	NT	0.091 ±0.021	0.121 ±0.003	Mixed Infection
DG LMP	NT	NT	0.020 ±0.005	0.079 ±0.0007	<i>E. dispar</i>
LIZ LMP	Negative	SLOW	0.010 ±0.002	0.195 ±0.004	<i>E. dispar</i>
LAB LMP	Negative	SLOW	0.007 ±0.004	0.066 ±0.003	<i>E. dispar</i>
MB LMP	NT	NT	0.004 ±0.007	0.097 ±0.005	<i>E. dispar</i>

IFAT MAb 20/7D: IMMUNOFLUORESCENCE WITH MONOCLONAL ANTIBODY 20/7D. NT: Not Tested
 HK: Hexokinase Migration. Slow: Characteristic migration for *E. histolytica*. Fast: Characteristic for *E. dispar*.
 OD: Optical Density reading at 410 nm.

Eh Break Point(BP)*: $\bar{x}+2SD= 0.0194+2(0.022)= 0.0639$

Ed BP*= $0.0188+2(0.021)= 0.0630$

*BP calculated with duplicate OD readings of water controls in 15 independent assays.

The comparison of PCR-SHELA for diagnosis of amoebic infection in pellets from cultures evidenced that the system for detection of *E. histolytica* DNA produced better OD readings than the system for detection of *E. dispar* DNA. It also evidenced that although no amplification was detected in the internal water controls included with each batch of PCR reactions, background contamination was present in the DNA obtained from the pellets of cultured material, the possible origin of which will be discussed later.

5.3.7 PCR Amplification of the SSUr DNA on Polyxenic Cultures

In order to evaluate the performance of PCR-SHELA when compared to a well known standard PCR system, 55 of the 60 DNAs tested in 5.3.6 were used to amplify a region of the small subunit of ribosomal DNA in *E. histolytica* and *E. dispar* with the corresponding specific primers according to the protocol described in 5.2.13, page 155. This amplification also intended to clarify (when possible) the results on the mixed infections detected by PCR-SHELA and on the apparently false positive *E. histolytica* isolates reported as zymodeme I in the collection obtained from Bangladesh.

The DNA from 25/28 of the cultures from Bangladesh was amplified using specific primers for the SSU rDNA and the results are found in the table below:

TABLE 5.10 COMPARISON OF PCR-SHELA AND SSU rDNA AMPLIFICATION ON CULTURES FROM BANGLADESH

SAMPLE ID	ZYMODEME	PCR-SHELA	rSSU PCR
1363050	II	<i>E. histolytica</i>	<i>E. histolytica</i>
2701	II	<i>E. histolytica</i>	<i>E. histolytica</i>
1351400	I	<i>E. histolytica</i>	<i>E. histolytica</i>
48286	II	<i>E. histolytica</i>	<i>E. histolytica</i>
44298	XIV	<i>E. histolytica</i>	<i>E. histolytica</i>
3771	II	Mixed Infection	<i>E. histolytica</i>
8691	XIV	<i>E. histolytica</i>	<i>E. histolytica</i>
4530	XIV	<i>E. histolytica</i>	<i>E. histolytica</i>
46385	XIV	<i>E. histolytica</i>	<i>E. histolytica</i>
6942	XIV	<i>E. histolytica</i>	<i>E. histolytica</i>
798	ND	<i>E. histolytica</i>	<i>E. histolytica</i>
144536	XIV	<i>E. histolytica</i>	<i>E. histolytica</i>
1381150	XIV	Mixed Infection	<i>E. histolytica</i>
1353600	XIV	<i>E. histolytica</i>	<i>E. histolytica</i>
1375975	II	<i>E. histolytica</i>	<i>E. histolytica</i>
1374300	II	Mixed Infection	<i>E. histolytica</i>

SAMPLE ID	ZYMODEME	PCR-SHELA	rSSU PCR
1339525	II	<i>E. histolytica</i>	<i>E. histolytica</i>
58996	XIV	<i>E. histolytica</i>	<i>E. histolytica</i>
1320300	II	<i>E. histolytica</i>	<i>E. histolytica</i>
1383875	I	Negative	<i>E. dispar</i>
6191	I	Mixed Infection	Mixed Infection
1378275	II	<i>E. histolytica</i>	<i>E. histolytica</i>
7098	XIV	<i>E. histolytica</i>	<i>E. histolytica</i>
616	I	<i>E. histolytica</i>	<i>E. histolytica</i>
478	II	Mixed Infection	<i>E. histolytica</i>

SSU rDNA amplification was achieved and agreed with the results obtained with PCR-SHELA in the DNA of 19 *E. histolytica* cultures and confirmed one (6191) mixed infection by amplification with both sets of primers in the material from Bangladesh. It also clarified further 4 mixed infections (3771, 1381150, 1374300 and 478) by amplification with the *E. histolytica* pair of primers only. Among the cultures with zymodeme I, as shown before, culture 6191 was confirmed as containing DNA from both species and cultures 1351400 and 616 amplified as *E. histolytica*, in agreement with the PCR-SHELA results. Culture 1383875 which did not amplify by PCR-SHELA was confirmed as *E. dispar*.

Overall, results on the amplifications of the DNA with PCR-SHELA and SSU rDNA specific sets of primers agreed in 24/25 cultures from Bangladesh, although results on 3 samples classified as mixed infections by PCR-SHELA were confirmed as containing DNA of *E. histolytica* only by amplification of the SSU rDNA.

In a similar fashion, it was possible to test 30 of the 32 colombian *E. dispar* isolates using specific primers for amplification of the SSU rDNA of *E. histolytica* and *E. dispar*. Amplification was achieved in DNA of 28 cultures and the results are listed in the table below:

TABLE 5.11 COMPARISON OF PCR-SHELA AND SSU rDNA AMPLIFICATION ON CULTURES FROM COLOMBIA

SAMPLE ID	HK	PCR-SHELA	rSSU PCR
11 HOSP FUT	SLOW	<i>E. dispar</i>	<i>E. dispar</i>
11 HOSP COL	NT	<i>E. dispar</i>	<i>E. dispar</i>
10 COL	SLOW	<i>E. dispar</i>	<i>E. dispar</i>
16 COL	SLOW	<i>E. dispar</i>	<i>E. dispar</i>
34 COL	SLOW	<i>E. dispar</i>	<i>E. dispar</i>
33 COL	NT	Negative	Negative
41 COL	SLOW	<i>E. dispar</i>	<i>E. dispar</i>
11 FUT	SLOW	Mixed Infection	<i>E. dispar</i>
12 FUT	NT	<i>E. dispar</i>	<i>E. dispar</i>
43 HOSP FUT	NT	<i>E. dispar</i>	<i>E. dispar</i>
7 FUT	SLOW	Mixed Infection	<i>E. dispar</i>
5 FUT	SLOW	Negative	<i>E. dispar</i>
19 FUT	NT	Mixed Infection	<i>E. dispar</i>
83 LMP	SLOW	<i>E. dispar</i>	<i>E. dispar</i>
217	NT	<i>E. dispar</i>	<i>E. dispar</i>
MAURICIO	NT	Negative	<i>E. dispar</i>
POL 9	NT	Mixed Infection	Negative
POL 10	NT	Negative	<i>E. dispar</i>
JULIA	SLOW	Mixed Infection	<i>E. dispar</i>
JENNIFER	NT	<i>E. dispar</i>	<i>E. dispar</i>
RENE	NT	Negative	<i>E. dispar</i>
9 LMP	SLOW	Mixed Infection	<i>E. dispar</i>
MIREYA	SLOW	Negative	<i>E. dispar</i>
LUISA	NT	<i>E. dispar</i>	<i>E. dispar</i>
MARCOS	NT	<i>E. dispar</i>	<i>E. dispar</i>
64 LMP	NT	Mixed Infection	<i>E. dispar</i>
DG LMP	NT	<i>E. dispar</i>	<i>E. dispar</i>
LIZ LMP	SLOW	<i>E. dispar</i>	<i>E. dispar</i>
LAB LMP	SLOW	<i>E. dispar</i>	<i>E. dispar</i>
MB LMP	NT	<i>E. dispar</i>	<i>E. dispar</i>

NT: NOT TESTED

HK: Hexokinase Migration

The rSSU PCR amplified 23 cultures as *E. dispar*, in agreement with the results obtained by PCR-SHELA. However, 6 mixed infections detected by PCR-SHELA were found to contain *E. dispar* DNA only by amplification of the SSU rDNA. One culture (33 col) remained negative with in both PCRs and one sample which amplified as a mixed infection detected by PCR-SHELA did not amplify with the rSSU PCR. Amplification with the primers for *E. dispar* rSSU was achieved in 5 of the cultures that were negative by PCR-SHELA. Overall, the *E. dispar* PCR-SHELA system amplified 25/30 samples, meanwhile the PCR for amplification of the *E. dispar* SSU rDNA amplified 29/30 samples tested (Table 5.11).

Bangladesh: 25 *E. histolytica* Cultures
Colombia: 30 *E. dispar* Cultures

<i>E. histolytica</i> SSU rDNA				<i>E. dispar</i> SSU rDNA			
	+	-	Total		+	-	Total
<i>E. histolytica</i> +	24	0	24	<i>E. dispar</i> +	24	1	25
PCR-SHELA -	1	30	31	PCR-SHELA -	4	26	30
Total	25	30	55	Total	28	27	55

PCR-SHELA Sensitivity: $[24/(24+1)] \times 100\% = 96\%$	PCR-SHELA Sensitivity: $[24/(24+4)] \times 100\% = 85.7\%$
PCR-SHELA Specificity: $[30/(30+0)] \times 100\% = 100\%$	PCR-SHELA Specificity: $[26/(26+1)] \times 100\% = 96.3\%$
Positive Predictive Value: $[24/(24+0)] \times 100\% = 100\%$	Positive Predictive Value: $[24/(24+1)] \times 100\% = 96\%$
Negative Predictive Value: $[30/(30+1)] \times 100\% = 96.8\%$	Negative Predictive Value: $[26/(26+4)] \times 100\% = 86.7\%$

From the above evaluation several aspects have to be discussed, firstly, it appears that the PCR-SHELA system for detection of *E. histolytica* is more sensitive than the system for detection of *E. dispar* in cultured material when both are compared to the rSSU PCR. Also, although mixed infections are likely to exist in nature, and mixed populations of trophozoites may be present in early cultures, their detection in long term cultured material is not likely, especially in the case of *E. histolytica* isolates, where its higher rate of multiplication should led to elimination of non-invasive populations present during long term subculture, however, it is still possible that a residual non-pathogenic population is passed on. PCR-SHELA detected 16 (26.6%) mixed infections in a sample of 60 cultures from endemic areas. Consequently, the presence of background DNA of either species in cultures can be explained by mechanical contamination through different sources: the first

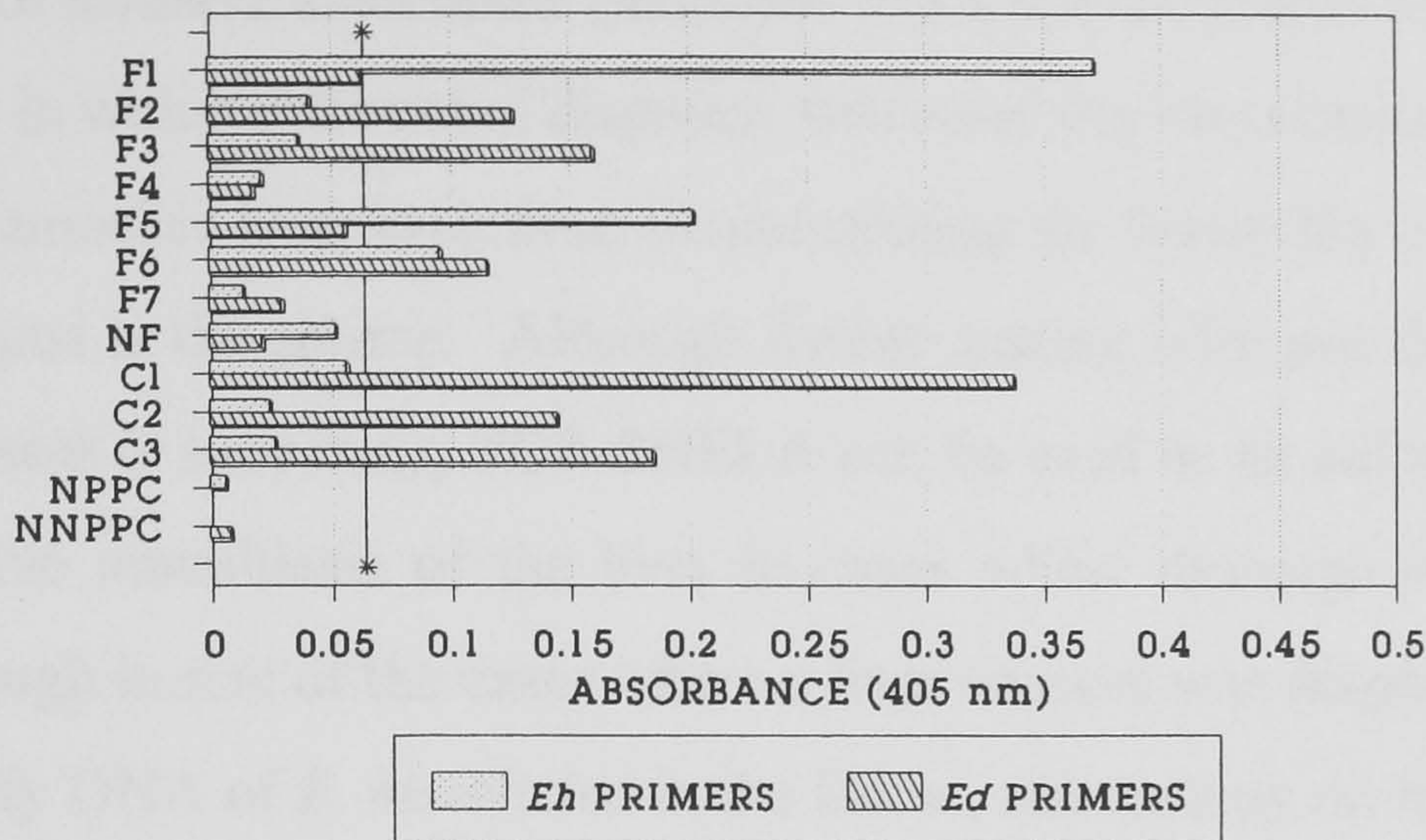
and more likely source is the process of subculture, when cross contamination between samples is possible if DNA residues from previous culturing are left in the glassware after washing, in particular in the reused glass bijoux bottles used for polyxenic Robinson's culture. The second source could be the production of aerosol droplets when adding the secondary Robinson's medium, in which case a few trophozoites would be enough to contaminate the original culture. This would be translated as different degrees of contamination in PCR reactions, ranging from slight amounts of background DNA to a total change in the species cultured. A third source could be the process of extraction of DNA itself, in which case, the multiple steps of the process (in particular the phenol-chloroform method) favoured cross contamination due to manipulation. An alternative explanation for the presence of background DNA after PCR from cultures is the possible non-specific priming. If this is the reason, a similar percentage of samples presenting background DNA would be expected after PCR-SHELA directly on uncultured stool samples from endemic areas and this will be examined later.

The analysis of the results presented in this section emphasises that diagnosis of amoebiasis ideally should not be based only on cultured material (WHO/PAHO/UNESCO, 1997) and that it is also necessary to detect intestinal amoebic infections directly in faecal samples, where diagnosis can be carried out promptly without the possibility of selection of parasite populations or contamination after successive subcultures.

5.3.8 Detection of *E. histolytica* and *E. dispar* by PCR-SHELA in Faecal Samples, Pus From Liver Abscess and Other Body Fluids

PCR-SHELA from 7 faecal samples collected at La Mesa, in Cundinamarca (Colombia) resulted in the detection of two *E. histolytica* and three *E. dispar* infections (Figure 5.9 A and B). No amplification was observed in samples harbouring *Entamoeba coli* or hookworm infections, nor in the controls containing water as sample replacement or parasite negative faeces (Aguirre *et al.*, 1995).

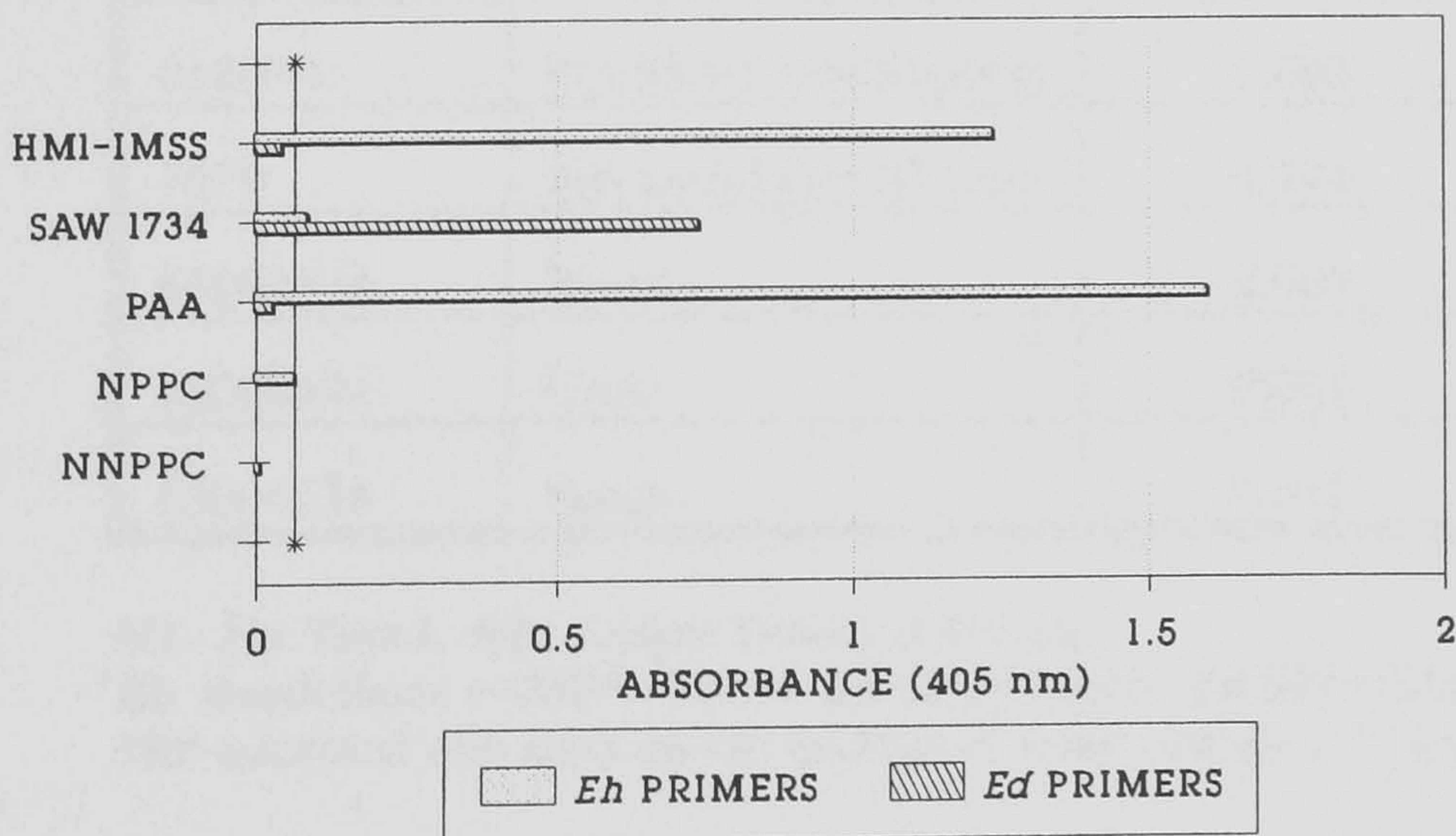
SAMPLES



Eh: *Entamoeba histolytica*
 Ed: *Entamoeba dispar*
 *Cut off Value: 0.063

FIGURE 5.9 A PCR-SHELA results from faeces (F), Robinson's cultures (C) and Primer Controls. NF:Negative faeces, NPPC:negative pathogenic primer control, NNPPC:negative non-pathogenic primer control. Microscopy results: **F1:***E. histolytica*, hematophagous troph., **F2:***Entamoeba sp*, trophozoites, **F3:***Entamoeba sp*, troph. and *Blastocystis hominis*, **F4:***E. coli*, cysts, **F5:***E. histolytica*, hematophagous trophozoites, **F6:***E. histolytica*, cysts, **F7:** Hookworm, Eggs. **C1:** *E. histolytica*, cysts, **C2:** *Entamoeba histolytica*, cysts, *E. coli*, cysts, *I. buetschlii*, cysts and *E. nana*, cysts; **C3:***E. histolytica*, cysts, *E. coli*, cysts and *I. buetschlii*, cysts.

SAMPLE



Eh: *Entamoeba histolytica*
 Ed: *Entamoeba dispar*
 *Cut Off Value: 0.063

FIGURE 5.9 B PCR-SHELA Results from Reference Strains, Amoebic Abscess and Primer Controls. HMI-IMSS: Reference *E. histolytica* strain, SAW 1734: Reference *E. dispar* strain. PAA: Pus Amoebic Abscess, NPPC: negative pathogenic primers control, NNPPC: negative non-pathogenic primers control.

The samples collected at HTD in London were processed blindly and PCR-SHELA results are found in Table 5.12. Included among the samples for evaluation, faeces from cases of invasive amoebiasis (intestinal and extra-intestinal) and from a case of ulcerative colitis in which differential diagnosis was necessary were included. PCR-SHELA detected three amoebic liver infections demonstrating the feasibility of amplification when DNA from pus is the source. Although further testing with pus derived from pyogenic liver abscesses is necessary, PCR-SHELA can be used as an aid to serology for diagnosis of invasive amoebiasis of the liver in cases where drainage of the abscess is indicated. Although in one of the cases where a liver abscess was diagnosed it was also possible to identify DNA of *E. histolytica* in the faeces, microscopy on faecal samples from patients with amoebic liver involvement is often negative.

TABLE 5.12 PCR-SHELA RESULTS ON HTD SPECIMENS

SAMPLE ID	SPECIMEN	PCR-SHELA <i>E. histolytica</i> OD	PCR-SHELA <i>E. dispar</i> OD
001472	Faeces	0.033	0.032
AW	Faeces	0.309	0.036
65004534	Faeces	0.783	-0.011
65004534	Pus from Liver Abscess	0.101	-0.001
018090	Pus from Liver Abscess	1.602	0.030
1079	Pus from Liver Abscess	0.744	0.004
65004534	Blood	0.009	0.010
65004534	Urine	0.063	-0.014
65004534	Serum	0.011	-0.008

NT: Not Tested. OD: Optical Density at 410 nm.

Eh Break Point $\bar{x}+2SD^*$ = 0.0194+2(0.022)= 0.0639. **Ed BP*** = 0.0188+2(0.021)= 0.0630

*BP calculated with duplicate OD readings of water controls in 15 independent assays.

In order to test the possibility of using other sources of DNA, red blood cells, serum, and urine in a case of amoebic liver abscess were screened for the presence of amoebic DNA. No amplification was achieved with any of those samples and further amplifications were not attempted.

5.3.9 Diagnosis of Intestinal Amoebiasis by PCR-SHELA in a Dutch Family

PCR-SHELA gave positive results with the *E. histolytica* specific system in four from the five microscopy positive samples (Table 5.4, page 162) and it was also positive in all four derived cultures. The results of further PCR amplifications on the false negative sample remained negative, evidencing again the need for an improvement in the methodology for extraction of DNA, which would bring sensitivity to the same level achieved by means of the use of the concentration methods (Table 5.13).

TABLE 5.13 PCR-SHELA RESULTS FROM FAECES AND CULTURES COMPARED TO ZYMODEME ANALYSIS AND SEROLOGY

SAMPLE ID	FAECES <i>Eh</i> PCR-SHELA OD	FAECES <i>Ed</i> PCR-SHELA OD	CULTURE <i>Eh</i> PCR-SHELA OD	CULTURE <i>Ed</i> PCR-SHELA OD	ZYMODEME	ELISA SEROLOGY
1H	0.031	0.027	NT	NT	NT	<1/40
2H	0.033	0.034	NT	NT	NT	NT
3H	0.143	0.035	0.340	0.036	IIα-	1/320
4H	0.323	0.036	NT	NT	NT	1/80
5H	0.266	0.035	0.591	0.041	IIα-	<1/40
6H	0.048	0.045	0.183	0.010	IIα-	1/320
7H	0.177	0.049	0.134	-0.003	IIα-	1/40

Eh: *Entamoeba histolytica*. *Ed*: *Entamoeba dispar*

OD: Optical Density at 410 nm.

Eh Break Point $\bar{x}+2SD^*$ = 0.0194+2(0.022)= 0.0639. **Ed BP*** = 0.0188+2(0.021)= 0.0630.

*BP calculated with duplicate OD readings of water controls in 15 independent assays.

NT: Not Tested

It is interesting to note that although the nanny (sample 1H) and her husband (sample 2H) were in close contact with the family, only the family members were infected, and from them, the mother (sample 3H), and the children (samples 4H, 6H and 7H), presented a positive serology. Only the index case (4H) presented haematophagous trophozoites. This family cluster highlights the importance of the concomitant use of several diagnostic techniques to achieve high sensitivity and specificity of diagnosis, which in this case involved microscopy, culture, serology and PCR-SHELA.

5.3.10 Detection of *E. histolytica* and *E. dispar* Intestinal Infections in Holland and Colombia

The results described in this section were obtained through collaborative work for the evaluation of PCR-SHELA with two types of samples: the material arriving to a reference laboratory setting at the University of Leiden, the Netherlands for confirmation of microscopy results (study in which I did not have a direct involvement other than training to the staff carrying out the study), and the material obtained after a screening of a sample of the general population in an “endemic” area in Colombia (in which access to the study material and results was kindly provided by Prof. Felipe Guhl and colleagues from Universidad de los Andes).

During the pilot evaluation at Leiden (Netherlands), PCR-SHELA on DNA from the stools detected 9 *E. histolytica* and 22 *E. dispar* cyst passers as confirmed by identical results obtained with the two PCR methods used as gold standard. In this case, specificity and sensitivity of PCR-SHELA were both 100% for the *E. histolytica* and *E. dispar* systems. The PCR-SHELA results of 30 of the corresponding cultures were in complete agreement with the hexokinase results. One culture harbouring *E. dispar* remained negative by PCR-SHELA. This negative culture was later amplified after dilution of the DNA when the test was repeated at the LSHTM. The sensitivity of the *E. dispar* system for cultures was 95%, with a specificity of 100% and positive and negative predictive values of 100% and 90% respectively.

After the pilot study described above, 369 faecal samples received for confirmation of diagnosis of amoebiasis by the reference laboratory of Parasitology at the University of Leiden were divided in two aliquots, one concentrated and the other was tested by PCR-SHELA. The samples belonged to patients with clinical suspicion of invasive amoebiasis, or with a strong positive antibody reaction in serological tests, and/or the presence of trophozoites of *E. histolytica/dispar* in the stools was observed when direct examination was carried out before referral to the reference laboratory.

For the analysis of results in PCR-SHELA, the samples were divided into two groups according to the results of the concentration method, which was used as the gold standard method: 246 samples(67%) were positive for *E. histolytica/dispar* cysts by concentration, and 123 samples (33%) were negative for cysts of *E. histolytica/dispar* by concentration. This group included samples where cysts of other amoebas were identified or no cysts at all were detected by the reference laboratory after concentration (Table 5.14).

TABLE 5.14 PCR-SHELA EVALUATION ON CLINICAL SAMPLES AT LEIDEN, NETHERLANDS

PCR-SHELA CLASSIFICATION	<i>E. histolytica/dispar</i> (Concentration)	NEGATIVES (Concentration)	TOTAL No. OF SAMPLES
<i>E. dispar</i>	221 (90%)	10 (8%)	231 (63%)
<i>E. histolytica</i>	23 (9%)	7 (6%)	30 (8%)
Negative	2 (0.8%)	106 (86%)	108 (29%)
TOTAL	246	123	369

PCR-SHELA detected 221 *E. dispar* and 23 *E. histolytica* infections in the group of samples in which cysts of *E. histolytica/dispar* had been identified after concentration, for a total of 244/246 positives (99%), with only two microscopy positive but PCR negative samples. From the samples on the former group, 90%(221/246) were *E. dispar* passers and more importantly 9%(23/246) of the cyst positives were due to *E. histolytica*. These results are in proportion with previous estimations on the epidemiology and distribution of invasive amoebiasis, in which approximately 10% of individuals identified as *E. histolytica/dispar* positive were, in fact, infected with *E. histolytica sensu stricto* (Walsh, 1986).

For the second group of 123 samples that were found negative for *E. histolytica/dispar* cysts after concentration, *E. histolytica* DNA was amplified in 7 and *E. dispar* DNA was amplified in 10, revealing that 14%(17/123) of the samples reported as not containing *E. histolytica/dispar* cysts after concentration were found positive for either *E. histolytica* or *E. dispar* DNA after PCR-SHELA.

According to the results described above, the following evaluation of PCR-SHELA is based in the formulas described in 5.2.17 (page 168):

		MICROSCOPY		
		+	-	
PCR SHELA	+	244	17	261
	-	2	106	108
		246	123	369

PCR-SHELA Sensitivity: $[244/(244+2)] \times 100\% = 99.2\%$

PCR-SHELA Specificity: $[(106/(106+17))] \times 100\% = 86.2\%$

Positive Predictive Value: $[244/(244+17)] \times 100\% = 93.5\%$

Negative Predictive Value: $[106/(106+2)] \times 100\% = 98.1\%$

Accuracy: $[(244+106)/(244+2+106+17)] \times 100\% = 94.8\%$

If we assume that false positives are likely to be true infections, and analyse the results with PCR-SHELA as the gold standard, the sensitivity of PCR-SHELA remains as **99.2%**, due to the two samples that were positive by concentration but negative by PCR-SHELA. However, the specificity of the test increased to **100%**, hence the positive predictive value of **100%**, and the same negative predictive value of **98.1%**. Since the accuracy depends on the sensitivity and specificity of the test, it increases to **99.4%**.

No mixed infections were found in 261 positives, which is remarkable when these results are compared to those in previous studies on samples from endemic areas using PCR. Another important conclusion after the analysis of these results is the fact that in 86%(106/123) of the samples in which there was clinical suspicion of amoebiasis but no *E. histolytica/dispar* cysts were found, other etiologies should be investigated and this is one of the justifications for the screening of those samples with very sensitive methods such as PCR, where a fast negative result makes way for a different diagnostic approach.

The overall consideration of PCR-SHELA results in Leiden, indicate that 71%(261/369) of the samples received by the reference laboratory with possible diagnosis of *E. histolytica/dispar* infection were correctly diagnosed and from them, 8%(30/369) contained *Entamoeba histolytica sensu stricto*. Remarkably, the 7 *E. histolytica* positive samples detected by PCR-SHELA in the group of negatives by concentration (Table 5.14) accounted for 23% (7/30) of the total of *E. histolytica* infections detected, which suggests that examination of stool samples using only a concentration method for confirmation of amoebic infection at the reference laboratory level involves the risk of missing potentially invasive infections.

The PCR-SHELA pilot study in an endemic area was carried out at Quibdo in Choco, Colombia (Molina *et al.*, 1998), and the results of that screening indicated that of 427 faecal samples analysed by microscopy 330 (77%) harboured at least one parasite species, indicating a high exposure to faecal contamination in the population. Among the most prevalent parasitic infections, *Giardia intestinalis* was present in 11.6% of the samples, *Bl. hominis* in 45%, *A. lumbricoides* in 22%, *T. trichiura* in 17%, *S. stercoralis* in 6% and Hookworm in 4%. Among the amoeba species, *E. histolytica/dispar* infections were detected in 40 (9.6%) of the samples, *E. hartmanni* in 2.8%, *E. coli* was present in 18% and *E. nana* in 39%.

A total of 52 samples including 40 microscopically cyst positive samples identified as *E. histolytica/dispar* carriers (6 mixed with *E. hartmanni*) and 12 samples identified as containing *E. hartmanni* but no *E. histolytica/dispar* were tested by rSSU DNA amplification and PCR-SHELA using the methodology described in 5.2.11 and 5.2.12 respectively.

PCR-SHELA tests on the 40 *E. histolytica/dispar* microscopy positive samples identified 3 as *E. histolytica* (confirmed when the samples were amplified with the PCR for the rSSU). In contrast, *E. dispar* was identified in 21/40 samples by PCR-SHELA (confirmed in 18 by the rSSU DNA PCR). Among the 12 “*E. hartmanni*” from Quibdo,

2 were identified as *E. histolytica* and other 2 as *E. dispar* by PCR-SHELA (and confirmed with the rSSU DNA PCR), bringing the prevalence for *E. histolytica* infections to 9.6% (5/52 samples) and for *E. dispar* infections to 44% (23/52 samples) among the group of positives. Thus, from the 12 *E. hartmanni* suspected samples, 8 did not amplify, and therefore could be *E. hartmanni*. DNA from 16 out of the 40 *E. histolytica/dispar* microscopy positives did not amplify despite extracting those samples twice and using the DNA extract undiluted, and diluted 1/10 and 1/100 in water for further PCRs. Because no amplification was achieved after dilution, it was assumed that those samples were misdiagnosed by microscopy and those could represent possibly *E. hartmanni* infections. However, for further screenings it was decided to increase the amount of faecal lysate to be extracted from 200 µl (as advised by the QIAGEN kit) to 1 ml to enhance detection and when dilution of the DNA was necessary, 5 µl of the extracted DNA (instead of 2µl) were used for each PCR reaction.

It is important to point out that one mixed infection was found and confirmed by both PCRs, which is interesting because Acuña-Soto and colleagues (1993) reported a prevalence of 12% *E. histolytica/dispar* infections in an indigenous population from Chiapas (Mexico) when faecal samples were screened by microscopy. From them, 36% were infected with *E. histolytica* and 56% were found to harbour mixed infections when tested by PCR (Acuña-Soto *et al.*, 1993). Whether those results reflect a particular case in which transmission of *E. histolytica* and *E. dispar* is being maintained in a closed population or it is the result of cross contamination of samples, is not clear. However, in a more recent study, Newton *et al.*, (1997) found mixed infections in 78% of the microscopically-positive faeces of 48 non-dysenteric Mexican children. Because the same methodology involving a cyst concentration technique before extraction of DNA was used in both Mexican studies, further studies using a different method involving less manipulation of the samples should be applied.

The results above discussed would indicate that in a sample of 427 subjects from the general population at Quibdo (Choco, Colombia), 1.2%(5/427) *E. histolytica* infections

were detected and these subjects were at risk of developing invasive disease and accordingly were treated. In contrast, 5.3% (23/427) had *E. dispar* infections without any risk of developing disease. Also, 8/427 (1.9%) infections could be suspected to be *E. hartmanni*. This will be confirmed only after the standardisation of a PCR system for amplification of the *E. hartmanni* rSSU with specific primers (sequence kindly provided by Dr G. Clark), which also will be used to test all the *E. histolytica/dispar* microscopy positive samples which did not amplify by *E. histolytica* or *E. dispar* PCR.

The feasibility of screening faecal samples by PCR in endemic areas was tested in Colombia and it was successful. However, because only one sample from each subject was collected and only microscopy positive samples were tested in PCR-SHELA during the pilot study at Quibdo, a new screening project in which DNA of a pool of three consecutive samples will be extracted (regardless of the microscopy result) will be carried out for a complete evaluation of PCR-SHELA in the field.

5.3.11 Assay Modifications

The need for a reduction in the length of the whole process to fit within a working day was the starting point for reductions in the times of PCR cycles, the hybridisation step and of the ELISA incubations. Also, a change in the method for extraction of DNA was critical, especially for the reduction of cross contamination during the simultaneous processing of several samples. The incorporation of a kit for purification of DNA from clinical samples made it possible to reduce the extraction to 20 minutes. The procedure which provided the best results was that advised for large volume samples by the manufacturers, but the starting temperature and lysis protocol used previously (described on page 148, section 5.2.2) were kept for the extraction of faecal samples:

1. Approximately 1 gram of faeces was diluted in 3 ml of sterile PBS pH 7.2, followed by three cycles of freezing in a mixture of dry ice-ethanol and thawing in water bath at room

temperature. This step was kept unaltered because it is a physical method efficient in breaking cyst walls and liberating their content.

2. From this step forward, the QIAamp Tissue kit for DNA extraction (QIAGEN® Cat. No.29304) was incorporated. 1 ml of the suspension ($\approx 1/3$ g faeces) containing the lysate (measured using tips with wide end) was transferred into a 1.5 ml screw capped eppendorff tube, given a short spin in the microfuge (a manual pulse) at 6500-8000 rpm and the supernatant was put into a new screw capped eppendorff avoiding any disturbance of the pellet. Addition of 25 microlitres of proteinase K (20mg/ml stock) per 200 microlitres of supernatant was followed by an equal volume of Buffer AL (QIAamp kit). The mixture was vortexed and incubated in a 70°C water bath for 10 minutes.

3. Then, 210 μ l of 96-100% Ethanol per 200 microlitres of sample were added and vortexed immediately. A QIAamp spin column (from the kit) was placed in a 2 ml collection tube (provided in the kit) and 635 μ l of the lysate were applied. The lid was carefully closed and then the column was centrifuged at 8000 rpm for 1 minute. A new collection tube was prepared for the column and after adding further 635 μ l or what was left of the remaining lysate, the column was centrifuged again and transferred to a clean collection tube. The filtrates from the centrifugations were all discarded.

4. The column was then washed with 500 microlitres of Buffer AW (QIAamp kit) followed by a spin at 8000 rpm for 1 minute. Again the filtrate was discarded and the column was transferred to a new collection tube. Further 500 microlitres of buffer AW (provided in the kit) were added and centrifuged at 14000 rpm or full speed.

5. Before discarding the filtrate, the column was placed in a 2 ml screw capped eppendorf (not provided in the kit) or in any screw capped centrifuge tube that fitted the column in order to elute the DNA with 200 microlitres of buffer AE (QIAamp kit) preheated at 70°C. An incubation of 5 minutes was followed by a spin at 8000 rpm for 1 minute. This step was repeated twice, for a final volume of 400 microlitres of DNA suspension. Thus, from

starting the DNA extraction with $\approx 1/3$ g of faeces (step 2 of the protocol), the final quantity of faeces in each PCR reaction was $[1/3 \text{ g } (2\mu\text{l}/400\mu\text{l})]= 0.0016 \text{ g } (1.6 \text{ mg})$ when 2 μl of the extracted DNA are tested. When 5 μl of a 1/10 dilution of the DNA are used, the equivalent amount of faeces used is 0.4 mg.

Protocol for Cultures:

The content of 1 to 5 culture bijoux bottles was collected, avoiding disturbance of the starch layer, and pelleted spinning at 700 g for 5-10 minutes. The supernatant was discarded and the pellet was taken into a 1.5 ml screw capped centrifuge tube. 25 microlitres of proteinase K (from a 25 mg/ml stock) per 200 microlitres of pellet were added, followed by 200 microlitres of buffer AL per 200 microlitres of pellet, vortexed and incubated in a 70°C water bath for 10 minutes. The same extraction protocol as for faeces was then followed from step 3.

Protocol for Pus:

200 microlitres of pus were mixed with 25 microlitres of proteinase K (25 mg/ml) and 200 microlitres of buffer AL. After vortexing, the mixture was incubated in a 70°C water bath for 10 minutes. Then, the faeces extraction protocol was followed from step 3.

Further modifications were introduced for the SHELA protocol by omitting the blocking step with salmon sperm DNA. In addition, the washing buffer was changed from TBS-T to PBS-T which is readily available in reference laboratories in the tropics and incubations of the ELISA steps at 37°C were introduced to accelerate the kinetics of the reactions. The final protocol for the ELISA follows:

Avidin coated (according to the protocol described in page 156, section 5.2.10) ELISA strips (Greiner, Germany) were washed with PBS-T, before addition of 100 μl of each diluted PCR product. Each sample was tested in duplicate wells and after washing, 100 μl of anti-digoxigenin alkaline phosphatase labelled antibody (Cat. No.1093 274 Boehringer Mannheim, GmbH, Germany) diluted 1/5000 in PBST/were added to each well,

followed by four washings (PBS-T) and (x1, PBS only). The ELISA was developed by adding 100 µl of p-nitro-phenyl-phosphate in carbonate buffer supplemented with 1mM MgCl₂. After stopping the reaction with 50 microliters 3M NaOH, absorbances were read at 405 nm.

5.3.12 Diagnosis of Intestinal Amoebiasis by PCR-SHELA in Travellers Returning From the Tropics to Barcelona, Spain

Screening of 57 samples collected between 1994 and 1997 by the laboratory of Parasitology from the Hospital Clinic i Provincial, Barcelona was carried out at the London School of Hygiene and Tropical Medicine (table 5.15). Although a first batch of 10 of the samples was extracted using the phenol-chloroform DNA purification method (5.2.2), a second batch of 47 was extracted using the QIAamp Kit for Tissue DNA from QIAGEN®. The result of the introduction of the kit was a reduction in the time for extraction and manipulation of samples. It is now possible to process batches of 10 samples in as little as 90 minutes (approximately 20 minutes for one sample only) with very low risk of cross contamination between them.

TABLE 5.15 PCR-SHELA ON FAECAL SAMPLES FROM TRAVELLERS RETURNING TO BARCELONA

SAMPLE ID	HK	<i>E. histolytica</i> PCR-SHELA OD	<i>E. dispar</i> PCR-SHELA OD	RESULT
2675	NT	NEGATIVE	NEGATIVE	NEGATIVE
2436	NT	0.001	0.146	<i>E. dispar</i>
219	S	0.011	0.174	<i>E. dispar</i>
622	S	0.010	0.262	<i>E. dispar</i>
819 20/3	S	0.004	0.128	<i>E. dispar</i>
2394	S	0.007	0.173	<i>E. dispar</i>
423	NT	0.004	0.125	<i>E. dispar</i>
505	S	0.003	0.145	<i>E. dispar</i>
1614	NT	0.009	0.317	<i>E. dispar</i>
695	NT	0.0015	0.184	<i>E. dispar</i>

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SAMPLE ID	HK	<i>E. histolytica</i> PCR-SHELA OD	<i>E. dispar</i> PCR-SHELA OD	RESULT
696	NT	-0.010	0.165	<i>E. dispar</i>
2635	S	0.004	0.255	<i>E. dispar</i>
2877	S	0.008	0.132	<i>E. dispar</i>
3210	F	1.041	-0.014	<i>E. histolytica</i>
3187	NT	-0.057	0.283	<i>E. dispar</i>
280	NT	0.007	0.911	<i>E. dispar</i>
910	NT	-0.052	0.774	<i>E. dispar</i>
1069	S	0.029	0.135	<i>E. dispar</i>
2529	NT	0.005	0.191	<i>E. dispar</i>
2103	NT	0.006	0.257	<i>E. dispar</i>
88	S	-0.0005	0.191	<i>E. dispar</i>
112	F	0.116	0.050	<i>E. histolytica</i>
764	S	-0.052	0.131	<i>E. dispar</i>
1628	NT	0.019	0.501	<i>E. dispar</i>
2333	NT	0.004	0.428	<i>E. dispar</i>
264	NT	0.027	0.354	<i>E. dispar</i>
2512	NT	-0.014	0.127	<i>E. dispar</i>
287	NT	0.087	0.383	<i>E. dispar</i>
2407	NT	-0.014	0.199	<i>E. dispar</i>
323	S	-0.001	0.135	<i>E. dispar</i>
2597	NT	0.091	0.031	<i>E. histolytica</i>
2015	S	0.003	0.269	<i>E. dispar</i>
2564	NT	0.054	0.281	<i>E. dispar</i>
3201	NT	0.054	0.138	<i>E. dispar</i>
746	NT	0.116	0.037	<i>E. histolytica</i>
963	NT	0.018	0.527	<i>E. dispar</i>
107	S	0.003	0.302	<i>E. dispar</i>
617	NT	0.016	0.527	<i>E. dispar</i>
2552	F	0.562	0.005	<i>E. histolytica</i>
971	NT	0.104	0.042	<i>E. histolytica</i>
2828	S	-0.007	0.141	<i>E. dispar</i>
1127	NT	0.038	0.334	<i>E. dispar</i>

SAMPLE ID	HK	<i>E. histolytica</i> PCR-SHELA OD	<i>E. dispar</i> PCR-SHELA OD	RESULT
277	NT	0.049	0.129	<i>E. dispar</i>
1198	NT	0.000	0.102	<i>E. dispar</i>
2399	S	0.051	0.596	<i>E. dispar</i>
2891	NT	0.030	0.973	<i>E. dispar</i>
822	F	0.434	0.017	<i>E. histolytica</i>
563	S	0.006	0.179	<i>E. dispar</i>
533	S	0.009	0.128	<i>E. dispar</i>
472	S	0.000	0.156	<i>E. dispar</i>
466	S	0.057	1.068	<i>E. dispar</i>
2024	F	0.335	0.018	<i>E. histolytica</i>
675	S	0.015	1.334	<i>E. dispar</i>
315	NT	0.011	0.133	<i>E. dispar</i>
1136	NT	0.011	0.934	<i>E. dispar</i>
2409	NT	0.008	0.350	<i>E. dispar</i>
428	NT	0.005	0.238	<i>E. dispar</i>

OD: Optical Density at 410 nm. Eh Break Point $\bar{x}+2SD^*= 0.0194+2(0.022)= 0.0639$

Ed BP*= 0.0188+2(0.021)= 0.0630

*BP calculated with duplicate OD readings of water controls in 15 independent assays.

HK: Hexokinase characterisation. F: Fast. S: Slow

NT: Not Tested

PCR-SHELA successfully classified 56 of the 57 faecal samples tested, from which 8 (14%) were identified as *Entamoeba histolytica* and 48 (84%) as *Entamoeba dispar* infections. The technique was evaluated after the last modification to the protocol for DNA extraction at the LSHTM with faecal samples from those travellers. All patients had a presumptive diagnosis of amoebiasis after positive microscopy. Hexokinase characterisation on 25 derived cultures (Figures 5.9 and 5.10) was in total agreement with the PCR-SHELA results. The sensitivity of the tests for faecal samples was 98% and the specificity was 100%. With one possible exception (sample 287), no mixed infections were detected. Only one sample did not amplify with either PCR-SHELA system and after retrospective analysis of the microscopy result of this specimen, an infection with *E. hartmanni* was detected.

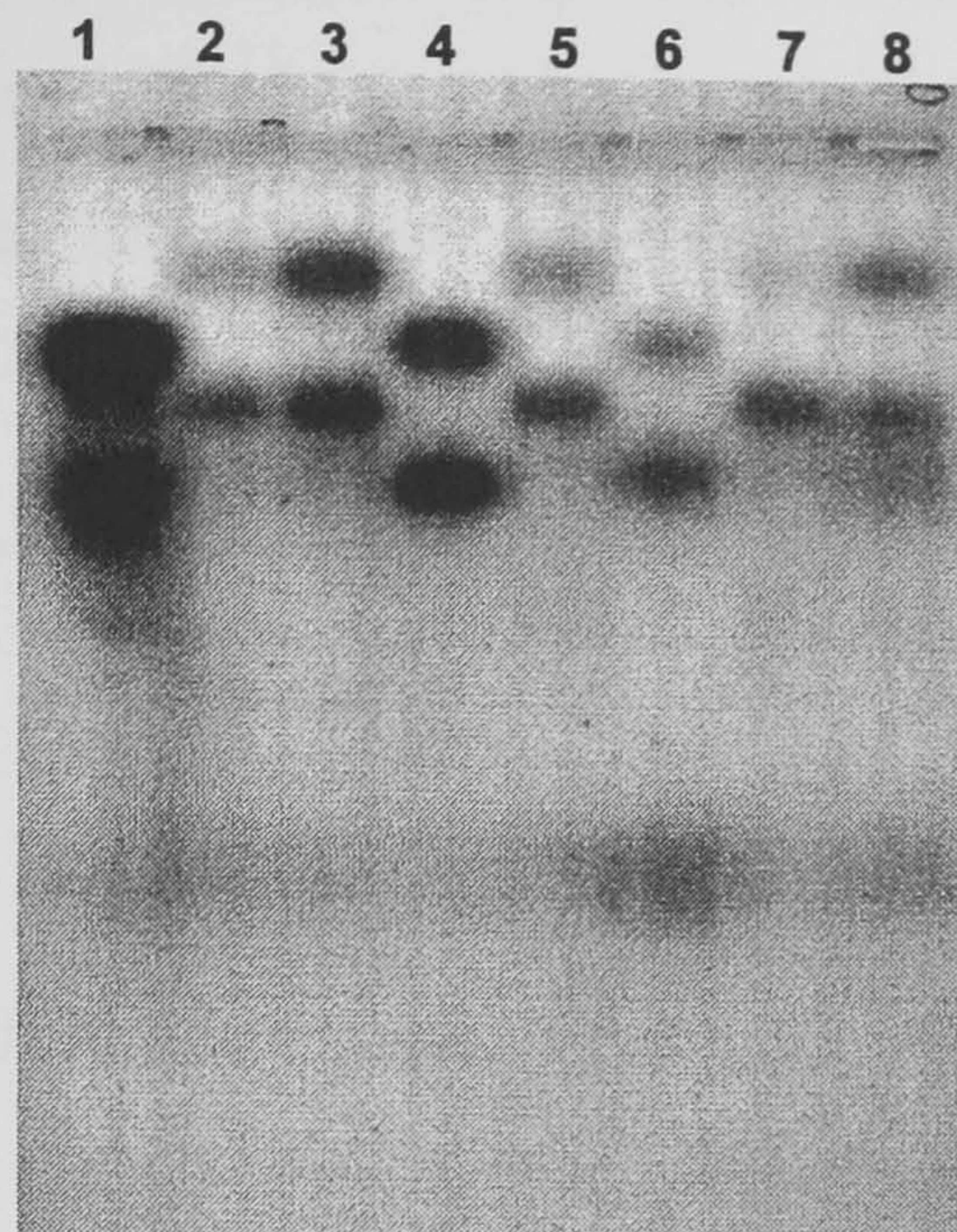


FIGURE 5.10A Hexokinase Characterisation of Strains from Barcelona and Controls. Lane 1: *E. histolytica*, Fast. Lane 2: *E. dispar*, Slow. Lane 3: 19, Slow. Lane 4: 822, Fast. Lane 5: 563, Slow. Lane 6: 25, Fast. Lane 7: 472, Slow. Lane 8: 533, Slow.

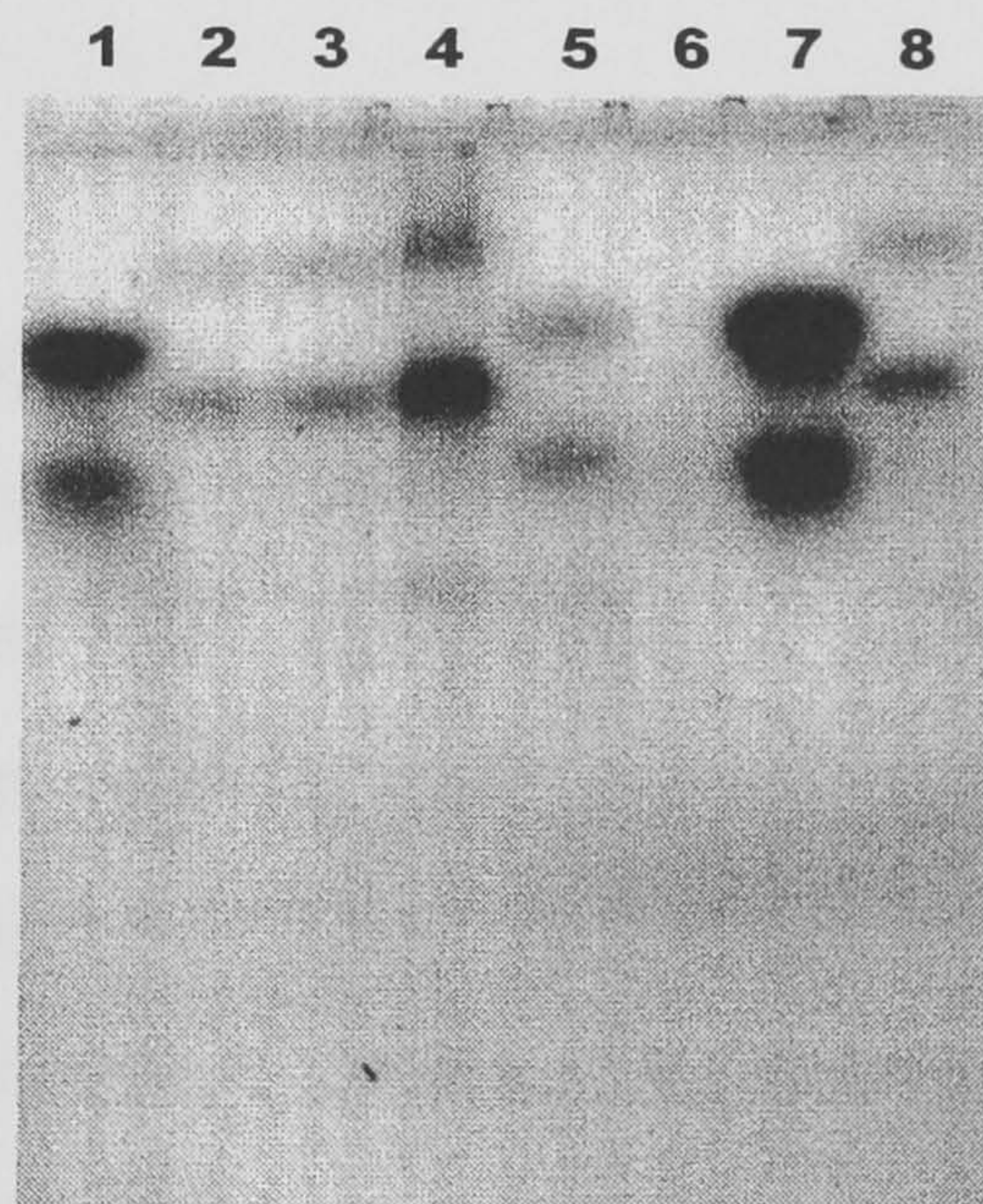


FIGURE 5.10B Hexokinase Characterisation of Strains from Barcelona and Controls. Lanes 1 and 7: *E. histolytica*, Fast. Lanes 2 and 8: *E. dispar*, Slow. Lane 3: 466, Slow. Lane 4: 159, Slow. Lane 5: 2024, Fast. Lane 6: 25, Negative.

The *E. histolytica* PCR system correctly identified the DNA of all five *E. histolytica* hexokinase positive samples, while the *E. dispar* PCR system gave negative reactions. Similarly, the *E. dispar* specific PCR system detected all 20 cyst-passers samples with *E. dispar* identified by hexokinase. The sensitivity and specificity on those samples was

100%, in agreement with the results found by Teig and colleagues at Leiden (Teig *et al.*, 1997). A further evaluation with the modified protocol for DNA isolation using fresh samples at Quibdo, (Choco, Colombia) is currently being carried out at the CIMPAT in Bogota.

5.4 DISCUSSION

The main aim of this work was to produce a very specific tool for differential diagnosis in cases where microscopy and/or clinical manifestations are indicative of invasive amoebiasis, and for analysis of asymptomatic carriers of amoebae. Thus, PCR-SHELA was standardised and evaluated as a new tool for differentiation between *E. histolytica* and *E. dispar* infections from diverse geographical origin. This tool was demonstrated to be useful for the differential diagnosis of intestinal and extraintestinal amoebic infection.

PCR-SHELA incorporates the use of a PCR technique for increased sensitivity together with a probe which provides total specificity by detecting specific internal regions on the sequences amplified. The introduction of this format enables non-radioactive detection of hybridized amplicons in a user and environmentally-friendly test in which electrophoresis in agarose gels and staining with ethidium bromide are replaced by ELISA, which is widely used in reference laboratories for the diagnosis of other infectious diseases such as tuberculosis and hepatitis.

The use of methodology involving the amplification of nucleic acids has been introduced for routine diagnosis of a number of clinical conditions and as such, costs of materials and equipment are slowly becoming affordable at laboratories in both developed and developing countries. Thus, several methods able to distinguish *E. histolytica* from *E. dispar* by PCR have been evolving in recent years in order to improve diagnosis in cultures, abscess-derived material and faeces. For instance, Tachibana *et al.*, (1992) developed a

PCR method based on specific sequences for the 30 kDa peroxidoxin from *E. histolytica* and from *E. dispar*. DNA from cultures was amplified and PCR products of 100 bp for *E. histolytica* and of 101 bp for *E. dispar* were detected by agarose gel electrophoresis. Also, the amplification of ribosomal genes of *E. histolytica* and *E. dispar* became the gold standard for PCR amplification after the sequences for both organisms were described as divergent (Bhattacharya *et al.*, 1988; Battacharya *et al.*, 1989; Huber *et al.*, 1989; Diamond and Clark, 1993). Furthermore, amplification of diverse regions of the small subunit of ribosomal RNA gene have been proposed for diagnostic purposes (Katzwinkel *et al.*, 1994, Troll *et al.*, 1997) and validation of antigen detection systems in ELISA format as compared to amplification of the SSU of rDNA have recently been published for cultures and faeces (Mirelman *et al.*, 1997 and Haque *et al.*, 1998).

After the evaluation of DNA derived from reference axenic and polyxenic cultures of *E. histolytica* and *E. dispar*, the *E. histolytica* kit from TechLab, USA (which detects the adherence lectin of the parasite), was 100 times less sensitive than PCR, with a detection limit of 1,000 *E. histolytica* trophozoites/well (Mirelman *et al.*, 1977). When specific nested PCR with restriction digestion of a 0.9 kb region of rSSU DNA (Katzwinkel *et al.*, 1994) was compared to culture of clinical specimens with isoenzyme analysis (Haque *et al.*, 1998), the sensitivity found was 96% (51 of 53 *E. histolytica* cultures), and when the corresponding fresh faecal samples were amplified using the same method, the sensitivity found was 87% (46 of 53 stools). The sensitivity of the *E. histolytica* Techlab kit after testing the same stool samples was similar, detecting 45 out of 53 samples as positive (85%). A further comparison of the Techlab kits with PCR-SHELA in stool samples from the reference laboratory at the University of Leiden reported that the *Entamoeba* kit from TechLab, which does not discriminate between *E. histolytica* and *E. dispar*, was positive in 100% of the *E. histolytica* infections and in 73% of the *E. dispar* infections detected by PCR-SHELA. However, the *E. histolytica* kit detected only 30% of the infections confirmed as *E. histolytica* by PCR-SHELA (Verweij *et al.*, 1998). The possible explanation for the poor sensitivity found in this case could be the effect of storage of samples for long periods of time at -20°C upon the antigen concerned. Interestingly, a

second generation *E. histolytica* specific ELISA by TechLab using the same format as the original test, but with an improved capture antibody, reported an improved sensitivity of 97% (compared with zymodeme analysis) when tested with 62 stool samples from Bangladesh (Eubanks *et al.*, 1998). TechLab's antigen detection tests are user friendly and rapid to perform however, when compared to PCR detection, the latter is more sensitive, detecting infections missed by other methods. It is also capable of specifically demonstrating the presence of *E. dispar* DNA and it can detect mixed infections.

The results of the first epidemiological study for differential diagnosis of *E. histolytica/E. dispar* in stool samples in endemic areas using PCR were published by Acuña-Soto *et al.*, (1993) working in a rural area in Mexico. Because the amplification of a target from faecal samples sometimes produced smears in agarose gels, this study incorporated filter-hybridisation of the PCR products with specific radioactive probes, in which positive reactions were revealed as dots. In this case, the specificity provided by the probes was added to the sensitivity of the PCR technique. However, the use of radioactive probes introduces an additional degree of complexity to the test, which linked to the difficulty for radioactive disposal made this methodology unsuitable in countries such as Colombia. The alternative was to use non-radioactive probes in solution hybridisation instead, with the added advantage that the reaction is more rapid (Wolcott, 1992). It was then possible to standardise the detection using immunoenzymatic assay (PCR-SHELA, Aguirre *et al.*, 1995).

The process of DNA extraction originally used in PCR-SHELA was time consuming and in consequence, it was successfully substituted by a commercially available kit for extraction of DNA. Batches of 10 samples can be extracted in a short time, limiting the risk of cross contamination during manipulation. An early modification to the method for DNA extraction was introduced by Britten *et al.*, (1997) and it was applied in an epidemiological study of *E. histolytica* in Turkey (Jetter *et al.*, 1997). Only *E. dispar* was detected by PCR-SHELA in 15 (20%) out of 79 faecal samples, including 5 samples that were negative by microscopy. Also, ten samples with positive microscopy did not amplify

by PCR. The samples were not cultured and it is not clear if differential diagnosis with *E. hartmanni* was carried out, and because no other PCR method was applied, it is difficult to evaluate if the false negatives according to microscopy are due to lack of sensitivity introduced by the modifications to the original methodology or are samples in which artifacts were mistaken for parasites.

Although the application of PCR for routine diagnosis remains controversial, contamination problems are minimal when adequate controls, and training with adequate materials for sample handling are provided to the relevant staff. This has been demonstrated by the elimination of cross contamination problems at Leiden, London, Barcelona and Bogota during this study. As a result, it was possible to evaluate PCR-SHELA in clinical environments at the reference laboratory level in Leiden and at the LSHTM with samples from travellers. This evaluation evidenced that as a clinical test, with samples that had been selected by their result in microscopy, PCR-SHELA is over 90% sensitive and specific. In contrast, in the pilot study for the evaluation of PCR-SHELA as an epidemiological tool which was carried out in an endemic area in Colombia, the test presented a high number of false negative samples which had been classified as positive according to microscopy. Although some of these results were probably due to incorrect diagnosis by microscopy, changes in the methodology in order to increase sensitivity were needed, for which an increment in the amount of sample lysate has now been introduced in the protocol for DNA extraction. Additionally, an increment in the amount of sample added to the PCR reactions was also suggested. These modifications are being tested in the field at the moment and their effect on the sensitivity of the test will be known in the near future.

In future studies, because of the low prevalence of *E. histolytica* in populations of healthy carriers and because of the subjectivity of microscopy for discrimination of morphological characteristics in samples with cysts and trophozoites of amoebas, negative results with PCR-SHELA will be confirmed by a different PCR system to eliminate the possibility of misdiagnosis by microscopy. Additionally, a new PCR system for the specific

amplification of the rSSU of *E. hartmanni* will be standardised in order to test all samples. This will explain some cases of false negative results for *E. histolytica/E. dispar* and also establish the true prevalence of the organism.

On the policy front, frequent updates on the morphological identification of parasitic infections by microscopy given to the relevant staff in diagnostic laboratories should be established as a measure for the improvement of the quality control in developing countries (Guhl *et al.*, 1996). This is necessary because microscopy is the most widely available tool for screening during diagnosis of intestinal parasitic infections, and because of its utility it should not be discarded (Gonzalez-Ruiz and Bendall, 1995).

GENERAL DISCUSSION AND PERSPECTIVES

Entamoeba histolytica and *Entamoeba dispar* although closely related and with identical morphology under light microscopy, have been separated in two different species (Clark and Diamond, 1991; Diamond and Clark, 1993). This separation has multiple implications, on one hand the study of the biology of *E. dispar* as an independent organism and its comparison to the homologous processes known in *E. histolytica* may provide a better understanding on the processes involved in the different levels of virulence associated to isolates of the latter organism. On the other hand, it is necessary to establish routine differential diagnostic methodologies for use in non-endemic and in endemic areas, where a differential test that permits the processing of a great number of samples in a short space of time will, in turn, contribute to the collection of new accurate data for epidemiological purposes.

The identification of index cases (symptomatic individuals) and their close contacts, particularly in travellers returning from tropical areas is important for the prevention of outbreaks of invasive disease in developed countries. Differentiation is also necessary for diagnosis by exclusion of, for example, liver or bowel carcinomas, or when considering immunosuppressive treatment for Crohn's disease or irritable bowel syndrome. However, for differentiation it is critical to provide clinicians with reliable tests to not only identify patients harbouring *E. histolytica* infections but also for those carrying *E. dispar*, which will avoid unnecessary treatment. Accordingly, WHO/PAHO (1997) has recommended that treatment should be provided only in cases where *E. histolytica* infection is proven or strongly suspected after exclusion of other entities with similar symptomatology (WHO/PAHO, 1997). These recommendations were drawn after it was shown that the risk of developing invasive disease for *E. dispar* carriers is very low (Ruiz-Palacios *et al.*, 1992; Garduño-Espinosa *et al.*, 1997). This fact, linked to the undesirable side effects of the treatment, and to the possibility that resistance may develop due to drug pressure, are convincing arguments in favour of this policy. However, widespread

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automedication with metronidazole is common in endemic areas such as Colombia and although metronidazole is effective against symptoms and tissue forms, luminal amoebae are less affected, generating transient healthy carriers of invasive strains, which are responsible for infection of new cases including small outbreaks of invasive disease mentioned above (Visser *et al.*, 1998).

The need for a test able to distinguish between *Entamoeba histolytica* and *Entamoeba dispar* infections in stool samples of symptomatic and asymptomatic individuals in Colombia originated the work described in this thesis. The starting point however, was the data on the prevalence of *E. histolytica/dispar* available then, which were based on the microscopy of stool samples, in which for example, very few studies had detected *E. hartmanni*. Wide variation in prevalence of intestinal *E. histolytica/dispar* infection was reported, and no reliable data were available for reference purposes. Nevertheless, the presence of high background antibody titres to *E. histolytica* in the population, and the occurrence of amoebic abscesses indicated that invasive strains were circulating in the population throughout the country. Thus, evaluating and later adapting systems to identify not only symptomatic individuals but also the carriers of *E. histolytica*, became one of the priorities for research in the field in Colombia. Culture of stool samples and serology (in volunteers) were added to the routine diagnosis by microscopy at the reference laboratory, and as standard methodology for field collection.

Chapter three refers to the results after the analysis of a small section of the population at Ibagu e and La Mesa using microscopy and culture. The endemicity of intestinal parasites and amoebal infections in particular was confirmed. The prevalence of *E. histolytica/E. dispar* was found to be 26% at Ibagu e and 17.4% at La Mesa. After culture, only the presence of *E. dispar* strains was proven, despite previous evidence of invasive episodes in the population at Ibagu e. The low rate of survival (10%) of the cultures from the field work at Ibagu e was linked to the possibility that most of these cultures contained *E. dispar* or other amoeba species, although the loss of these cultures could have been also due to their susceptibility to changes in the environment, specially the temperature fluctuations during their transportation to the reference laboratory in Bogota. The efficiency

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of the culture technique was improved at La Mesa (58%), although no invasive strains were isolated either, as confirmed by isoenzyme analysis and immunofluorescence with MAb 20/7D. The possibility for the evaluation of the potential invasiveness of cultured isolates with MAb 20/7D was a considerable step forward for strain characterisation, thanks to its extremely high sensitivity and specificity when used in IFAT (Gonzalez-Ruiz *et al.*, 1992). However, there were no amoebic dysentery cases among the individuals tested during the field work, although the serological data indicated that a small percentage of the population tested at Ibaguè had been or were currently infected with *E. histolytica*. Detection of *E. histolytica* directly in the faecal specimens was needed.

The MAb 20/7D had been adapted into a capture ELISA format (FAC-ELISA) and it produced encouraging results when testing cases of amoebic dysentery and colitis (Gonzalez *et al.*, 1994). However, as discussed in Chapter 4, the low titre of the MAb 20/7D produced made it necessary to reclone the original hybridoma. A clone with high titre was obtained, although the antibody product of this clone, as did the original, also reacted with an homologous protein in *E. dispar* by western blot under non-reducing conditions. Similar reactions were observed in several studies using other MAbs (Petri *et al.*, 1990; Walderich *et al.*, 1998). Also, although *E. histolytica* and *E. dispar* are different enough to be classified as separate species (Clark, 1998), there are considerable antigenic similarities between them. This could explain the reaction of MAb 20/7D with proteins in lysates of both parasites. Further study of the interaction of the MAb 20/7D with lysates of *E. histolytica* and *E. dispar* revealed an affinity of approximately one order of magnitude greater toward *E. histolytica*. It was concluded that this small difference would limit the use of the FAC-ELISA in endemic areas due to the presence of variable concentrations of soluble antigen of either species in the faecal samples, which could lead to identification of heavy *E. dispar* infection as *E. histolytica*.

Whereas it was decided to continue to use MAb 20/7D to characterise cultured isolates by IFAT and to keep the original format of the FAC-ELISA test for detection of amoebic dysentery and colitis, its adaptation as a routine tool for soluble antigen detection in asymptomatic individuals was abandoned. Chapter 5 refers to the alternative approach

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followed: Differentiation at the nucleic acid level, where clearly distinct sequences of *E. histolytica* and *E. dispar* had been recognised. Successful PCR amplification of the 145bp and 133 bp specific repeats located between the ribosomal DNA sequences of *E. histolytica* and *E. dispar* respectively was achieved using DNA obtained from faecal samples, cultures and body fluids (Aguirre *et al.*, 1997). Samples can be sent unpreserved by post (Verweij *et al.*, submitted) or kept frozen for long periods of time to be transported to the reference laboratory. Alternatively, DNA can be extracted in the field on collection of the sample, since this process has been facilitated by the introduction of a commercially available kit, which also reduces the possibility of cross contamination between samples. After PCR, the ELISA format of PCR-SHELA allows the analysis of results according to clear-cut colorimetric differentiation, and with high sensitivity and specificity.

The presence of “false positive or negative” results when evaluating PCR-SHELA against microscopy highlights the unreliability of the diagnosis based on the morphology of the organisms found. Cysts of other species of amoebas with similar features, cells and debris can be misidentified as *E. histolytica/dispar* even by experienced microscopists. Also, the lability of the morphological characteristics of trophozoites contributes to the high amount of false negative results by microscopy in samples examined at the reference laboratory level. However, false negative results on samples from the field could also be the consequence of sequence variation in the intergenic spacers of *E. histolytica* and *E. dispar* amplified for PCR-SHELA. This possibility has to be considered in view of the restriction length polymorphisms found in the *Dra*I and *Hin*fl repeats of the episomal DNA of *E. histolytica* (Bhattacharya and Bhattacharya, 1998). For these cases, amplification of a different target such as the DNA sequences for the small subunit of the ribosomes (SSU) might help to clarify results. The further analysis on the variability of these sequences will be useful for the interpretation of epidemiological data and genetic characterisation of *E. histolytica* and could be important in determining prognosis in cases of invasive disease (Tang *et al.*, 1997, Whelen and Persing, 1996). For instance, the parallel application of two or more PCR techniques for intestinal *E. histolytica* infections could be used as confirmatory tests in areas where high prevalence of mixed infections has been reported such as Mexico and Bangladesh (Acuña-Soto *et al.*, 1993; Newton *et al.*, 1997; Haque *et*

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al., 1998). These observations contrast with the results obtained using PCR for amplification of SSU rDNA and PCR-SHELA in samples from an endemic area in Colombia and in samples from travellers returning to Barcelona and Leiden.

The prevalence of *E. histolytica* infections detected by PCR-SHELA in the samples collected at Quibdo in Choco (Colombia) was 1.2% or 5 positives from 40 *E. histolytica/dispar* microscopy positives in 427 samples. According to the latest estimates from the World Bank, the population of the country for mid-1997 was 38.1 million, 24% of them (approx. 9.1 million people) did not have access to safe water. Assuming a baseline prevalence of 1.2%, approximately 457,200 people in the country could be at risk of developing invasive disease. Surveys aiming for the specific identification and treatment of these individuals will probably have a major impact on the control of the transmission of *E. histolytica* in Colombia.

The objective of this thesis was the evaluation and adaptation of tools for the identification and differentiation of *E. histolytica* and *E. dispar* in faecal samples from symptomatic and asymptomatic individuals. PCR-SHELA has been validated as such a tool and differentiation is used as an addition to microscopy at Leiden, Barcelona and Bogota at the reference laboratory level. The problem of costs and complexity of PCR and ELISA as limiting factors for their application in developing countries is being slowly overcome. The local production of reagents has improved the availability of primers and *Taq* polymerase in Colombia, and adequate laboratory facilities for procedures involving molecular techniques are being established in endemic regions, as it is the case at the Universidad del Tolima in Ibague. Hence, the application of PCR-SHELA for diagnostic and epidemiologic purposes at Ibague and surrounding areas will be carried out locally, providing an accessible reference laboratory for confirmation in cases of suspected amoebic infection or when cysts and/or trophozoites are detected by microscopy.

Clarification of the distribution of *E. histolytica* and *E. dispar* infections in the world is on its way, meanwhile, many questions remain to be answered in amoebiasis: Which are the transmission dynamics of *E. histolytica* and *E. dispar*?, Are mixed infections

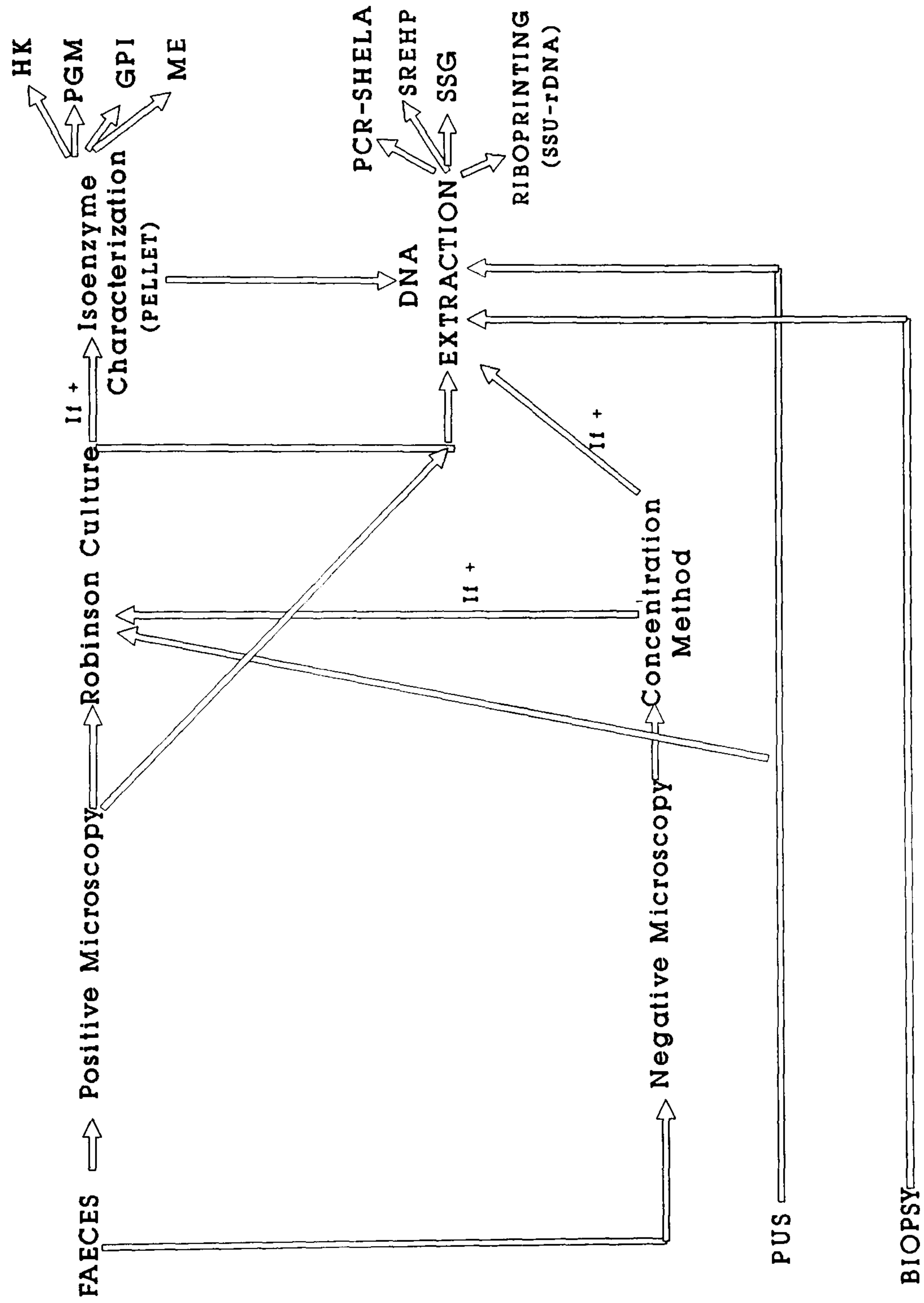
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a common feature in some endemic areas only?, Does *E. dispar* carriage protect against *E. histolytica* infection?, Does the degree of virulence or type of pathogenesis vary between strains of *E. histolytica*? What is the relationship between parasite density and disease?, Does prior exposure to *E. histolytica* infection favour the “asymptomatic cyst passer” state? Is there acquired immunity to infection or to type of pathology?, Does drug pressure by generalised use of commonly available remedies in endemic areas favour resistance to treatment in *E. histolytica*?

It is possible that the creation of DNA banks from well characterised sources and from wide geographic origins and the setting up of multicentre studies using standardised methodologies will help to find answers to some of the above questions. The continuation of the collaborative work described in this thesis involves the further characterisation of *E. histolytica* and *E. dispar* DNAs. Ribotyping (Clark and Diamond, 1992) of isolates from Colombia, The Netherlands, Spain and England will be pursued, in addition to the detection of strain polymorphisms by amplification of the serine-rich antigen gene (SREHP) (Stanley *et al.*, 1990) and of the strain specific gene (SSG) (Burch *et al.*, 1991) in order to incorporate species-specific and strain-specific molecular markers into the study (Figure 6.1, page 211). Also, a database on travellers returning from the tropics to the centres participating in the study has been initiated, aiming to collect data with epidemiologic purposes.

The analyses of results gathered using traditional and novel diagnostic tools are expected to help provide guidelines for the effective treatment and control of invasive amoebiasis in places where the lack of proper sanitation and limited access to clean water make control much more difficult than in developed areas.

FIGURE 6.1 PROTOCOL FOR CHARACTERISATION OF SAMPLES CONTAINING *E. histolytica* AND *E. dispar*



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APPENDIX 1

REAGENTS, CULTURES AND BUFFERS

A. SOLUTIONS FOR COPROLOGY

Iodine Stock Solution

Iodine	4g
Potassium iodide	8g
Distilled water	200ml

The iodine was added to the potassium iodide solution. Stored at room temperature in a brown bottle.

Iodine working solution

25% v/v Acetic acid	10ml
Lugol's Iodine	10ml

Isotonic Saline (0.85% w/v)

NaCl	0.85g
Distilled Water	100ml

Store at room temperature.

Zinc Sulphate Solution (33% w/v)

Zinc Sulphate	165g
Distilled water	500ml

Dissolved in a hot water bath, mixing until completely dissolved. Density was measured with a hydrometer until correct density of 1.180 was reached. Stored at room temperature.

Stock Schaudinn's Fixative

Mercuric chloride	35g
Ethanol (95% w/v)	250ml
Distilled water	500ml

Mercuric chloride was dissolved in water using a hot water bath, then the solution was cooled and decanted and measured. Ethanol was added in a proportion of 1 part to two parts of mercuric chloride solution.

Working Solution

Stock Schaudinn's solution	95ml
Glacial acetic acid	5ml

Trichrome Stain

Chromotrope 2R	0.6g
Light Green SF	0.15g
Fast Green	0.15g
Phosphotungstic acid	0.7g
Glacial acetic acid	1ml
Distilled water	100ml

The staining components were mixed with the acetic acid before adding the water. Stored at room temperature.

B. ISOLATION OF *ENTAMOEBAS* SPP IN POLYXENIC CULTURE MEDIA

ROBINSON'S CULTURE MEDIUM (Robinson, 1968)

Saline Agar Slopes

Agar base	1.5g
NaCl	0.7g
dH ₂ O	100ml

the agar and the NaCl were dissolved in the water and dispensed in 2.5 ml aliquots in bijoux bottles. Autoclaved at 121 °C and 15 lb of pressure for 15 minutes. Sloped and stored at room temperature.

Erythromycin Solution

2g of Erythromycin base (Sigma E-5389) were suspended in 10 ml of ETOH (20% w/v) in a sterile bottle, after 2 hours the stock was diluted 40X to 0.5% with sterile distilled water (adding ETOH to water dropwise). Stored at 4°C.

Bactopeptone 20% Stock Solution

20g Bactopeptone dissolved in 100 ml of water. Distributed in aliquots and autoclaved. Stored at 4°C.

Phthalate 0.5M Stock Solution

204g Potassium phthalate (Phthalic acid Sigma P-6758) were dissolved in 100 ml of 40% NaOH. Made up to 2 litres and pH was adjusted to 6.3. Dispensed in universal bottles (20 ml aliquots) and autoclaved. Stored at room temperature. A 1/10 dilution with sterile distilled water was used as working dilution.

R Stock Medium

NaCl	125 g
Citric Acid	50 g
KH ₂ PO ₄	12.5g
Ammonium sulphate	25 g
Magnesium sulphate.7H ₂ O	1.25 g
Lactic acid(90%)	100 ml

Made up to 2.5l with distilled water.

For the working solution, 100 ml of stock were diluted with 7.5 ml of 40% NaOH and 2.5ml of a 0.04% bromothymol blue solution and made up to 1 litre with distilled water. Adjusted to pH 7.5, and autoclaved.

BR Culture Medium

Escherichia coli strain B was grown in 0.5% bactopeptone and added to 1l of R, incubated at 37°C for 48 hours or overnight. Stored at room temperature up to 2 months. BR was used to set primary cultures up.

BRS Medium

Equal volumes of bovine serum Sigma B-2771 (heat inactivated at 56°C for 30 minutes) were added to the bottles of BR and incubated at 37°C for 4 hours. Stored at 4°C.

BRS-Phthalate Medium

Equal volumes of phthalate working solution and BRS were mixed and kept at 4°C ready to use in secondary cultures and further subcultures.

B. METHODS

Primary Culture

A bijoux bottle containing the agar slope and BR to about 2/3 of the bottle was inoculated with a small piece of faeces, 4 drops of erythromycin solution and approximately 10 mg of rice starch (BDH Chemicals Ltd. No.30263). Incubated at 37°C for 24 hours.

Secondary Culture

After 24 hours, the supernatant of the culture was discarded (without disturbing the starch-faecal layer) and replaced by BRS-phthalate to 2/3 of the bottle, two drops of 20% bactopectone, two drops of 0.5% erythromycin and more starch. Incubated for further 24 hours at 37°C.

Reading of Cultures

A wet preparation of the starch-faecal layer and Lugol's Iodine was examined for the presence of trophozoites after 48 hours of inoculation of the sample. Further readings were made every 48 hours during one week for negative cultures before discarding them.

Subcultures

Subcultures from positive Robinson's were made every 48 hours to a fresh bijoux slope using secondary culture medium. Amplification of cultures for further assays such as DNA extraction and isoenzyme lysates were carried out in 25 cm³ plastic flasks in liquid secondary Robinson's medium.

Liquid Robinson's Medium

BRS	100 ml
K-phthalate 0.5M Stock	3 ml
Erythromycine 0.5%	7.5ml
Bactopeptone 20%	7.5 ml

The volume was made up to 400 ml with steril distilled water. Distributed in 25cm³ falcon flasks or 12 ml flat-bottomed tubes and starch layers from positive bijoux bottles were added. Incubated at 37°C for 48 hours and examined under the inverted microscope.

C. CULTURE MEDIUM FOR AXENIC CULTURE OF REFERENCE STRAINS

TYI-S-33 Axenic Culture Medium

TYI Broth

The following ingredients were dissolved in 500 ml of distilled water and brought to 870 ml:

Trypticase (BBL)	20g
Yeast Extract(BBL/Difco)	10g
Glucose	10g
NaCl	2g
K ₂ HPO ₄	1g
KH ₂ PO ₄	0.6g
L-Cysteine HCl(Sigma C-1276)	1g
L-Ascorbic acid	0.2g
Ferric Ammonium Citrate(Sigma F-5879)	2.28mg

pH was adjusted to 6.8 with 1N NaOH, clarified by filtering by Whatman No.1 filter paper and autoclaved for 15 min. at 121°C and 15 lb. Cool at room temperature and store up to 3 months in the freezer.

Vitamin Mixture Stocks

Solution A: Vitamin mixture NCTC 107

1. Water soluble B vitamins

A:

Niacin Sigma (N-4126)	62.5mg
PABA Sigma (A-3659)	125mg

Dissolved in boiling water and brought to a final volume of 150 ml.

B:

Niacinamide Sigma (N-0636)	62.5mg
Pyridoxine HCl Sigma (P-6280)	62.5mg
Pyridoxal HCl Sigma (P-6155)	62.5mg
Thiamine HCL Sigma (T-1270)	25 mg
Ca Pantothenate Sigma (P-5155)	25mg
i-Inositol Sigma (I-7508)	125mg
Choline Chloride Sigma (C-7527)	1.25g

Dissolve in distilled water and brought to a final volume of 150 ml.

C:

Riboflavin Sigma (R-9504)	25mg
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Added to 75 ml of distilled water and dissolved with drops of 0.1N NaOH (until solution became transparent). Brought to a final volume of 100 ml.

Combined A, B and C were brought to a final volume of 500 ml with distilled water.

2. d-Biotin Sigma B-4501	30mg
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Dissolved in 200 ml distilled water with the aid of 0.1N NaOH

3. Folic Acid Sigma F-7876	30mg
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Dissolved in 300 ml of distilled water.

4. ADK Lipid Soluble Vitamins

a. Calciferol Sigma E-5750	300mg
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Vitamin A alcohol (Retinol)Sigma R-7632	300mg
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Appendix 1 Reagents, Cultures and Buffers

Dissolved in 63 ml of 95% ETOH

b. Menadione sodium bisulfite Sigma M-5750 60mg

Dissolved in 300 ml of 5% aqueous Tween 80

Combined a and b and brought to 3000ml with distilled water

5. α -tocopherol acetate Sigma T-3001 25mg

Dissolved in 250 ml distilled water.

Vitamin Mixture 107 was made up as follows:

B-vitamins	500ml
Biotin	250ml
Folic acid	250ml
ADK vitamins	2500ml
Vitamin E	250ml

Filter sterilised as a single volume of 3750 ml. Stored in the freezer wrapped in foil.

Solution B

Vitamin B₁₂ Sigma(V-2876) 40mg

Dissolved in 100 ml of distilled water

Solution C

DL-6,8 Thioctic acid Sigma (T-1395) 100mg

Dissolved in absolute ETOH and brought to a final volume of 100 ml

Solution D

Tween 80 Sigma (P-1754) 50g

Dissolved in ETOH and brought to a final volume of 100 ml with distilled water.

Working solution:

500 ml of A
6 ml of B
2 ml of C
2 ml of D

were combined and after addition of 90 ml of distilled water, filter sterilised through 0.2 µm filters. Dispensed in 15 ml aliquots and stored at -20°C in the dark.

Bovine Serum

Inactivated at 56°C for 30 min. before use. Kept at -20°C.

Antibiotic Mixture:

Streptomycin Sigma (S-9137)	200mg/ml
Ampicillin Sigma (A-0166)	100mg/ml

Complete Medium

500 ml of complete medium were prepared by addition of:

Vitamin-Tween 80 mixture	15ml
Bovine Serum	50ml
TYI Broth	435ml
Antibiotic mixture	1.0ml

Stored in the fridge in the dark for up to ten days. 12 ml flat-sided plastic test tubes (Nunc Cat. No.1-56758) and standard 25cm³ flat bottomed Falcon flasks were used for culture of trophozoites.

Subcultures

Once monolayers of trophozoites were observed under the inverted microscope, subculture was carried out by first releasing the growing amoebas from the donor tube by chilling on ice for about 20 minutes. Then, centrifugation at 700g for 10 minutes, keeping only 1 ml of the supernatant and the corresponding pellet. Fresh cultures in plastic tubes were inoculated with 0.1 to 0.2 ml of the suspension which contained at least 5000 organisms per ml. About 40 ml of fresh complete medium were used in flasks. Medium was added to the vessels to fill them completely.

MRC Ringer for Cryopreservation

NaCl	9 g
KCl	0.2 g
CaCl ₂	0.2 g
H ₂ O	1000 ml

D. BUFFERS FOR ISOENZYME ANALYSIS

200 mM Stock Stabilising Buffer: Parasite extracts for isoenzymes

Dithiothreitol (DTT) Sigma D-5545	74mg
Σ-amino-n-caproic acid Sigma A-7824	26mg
Na ₂ -EDTA pH 7.0	31mg
Distilled water	1ml

The water was first added to the EDTA and then the other inhibitors. Stored at 4°C for up to two weeks.

Working Solution

The stock solution was diluted to 2 mM in distilled water immediately before use. An equal volume was added to the volume of the pellet to be lysed.

Tank buffer Tris Maleate 0.1M for PGM and HK

Tris HCl	12.14g
Maleic acid	11.61g
MgCl ₂ .6H ₂ O	2.03g
EDTA	3.73g
NaOH	4.872g

Brought to 1l with distilled water. pH was adjusted to 7.4. A 3:20 dilution (0.015M) of the tank buffer was used for the corresponding gel buffers.

Tank buffer Phosphate 0.2M for ME and GPI

NaH ₂ PO ₄ .2H ₂ O	12.15g
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Appendix 1 Reagents, Cultures and Buffers

Na₂HPO₄ anhydrous

17.4g

Diluted to 1l with distilled water. pH adjusted to 7.0 and a 3:40 dilution (0.015M) was used for the corresponding gels

Isoenzyme Developer Solutions

PGM

NADP

10mg/ml (use 0.5ml)

MTT

30mg/ml ETOH (use 1ml)

PMS

10mg/10ml (use 1ml)

MgCl₂

0.1M (use 2ml)

G6PD

1iu/μl (use 5μl)

G1P

30.4mg/ml (use 1ml)

Agar

15mg/ml (use 10ml)

Tris HCl pH 8.0

0.3M (use 10ml)

Prepared immediately before use in a foil covered container.

HK

NADP

10mg/ml (use 2ml)

Glucose

1g/10ml (use 1ml)

MTT

30mg/ml ETOH (use 1ml)

PMS

10mg/10ml (use 1ml)

ATP

360mg/ml (use 1 ml)

MgCl₂

0.1M (use 2ml)

G6PD

1iu/μl (use 5μl)

Agar

15mg/ml (use 10ml)

Tris HCl pH 7.4

0.3M (use 10ml)

Prepared immediately before use in a foil covered container.

ME

NADP

10mg/ml (use 0.5ml)

MTT

30mg/ml ETOH (use 1ml)

PMS

10mg/10ml (use 1ml)

Na Malate

22.3mg/ml (use 1ml)

MgCl₂

0.1M (use 2ml)

Agar

15mg/ml (use 10ml)

Appendix 1 Reagents, Cultures and Buffers

Tris HCl pH 7.4 0.3M (use 10ml)

Prepared immediately before use in a foil covered container.

GPI

NADP	10mg/ml (use 0.5ml)
MTT	30mg/ml ETOH (use 1ml)
PMS	10mg/10ml (use 1ml)
MgCl ₂	0.1M (use 2ml)
F6P	6mg/ml (use 0.5ml)
G6PD	1iu/ μ l (use 5 μ l)
Agar	15mg/ml (use 10ml)
Tris HCl pH 8.0	0.3M (use 10ml)

Prepared immediately before use in a foil covered container.

E. BUFFERS AND SOLUTIONS FOR SDS-PAGE

Stock Acrylamide (30%) Sigma A-4058

Acrylamide (28.6%)	71.5g
N,N'-Methylene-bis-acrylamide(1.4%) Sigma M-725	63.5g
Distilled water to	250ml

10X Resolving Buffer pH 8.8

1M HCL	48ml
Tris Base	36.3g
Distilled water to	100ml

8X stacking Buffer pH 6.8

1M Orthophosphoric Acid	26.6ml
Tris Base	5.7g
Distilled water to	100ml

Appendix 1 Reagents, Cultures and Buffers

Ammonium Persulphate (APS) Solution

APS	0.1g
Distilled water to	1ml

Electrode Buffer pH 8.7-8.9

Tris Base	8g
Glycine	28.8g
Sodium Dodecyl Sulphate	1g
Distilled water to	1000ml

5X Sample Buffer (without 2-Mercaptoethanol)

Sodium Dodecyl Sulphate	1g
8X Stacking Buffer	6.25ml
Distilled water to	10ml

8% Resolving Gel

30% Acrylamide Stock	3.2 ml
10X Resolving Gel Buffer	1.25 ml
Distilled Water	7.15
Amonium Persulfate Solution (APS, 10% w/v)	50 μ l
TEMED (N,N,N',N'-tetramethylethylenediamine)	5 μ l

Stacking Gel

30% Acrylamide Stock	0.675 ml
5X stacking Gel Buffer	0.75 ml
Distilled water	4.58
APS	15 μ l
TEMED	15 μ l

Bromophenol Blue

Bromophenol Blue	0.05g
50% v/v Glycerol in water	20ml

Appendix 1 Reagents, Cultures and Buffers

Molecular Weight Markers	(kDa)
Myosin (Sigma C-3889)	200
Phosphorylase B (Sigma P-4649)	97.4
Bovine Albumin (Sigma A-7517)	66
Albumin (A-7642)	45
Carbonic Anhydrase (C-2273)	29

Gel Fixative

Methanol	200 ml
Acetic Acid	70ml
Distilled water	200ml

Gel Staining Solution

Coomassie Brilliant Blue R250	0.625g
Gel Fixative	250ml

Gel Destaining Solution

Methanol	40ml
Acetic Acid	60ml
Distilled water	700ml

F. BUFFERS AND SOLUTIONS FOR WESTERN BLOTTING

10X Blotting Buffer pH 9.2

Tris Base	0.48M
Glycine	0.39M
Distilled water to	1000ml

1X Blotting Buffer (Working Solution)

10X Blotting buffer	20ml
Methanol	40ml
Distilled water	160ml

Blocking Solution

Marvel fat-free milk (Cadburys, UK)	3g
Sodium Azide	0.02g
PBS to	100ml

Antibody Diluent

0.05% Tween 20/Blocking Solution

Substrate Solution

3,3' diaminobenzidine HCL (DAB)*	10mg
4-Chloro-1-Naphthol (CN)	30mg
30% Hydrogen Peroxide	10 μ l
PBS	40 ml

*Dissolve the mixture DAB/CN in 5ml methanol prior to PBS

G. BUFFERS AND SOLUTIONS FOR ELISA

10X Coating Buffer

Sodium Carbonate (0.15M)	7.95g
Sodium Bicarbonate(0.35M)	14.65g
Distilled water to	500ml

20X Phosphate Buffered Saline (PBS)

NaCl(2.74M)	800g
KH ₂ PO ₄ (0.03M)	20g
Na ₂ HPO ₄ (0.016M)	115g
KCl(0.054M)	20g
Distilled water to	5000ml

pH was adjusted to 7.2.

Washing Solution (PBS/T)

1X PBS pH 7.2-7.4 containing 0.05% Tween 20

Blocking Solution

1X PBS pH 7.2-7.4 containing 0.5% casein for serology

1X PBS pH 7.2-7.4 containing 1% gelatin for monoclonal antibody ELISAS

Antibody Diluting Solution

PBS/T containing 0.25% w/v gelatin (PBS/T/G) for ELISA

PBS/T/G/50% Foetal Calf Serum (PBS/T/G/FCS) for FAC-ELISA

Stock Substrate Buffer

Citric Acid	0.1M
Na ₂ HPO ₄	0.2M

OPD Substrate Solution

Stock Citric Acid Solution	25ml
Stock Na ₂ HPO ₄ Solution	25ml
Distilled water	50ml
o-Phenylene Diamine (OPD P-1526 Sigma, Uk)	40mg
30% Hydrogen Peroxide (H-1009 Sigma, Uk)	40µl

Stopping Solution

HCL 2.5 M	50 µl/well
H ₂ SO ₄ 2.5 M	50 µl/well

H. MEDIA AND SOLUTIONS FOR MONOCLONAL ANTIBODIES

RPMI (Rosewell Park Memorial Institute) medium (Gibco) was made to 1X containing 2g/l of sodium bicarbonate, adjusted to pH 7.3 and filter sterilized through a 0.2µm membrane (Millipore, UK). 500 ml bottles were stored at 4°C.

100X PGAB Solution

Pyruvic Acid (Sigma P-5280)	2.2g
L-Glutamine (Sigma G-6392)	5.84g
Penicillin G (Sigma P-3032)	4x10 ⁶ iu
Streptomycin Sulphate (Sigma S-9137)	4g
Distilled water to	200 ml

Filtered through a 0.2 µm membrane and dispensed in 5 ml aliquots stored at -20°C.

Cell Growth Medium

RPMI Medium	100ml
PGAB	1ml
Foetal Calf Serum (FCS) Sigma F-4135	20ml

I. BUFFERS FOR IMMUNOAFFINITY PURIFICATION

TNE Buffer (Loading Buffer)

Tris/HCl pH 7.4	20mM
NaCl	0.3M
EDTA	0.15M

This buffer was used to dilute and load antibodies and proteins through affinity columns overnight.

Coupling buffer

NaHCO ₃	0.1M
NaCl	0.5M
pH 8.5-9.0	

Blocking Buffer

Na ₂ HPO ₄	0.1M
Glycine	0.2M
pH 8.0	

Washing Buffer (pH oscillation)

Acetate Buffer	0.1M
0.5M NaCl	0.5M
pH 4.0	

Alternating with coupling buffer

Elution Buffer

Glycine/HCl	0.1M
pH 2.5	

Diethylamine (DEA) Buffer

Diethylamine	0.02M
TNE Buffer	50 ml

104 μ l of a 9.58 M stock are added to 50 ml of TNE. Adjusted to pH 11.0 for elution of *E. histolytica* and *E. dispar* 20/7D proteins from columns.

Protease Inhibitors

	Stock:	Use:
Chymostatin (C-7268 Sigma, UK)	5mg/ml DMSO	10 μ l
Antipain (A-6191 Sigma, UK)	5mg/ml Water	10 μ l
Pepstatin A (P-4265 Sigma, UK)	5mg/ml Methanol	10 μ l
PMSF (P-7626 Sigma, UK)	100 mM Ethanol	0.5 ml
Bestatin (B-8385 Sigma, UK)	1mg/ml Methanol	10 μ l
TLCK (T-7254 Sigma, UK)	12.5mg/ml Water	40 μ l
EDTA	10 μ g/ml Water	10 μ l
E64 (E-8640 Sigma, UK)	1mM Water	20 μ l

Added to 50 ml of TNE Buffer.

J. BUFFERS AND SOLUTIONS FOR DNA

Lysis Buffer for DNA Extraction (for phenol Chloroform method)

Stocks (100 ml of each):

NaCl	1M
EDTA	0.5M
SDS	10 g
Tris/HCl pH 8.0	1M

Working Solution (500 ml):

		Stock
NaCl	50 mM	25 ml
EDTA	50 mM	50 ml
SDS	1%	50 ml
Tris pH 8.0	50 mM	25 ml
Distilled water		350 ml

Samples were diluted 1:1 v/v with lysis buffer.

Proteinase K Buffer:

0.01M Tris pH 7.8
0.005M EDTA
0.5% SDS

10 mg of Proteinase K are added per ml of buffer.

Sodium Acetate Solution

3M Sodium Acetate pH 5.2

DNA Sample Buffer For Agarose Gel Electrophoresis

6X stock:

0.25% (v/v) bromophenol blue
0.25% (v/v) xylene cyanol FF
30% (v/v) glycerol

TAE Buffer:

0.04 M Tris-acetate
0.001 EDTA

TBE Buffer (10X) Electrophoresis of DNA

90mM Tris base
90mM Boric acid
2 mM EDTA
pH 8.0

TE Buffer to dissolve DNA

10mM Tris HCl pH 7.2
1mM EDTA

Low TE Buffer for preparation of G-50 sephadex columns

100 ml TE buffer pH 7.4
900 ml Distilled water

Denhardt's Solution for Blot prehybridization and hybridization

100X stock:
10 g Ficoll type 400
10 g Polyvinylpyrrolidone
10 g Bovine Serum Albumin (BSA) Fraction V
Distilled water to 500 ml

Filtered through a Nalgene filter, stored at -20°C

20X SSC Buffer

3M NaCl
0.3M Sodium citrate
pH 7.0

Prehybridisation Solution for Nylon membranes

50% (V/V) formamide
5X Denhardt's solution
5X SSC
0.5 mg/ml salmon sperm DNA (Sheared and denatured)

Hybridisation Buffer for nucleic acids to membranes

50% (v/v) formamide
5X SSC
1X Denhardt's Solution
0.1% (w/v) SDS
100 μ g/ml Salmon Sperm DNA (Denatured and sheared)

Denaturing Buffer (South 1) for DNA transfer to membranes

1.5M NaCl
0.5M NaOH
pH 14.0

Neutralising Buffer (South 2) DNA transfer to membranes

1M Tris HCl pH 7.2
1.5M NaCl

APPENDIX 2

Aguirre *et al.*, 1995. Polymerase Chain Reaction-Solution Hybridization Enzyme-Linked Immunoassay (PCR-SHELA) for the Differential Diagnosis of Pathogenic and Non-pathogenic *Entamoeba histolytica*.

APPENDIX 3

Aguirre *et al.*, 1997. Diagnosis of *Entamoeba histolytica* and *Entamoeba dispar* in Clinical Specimens by PCR-SHELA.



Short Report

Polymerase chain reaction-solution hybridization enzyme-linked immunoassay (PCR-SHELA) for the differential diagnosis of pathogenic and non-pathogenic *Entamoeba histolytica*

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Keywords: *Entamoeba histolytica*, polymerase chain reaction, enzyme-linked immunosorbent assay, differential diagnosis

This preliminary report describes the adaptation and simplification of recently described polymerase chain reaction (PCR) procedures (ROMERO *et al.*, 1992; ACUÑA-SOTO *et al.*, 1993) to enable the differential diagnosis of pathogenic and non-pathogenic *Entamoeba histolytica*. We have used the system described by WILSON *et al.* (1993) and QIAO *et al.* (in press), in which products are detected using a colorimetric method based on an enzyme-linked immunosorbent assay (ELISA). This enables the PCR results to be read either by spectrophotometry or, more importantly, directly by eye, and obviates the need for electrophoresis, ultra-violet light or photographic equipment.

Two reference strains of *E. histolytica*, HMI-IMSS (a pathogenic zymodeme) and SAW 1734 (a non-pathogenic zymodeme), 3 non-pathogenic isolates in Robinson's medium, and pus aspirated from a hepatic amoebic abscess were examined. Seven additional faecal samples (from the Hospital Pedro Leon Alvarez, La Mesa, Cundinamarca, Colombia), in which intestinal parasites had been detected by microscopy, were also examined.

Approximately 1 g of each faecal sample was suspended in phosphate-buffered saline, sieved (425 µm mesh) to eliminate large particles, and frozen/thawed 3 times with dry ice-ethanol/water at room temperature. The mixture was then centrifuged (1 min, 2500 g) and the supernatants were diluted 1:1 in lysis buffer (50 mM NaCl, 50 mM ethylenediaminetetraacetic acid, 1% sodium dodecyl sulphate and 50 mM Tris-HCl, pH 8.0) containing 100 µg/mL of proteinase K. Overnight incubation at 37°C was followed by extraction using the standard phenol-chloroform method and ethanol precipitation.

Specific primer pairs (P1/P2 [5'...DIG-TCA AAA TGG TCG TCG TCT AGG C...3'/5'...CAG TTA GAA ATT ATT GTA CTT TGT A...3'] and NP1/NP2 [5'...DIG-GGA TCC TCC AAA AAA TAA AGT TT...3'*/5'...ACA GAA CGA TAT TGG ATA CCT AGT A...3']) were used to amplify a 125 base pair (bp) and a 133 bp repetitive region from extrachromosomal circular deoxyribonucleic acid (DNA) in pathogenic and non-pathogenic strains respectively. One primer of each pair was labelled with digoxigenin.

Each 100 µL of reaction mix contained 4mM MgCl₂, Promega® (M-1861) *Taq* buffer (500 mM KCl, 100 mM Tris-HCl, 1% Triton X-100®), 0.2 mM of each deoxynucleoside triphosphate, and 5 units of Promega® *Taq* polymerase. 0.5-1 ng of DNA was added to 40 µL of

PCR reaction mix containing 25 pmol of each primer and 1 pellet of wax. Between 0.5 and 10 ng of reference pathogenic and non-pathogenic strain DNA were used in control PCR reactions. *Escherichia coli* DNA, DNA extracted from parasite-free faeces, and water as a sample replacement, were used as negative controls. Each DNA sample was tested with pathogenic (P) and non-pathogenic (NP) primer pairs. Amplification conditions were 1 min at 94°C, 30 s at 94°C (×1), 1 min at 55°C, and 2 min at 72°C (35 cycles).

Specific internal sequences in the PCR products were detected by a solution hybridization procedure after PCR (the annealing temperature of the probes was the same as that used for the amplification primers). Five pmol of 5'-biotin-conjugated probes specific for pathogenic (5'...GAG GTT CTT AGG AAA TCG AAA A...3') and non-pathogenic (5'...GGT GAG GTT GTA GCA GAG ATA TT...3') *E. histolytica* (see ROMERO *et al.*, 1992) were added to 20 µL of PCR product before denaturation (99°C, 10 min), annealing (55°C, 90 min), dilution with 200 µL of Tris-buffered saline (TBS) containing 0.05% Tween-20® (TBS-T); TBS is 0.02 M Tris and 0.15 M NaCl, pH 7.2.

Microwell strips (Greiner, Germany) coated for 1 h at 37°C with avidin (Sigma: 0.1 µg/mL in 50 mM carbonate buffer, pH 9.6) were washed 3 times with TBS-T, blocked with 0.02 ng/mL of salmon sperm DNA for 60 min, and rewashed 3 times with TBS-T. 100 µL of each diluted PCR product were added to duplicate wells and incubated (room temperature, 60 min). After washing 3

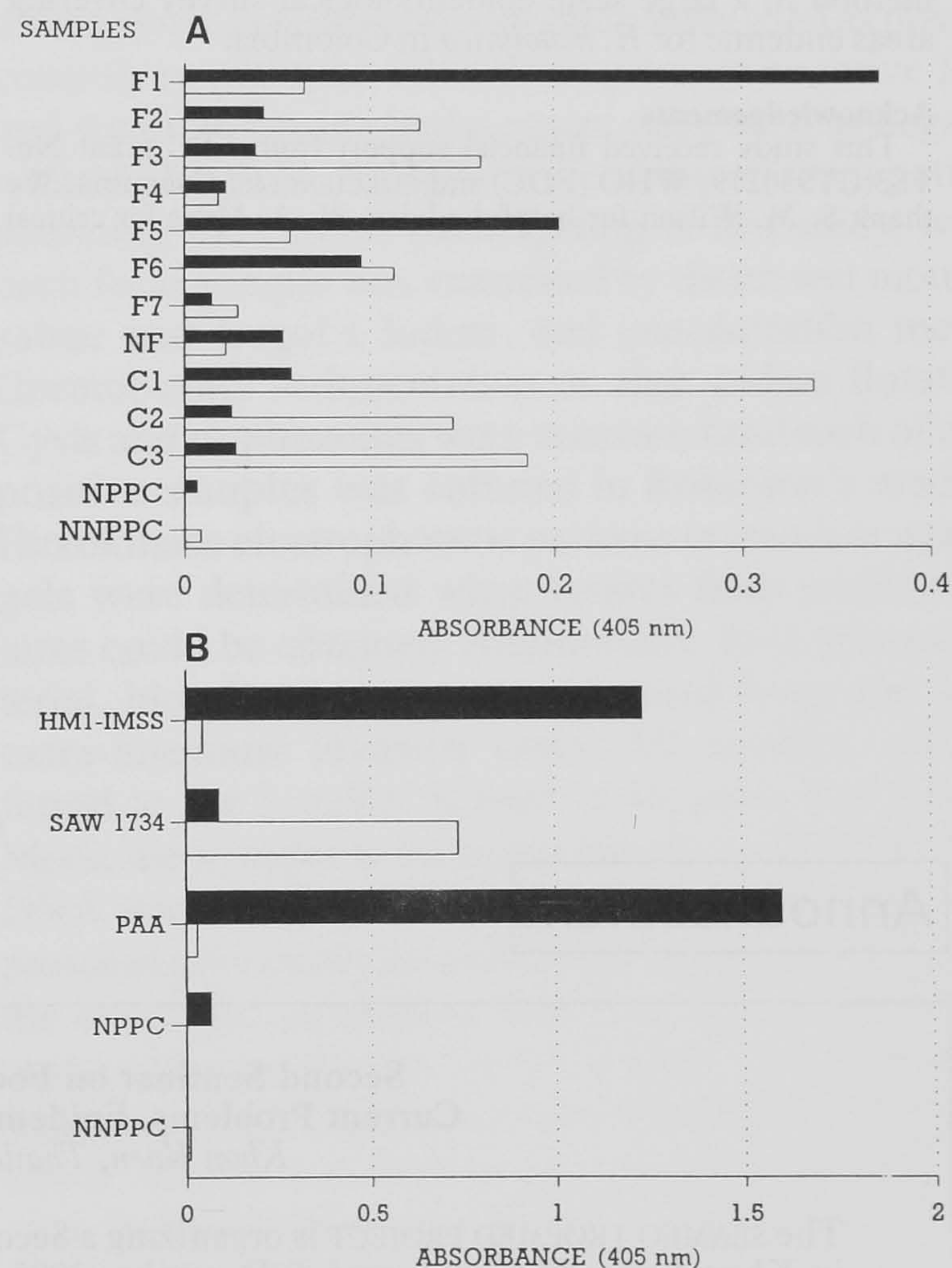


Figure. PCR-SHELA results. A. Faeces (F), Robinson's cultures (C) and controls: NF, negative faeces; NPPC, negative pathogenic primer control; NNPPC, negative non-pathogenic primer control. F1, *E. histolytica* haematophagous trophozoites; F2, *Entamoeba* sp. trophozoites; F3, *Entamoeba* sp. trophozoites and *Blastocystis hominis*; F4, *E. coli* cysts; F5, *E. histolytica* haematophagous trophozoites; F6, *E. histolytica* cysts; F7, hookworm eggs; C1, *E. histolytica* cysts; C2, *E. histolytica* cysts, *E. coli* cysts, *Iodamoeba buetschlii* cysts and *Endolimax nana* cysts; C3, *E. histolytica* cysts, *E. coli* cysts and *I. buetschlii* cysts. B. Reference strains, amoebic abscess and controls. HMI-IMSS, reference pathogenic strain; SAW 1734, reference non-pathogenic strain; PAA, 'pus' from amoebic abscess; NPPC, negative pathogenic primers control; NNPPC, negative non-pathogenic primers control. ■, Pathogenic primers; □, non-pathogenic primers.

*Modified from ACUÑA-SOTO *et al.* (1993) to show the correct orientation of the sequence.

times with TBS-T, 100 µL of 1 5000 anti-digoxigenin alkaline phosphatase-labelled antibody (Fab fragment: Boehringer Mannheim, Germany) were added to each well (room temperature, 60 min) followed by 4 washes (3 with TBS-T, one with TBS). The ELISA was developed by adding 100 µL of p-nitrophenyl phosphate (1 mg/mL in 50 mM carbonate buffer, pH 9.6, containing 1 mM MgCl₂). After 1 h absorbance was read at 405 nm. No significant change in absorbance took place during the plate reading period of 1 min. The assay results are shown in the Figure.

Whilst the PCR has been used successfully to differentiate between pathogenic and non-pathogenic *E. histolytica* by ROMERO *et al.* (1992) and ACUÑA-SOTO *et al.* (1993), the simplification of the procedure described here is a significant step forward in the differential diagnosis of this parasite in central diagnostic laboratories. The assay can be used on DNA extracted directly from stool samples or on DNA obtained from trophozoites in short-term cultures in Robinson's medium. The extraction method allows DNA to be obtained from both cysts and trophozoites. Concentration techniques previously described (FAUST *et al.*, 1938; ALLEN *et al.*, 1970) and used by ACUÑA-SOTO *et al.* (1993) lose trophozoites and therefore make the detection of pathogenic *E. histolytica* less likely. Results are obtained within 48 h, which is more rapid than culturing followed by isoenzyme characterization and more informative than microscopy. This technique, which might be improved by using the DNA extraction method of KATZWINKEL-WLADARSCH *et al.* (1994), is a useful adjunct to microscopy and isoenzyme analysis. It is currently being evaluated as a diagnostic method in a large scale epidemiological survey covering areas endemic for *E. histolytica* in Colombia.

Acknowledgements

This study received financial support from EEC grant No. TS3:CT930219, WHO (PDC) and COLCIENCIAS, Colombia. We thank S. M. Wilson for helpful advice, M. A. Miles for critical

reading of the manuscript, and Alvaro Rojas, the director, and staff of the Hospital Pedro Leon Alvarez in La Mesa for their help.

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Received 10 October 1994; accepted for publication 8 November 1994

Announcement

Second Seminar on Food-borne Parasitic Zoonoses: Current Problems, Epidemiology, Food Safety and Control Khon Kaen, Thailand, 6–9 December 1995

The SEAMEO TROPED PROJECT is organizing a Second Seminar on Food-borne Parasitic Zoonoses to be held in Khon Kaen, Thailand, on 6–9 December 1995. In addition to scientific sessions a one day trip will be made into Laos. Additional information can be obtained from the SEAMEO TROPED PROJECT, Faculty of Tropical Medicine, Mahidol University, 420/6 Rajvithi Road, Bangkok 10400, Thailand or from Dr John H. Cross, Uniformed Services University of The Health Sciences, Bethesda, MD 20814, USA. Telephone +1 (301) 295-3139; Fax +1 (301) 295-1971.

Diagnosis of *Entamoeba histolytica* and *Entamoeba dispar* in Clinical Specimens by PCR-SHELA¹

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Introduction

Amebiasis has been regarded as the third cause of morbidity and mortality among parasitic diseases in the world after malaria and schistosomiasis (1). This fact, linked to the growing evidence for the separation of invasive and non-invasive *Entamoeba histolytica* sensu lato in two different species (*E. histolytica* sensu stricto and *E. dispar* respectively), pointed out the need for the development of sensitive and specific tools for accurate differential diagnosis and the epidemiology of amebiasis in endemic and non-endemic areas (2). The PCR-SHELA system combines specific DNA amplification and hybridization steps, together with a colorimetric detection of PCR products by ELISA in a robust test where high sensitivity and specificity are achieved (3).

Materials and Methods

Reference *E. histolytica* axenic strains HM1-IMSS, 200-NIH cultured in TYI-S33 medium, SD 184, Eh 157 cultured in YI-S medium and polyxenic *E. dispar* SAW

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¹This project was supported by the Commission of the European Community grant No. TS3:CT930219, WHO(PDC) and COLCIENCIAS, Colombia.

1734 grown in Robinson's medium were used as positive controls in the tests. DNA from parasite negative feces and water as sample replacement were used as negative controls for PCR. Samples were obtained from patients attending the centers participating in this study, where each fecal sample was examined by direct wet mount in saline and Lugol's Iodine, and concentration method (formol-ether sedimentation or zinc sulfate flotation). Cysts and trophozoites were measured and each of those positive samples was cultured in Robinson's medium. Hexokinase electrophoretic patterns in starch or agarose gels were determined when lysates from positive cultures could be obtained. Additionally, liver abscess material, blood and urine were obtained from one of the extra-intestinal invasive cases. All samples were referred to the London School of Hygiene and Tropical Medicine in order to be tested blindly by PCR-SHELA. DNA was extracted from all samples according to the protocol previously described by Aguirre et al. (3) where the overnight incubation with lysis buffer at 37°C was replaced by incubation for 2 h at 55°C. The PCR amplification of the 25 Kb episomal rDNA from *E. histolytica* and *E. dispar* was kept as previously described. However, hybridization time to the corresponding internal probe was reduced from 90 to 15 min.

Results and Discussion

Clinical samples from 14 patients were tested blindly by PCR-SHELA for the presence of *E. histolytica* and *E.*

Table 1
PCR-SHELA Results on Clinical Specimens

SAMPLE	Specimen	Clinical data	Microscopy	EH OD	ED OD
4534	Blood		NT	0.009	0.010
4534	Pus	Liver abscess	Negative	0.101	-0.001
4534	Feces		Negative	0.783	-0.011
4534	Urine		NT	0.063	-0.014
4534	Serum		NT	0.011	-0.008
1204	Feces	Asymptomatic	<i>D. fragilis</i> , <i>Bl. hominis</i>	0.031	0.027
1206	Feces	Asymptomatic	Negative	0.033	0.034
1203	Feces	Liver amebic abscess 3 years before	<i>E. histolytica</i> and <i>E. coli</i> cysts, <i>D. fragilis</i> , <i>Bl. hominis</i>	0.612	-0.016
1187	Feces	Asymptomatic cyst carrier	<i>E. histolytica</i> and <i>E. coli</i> cysts, <i>Bl. hominis</i>	0.757	0.023
1205	Feces	Asymptomatic cyst carrier	<i>E. histolytica</i> and <i>E. coli</i> cysts, <i>Bl. hominis</i>	0.413	-0.014
1095	Feces	Asymptomatic	<i>E. histolytica</i> cysts and trophozoites	-0.001	-0.008
1096	Feces	Asymptomatic	<i>E. histolytica</i> cysts and trophozoites	0.134	-0.003
822	Feces	Dysentery	<i>E. histolytica</i> cysts and trophozoites	0.434	0.017
675	Feces	Salmonellosis	<i>E. histolytica</i> cysts and trophozoites	0.051	0.262
1472	Feces	Ulcerative colitis	Negative	0.033	0.032
1079	Pus	Hepatic abscess	Negative	0.744	0.004
ANTONY WIHT	Feces	Dysentery	Hematophagous trophozoites	0.309	0.036

E. histolytica and *E. dispar* optical densities were determined for each sample.

NT: Not tested.

EH OD: *Entamoeba histolytica* Optical Density.

ED OD: *Entamoeba dispar* Optical Density.

dispar DNA. Parasite-negative fecal specimens were tested as controls (mean OD values 0.029 and 0.031, respectively), and titration of cultured trophozoites-spiked feces gave a lower detection limit of ten trophozoites per gram of feces (data not shown). From 12 fecal specimens examined, PCR-SHELA was able to correctly assess 11 and all five positive cultures derived from the fecal samples were correctly identified, including the specimen undetected directly from the fecal sample. It was also possible to establish a differential diagnosis in a case of salmonellosis, where cysts mor-

phologically compatible with *E. histolytica* sensu lato were detected by microscopy. Pus material from abscesses is also a good source of DNA for PCR amplification in cases where drainage is indicated. However, it was not possible to identify *E. histolytica* DNA from blood, urine or serum samples from an extraintestinal invasive case. In order to improve the sensitivity of the system, collaborative work is being carried out together with Ackers and colleagues, modifying the DNA extraction step, and also shortening the processing times to obtain results within one working day.

Table 2
PCR-SHELA Results on Cultures From Clinical Specimens.

Culture	HK	EH OD	ED OD
1203	Fast	0.299	0.006
1095	Fast	0.877	0.006
1096	Fast	0.229	0.028
822	Fast	0.859	0.024
675	Slow	0.054	0.238
2024	Fast	0.422	0.007
EH SD184	Fast	0.942	-0.009
EH 157	Fast	0.863	-0.009
NIH-200	Fast	0.285	0.038
SAW 1734	Slow	-0.001	0.603

E. histolytica and *E. dispar* optical densities were determined for each specimen. Results on reference strains are included.

NT: Not tested

EH OD: *Entamoeba histolytica* Optical Density.

ED OD: *Entamoeba dispar* Optical Density.

In conclusion, the availability of a sensitive and specific diagnostic tool permits the rapid simultaneous screening of great numbers of samples facilitating future worldwide multicentric studies for the review of the epidemiology of amebiasis, where homogeneous methodology should be applied. However, it is our view that

direct microscopy with micrometric measurement and concentration methods remain as first hand routine tools for the diagnosis of amebiasis.

Acknowledgments

We are indebted to Mr. J. Williams for his advice in the zymodeme characterization of cultures and to Dawn Britten for providing *E. histolytica* strains SD 157 and SD 184.

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KEY WORDS: *Entamoeba histolytica*; *E. dispar*; Diagnosis; PCR-SHELA.