ISOENZYME POLYMORPHISM IN *ENTAMOEBA HISTOLYTICA*

AN EPIDEMIOLOGICAL SURVEY IN A

RURAL SOUTH AFRICAN POPULATION

BY

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PREFACE

One of the controversial issues regarding the epidemiology of amoebiasis is the status of the cyst passer. These carriers of Entamoeba histolytica are usually asymptomatic and the decision to treat them or not remains unresolved. At the present time, one group of scientists subscribe to the hypothesis that E. histolytica is normally a harmless human commensal which, under appropriate conditions, has the potential to become pathogenic and invade its host's tissues. It is therefore argued that all carriers of the parasite should be treated. Another group believe that there are two distinct strains or even sub-species of E. histolytica that are morphologically identical but vary in their pathogenicity, one being pathogenic and the other non-pathogenic. Thus it is considered that only those carriers of the pathogenic variety require treatment.

Isoenzyme characterisations of E. histolytica has revealed many distinct polymorphic forms of the parasite. However, there is a high degree of homogeneity in the isoenzymes of hexokinase and phosphoglucomutase of the pathogenic as well as the non-pathogenic strains. Isoenzymes are an expression of genetic codes within the DNA of the parasite and are therefore a convenient way of 'fingerprinting' the different strains of E. histolytica. With the development of DNA hybridisation techniques, polymorphism at the genetic level has recently been described and genetic differences between pathogenic and non-pathogenic strains confirmed.

This thesis is the product of 5 years research work during which isoenzyme characterisation of E. histolytica was employed to reappraise the

epidemiology of the parasite and the serology of amoebiasis. The stability of isoenzyme typing in distinguishing the pathogenic from non-pathogenic forms was verified and other pathophysiological differences between these two strains were identified which indicated that these two forms of E. histolytica may well be distinct subspecies of the organism.

This thesis contains the candidate's original work and has not been submitted in any form to another university.

Selected results from this thesis have been published in scientific journals. Research workers who were closely associated in these studies are co-authors in these publications:

1 GATHIRAM V, SIMJEE AE, BHAMJEE A, JACKSON TFHG, PILLAY LV, ANDERSON CB. Concomitant and secondary bacterial infection of the pus in hepatic amoebiasis. South African Medical Journal 1984; 65: 951-953.

2 GATHIRAM V, JACKSON TFHG. Frequency distribution of Entamoeba histolytica zymodemes in a rural South African Population. Lancet 1985; i: 719-721.

3 JACKSON TFHG, GATHIRAM V, SIMJEE AE. Seroepidemiological study of antibody responses to the zymodemes of Entamoeba histolytica. Lancet 1985; i: 716-719.

4 GATHIRAM V, JACKSON TFHG. A longitudinal study of asymptomatic carriers of pathogenic zymodemes of Entamoeba histolytica. South African Medical Journal 1987; 72: 669-672.

V. GATHIRAM

ETHICAL CONSIDERATIONS

Informed consent was obtained from all participants in this study. Where the patient was a minor, informed consent was obtained from the parent or legal guardian.

All research protocols relating to this study were approved by the Ethics Committee of the Faculty of Medicine, University of Natal.

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ABSTRACT

Isoenzyme characterisation of Entamoeba histolytica into pathogenic and non-pathogenic zymodemes substantiated previously held views that this parasite constitutes two distinct strains or even sub-species that are morphologically identical but vary in their pathogenicity. A reappraisal of the epidemiology of amoebiasis and investigation of the patho-physiological relationships between these pathogenic and non-pathogenic zymodemes and their host was therefore indicated.

Only pathogenic zymodemes were isolated from hospitalised patients with amoebic liver abscess (ALA) and amoebic dysentery (AD). In the amoebiasis endemic peri-urban population of Durban, E. histolytica occurred at an overall prevalence of 10%. Carriers of non-pathogenic zymodemes constituted 9% of the population. A key observation was that asymptomatic infections with pathogenic zymodemes occurred at a prevalence of 1%. Higher prevalence of E. histolytica occurred in association with poor sanitary conditions. Furthermore, both pathogenic and non-pathogenic zymodemes tended to cluster into family units suggesting person-to-person transmission of the parasite by the faecal-oral route. Although invasive amoebiasis occurs far more frequently in males than females (8:1) both pathogenic and non-pathogenic zymodemes are equally distributed in male and female E. histolytica cyst passers.

Ninety percent of carriers of pathogenic zymodemes spontaneously cleared their infections and remained asymptomatic throughout the study period of 2 years while 10% developed AD which required treatment with

metronidazole. No spontaneous changes in zymodemes from the non-pathogenic to the pathogenic type was observed in a longitudinal study.

The serological response of asymptomatic carriers of pathogenic zymodemes (100% seropositive) was identical to that of patients with ALA or AD with a high proportion (94-100%) of them being strongly seropositive. The prevalence of seropositivity amongst subjects who were not infected by E. histolytica (13% seropositive) was not statistically different ($p > 0,5$) from that of the random population of this endemic area (19% seropositive) and carriers of non-pathogenic zymodemes (21% positive); the prevalence of strongly seropositive reactions among this group was only between 2-4%. It is concluded that a positive serological response is directly due to past or present contact with pathogenic zymodemes. This is further substantiated by the observation that the proportion of seropositive subjects was found to increase dramatically in a population near Cape Town where an outbreak of invasive amoebiasis (ALA and AD) occurred indicating a high prevalence of pathogenic zymodemes in this community. Another community in northern Transvaal (Gazankulu) where ALA and AD does not occur was, as expected, uniformly seronegative.

Axenic growth of pathogenic zymodemes was possible but could not be accomplished with the non-pathogenic zymodemes. Even though monaxenic growth together with Trypanosoma cruzi was possible with both strains, the pathogenic zymodemes tended to grow more prolificly. No zymodeme changes from non-pathogenic to pathogenic and vice versa were observed with such changes in culture conditions.

Cyst production by the pathogenic zymodemes *in vivo* was confirmed experimentally, thereby demonstrating the ability of pathogenic *E. histolytica* to independently complete their life-cycle thus giving it the ability to propagate itself successfully as a sub-species.

CHAPTER 1 GENERAL INTRODUCTION

1.1 PREAMBLE

Entamoeba histolytica, the causative organism in invasive amoebiasis has since the time of its discovery in 1875 (Lösch, 1875), been the subject of much research and controversy. Most of the debate has centered on the pathogenesis of the disease; pivotal to this is the marked discrepancy between the often high prevalence of the parasite in endemic areas on the one hand associated with a relatively low prevalence of invasive disease on the other. Theories put forward to explain this anomaly have generated copious research effort but have not as yet resulted in the formulation of an irrefutable mechanism of the pathogenesis of amoebiasis. An overview of the hypotheses that have emerged and some of the earlier work used to substantiate these are outlined below as a background to the present study since these resulted in the production of scientific evidence that will contribute to the solution of this controversial topic.

1.2 HISTORICAL BACKGROUND AND TAXONOMIC CONSIDERATIONS

In 1875 Dr Friedrich Alexandravich Lösch (1875) described the presence of motile trophozoites in the dysenteric stools of a

Pietersburg (now Leningrad) labourer which he named 'amoeba coli'. Additionally, he successfully reproduced the clinical picture of dysentery in 4 out of 5 dogs by inoculating the amoebae obtained from his patient into the rectum of these experimental animals; furthermore, he demonstrated ulcerative lesions teeming with 'amoeba coli' in the colons of these dogs at autopsy. These lesions were identical to those found in his patient on post-mortem examination. In spite of all this evidence Lösch remained unconvinced that the amoeba was of any aetiological importance in the causation of dysentery; he believed that bacteria were the primary aetiological agents in the ulcerative process resulting in dysentery and that the presence of 'amoeba coli' served merely to perpetuate the inflammatory reaction. One gets the impression on reading his paper that dysentery is a specific disease entity caused by bacteria and not a symptom complex of blood and mucous in the stools as we understand it today.

A few years later, Cunningham (1881) described amoebae isolated from both apparently healthy individuals and patients with cholera. Cunningham failed to distinguish them from the 'amoeba coli' of Lösch (1875). He (Cunningham, 1881) also believed that these amoebae were quite innocuous and that the association of the parasite with intestinal disorders appeared to be dependent on the abnormal condition of the intestinal contents allowing the rapid multiplication of the reproductive elements of the amoebae which gained access to them. From the descriptions recorded by

Cunningham, these amoebae were later identified as Entamoeba coli by Dobell (1919).

In the intervening years, up to 1891 several other workers viz Kartulis (1887), Hlava (1887), Osler (1890) and Dock (1891), also demonstrated the presence of amoebae, similar to those described by Lösch, in colonic ulcers of dysenteric patients and in liver abscesses. The association between amoebae and dysentery, however, still remained in doubt. In the opinion of Dobell (1919) there were two chief reasons for this: firstly, the failure to recognise that amoebae constitute a large group of organisms containing many species belonging to different genera - of which man harbours several different kinds only one of which is pathogenic and secondly, that dysentery is not just one disease but a symptom of several pathological conditions; he (Dobell, 1919) found it inconceivable that one could have ruled out amoebae as a cause of dysentery by simply demonstrating that certain bacteria can also cause it.

Councilman & Lafleur (1891) in their classic study "Johns Hopkins Hospital Reports : amoebic dysentery" described the presence of 'amoeba coli' in bacteriologically sterile liver abscess and thus demonstrated the pathogenic potential of the organism independent of concomitant bacteria. They called the parasite "amoeba dysenteriae" and concluded that "... amoeba dysenteriae has been shown to be the cause (of disease) by its constant presence in the stools and in the anatomical lesions".

It was the demonstration of cyst production by the pathogenic amoeba by Quincke & Roos (1893) that finally completed the picture of the life-cycle of "amoeba coli" described by Lösch (1875). They failed, however, to give an adequate description of the number and specific characteristics of the nuclei. Also of importance was their discovery that dysentery may be produced in cats not only by the inoculation of trophozoites into the rectum but also by feeding them with cysts derived from dysenteric patients.

According to Elsdon-Dew (1968) Casagrandi & Barbagallo (1895) described Entamoeba coli from healthy people in 1895, thus establishing the generic name Entamoeba. In 1903 Schaudinn renamed the dysentery producing amoeba Entamoeba histolytica, reserving the name Entamoeba coli for the non-pathogenic species of amoeba known at that time.

According to Dobell (1919) the quadrinucleate cysts of E. histolytica were rediscovered by Huber (1903) who showed them to Schaudinn; the latter, apparently would not admit that they belonged to the species E. histolytica and convinced Huber that these cysts belonged to a separate species which he (Schaudinn) called E. tetragena. Viereck (1907) adopted the name E. tetragena for the quadrinucleate cysts that he observed and like Schaudinn, was convinced that they belonged to a separate species. Elmassian (1909) observed a small species of amoeba associated with E. tetragena cysts in the dysenteric stools of a man. He apparently

thought that these belonged to yet another species (Elsdon-Dew, 1968) and named them Entamoeba minuta.

Kuenen & Swellengrebel (1913) working in Sumatra observed different stages in the life-cycle of what was then called E. tetragena. The "minuta" stage of Elmassian (1909) was usually present in normal stools or during convalescence from amoebic dysentery; in their opinion this stage was saprozoic feeding on bacteria and faecal material; in addition a the "histolytica" stage occurred in which the trophozoites were larger (magna forms) and which were always found in conjunction with dysenteric stools. They (Kueunen & Swellengrebel, 1913), also observed that the third or cystic stage was invariably associated with the minuta forms of the parasite. These authors hypothesised that the cystic stage was the progeny of the "minuta" forms and this form of the parasite, they proposed, was responsible for propagation of the species. Kuenen & Swellengrebel (1913) also considered the tissue or "histolytica" phase to be an offshoot of the minuta forms concluding that the "histolytica" phase did not have any part in the life-cycle of the parasite (Fig 1.1).

In 1913, Walker & Sellards conducted their classic experiments in which cysts and trophozoites of E. histolytica-Schaudinn (1903), E. coli-Schaudinn (1903) and free-living amoebae isolated from the Manilla (Phillipines) water supply were fed to human volunteers.

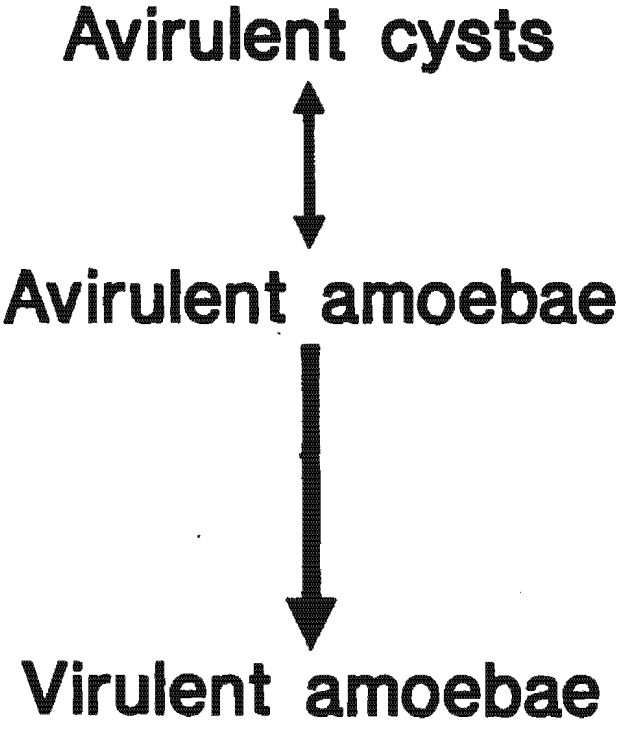

Figure 1.1 Life-cycle of E. histolytica according to Kuenen & Swellengrebel (1913).

They proved conclusively that both free-living amoebae and E. coli were non-pathogenic to humans. Of 20 men fed different strains of E. coli, 17 became parasitised and none developed any symptoms. They concluded that "E. coli is an obligatory parasite ... and that it is non-pathogenic and consequently plays no role in the aetiology of entamoebic dysentery". As a result of their experiments with E. tetragena-Viereck (1907) and E. histolytica-Schaudinn (1903), they concluded that E. tetragena-Viereck (1907) is identical to E. histolytica-Schaudinn (1903) and that E. minuta-Elmassian (1909) is the pre-encysted stage of E. histolytica-Schaudinn (1903). Of all their feeding experiments the most important one is summarised in Figure 1.2 and constitutes the most definitive study ever conducted on this topic.

By feeding cysts (Strain A) obtained from a convalescent carrier (Fig 1.2) they were able to produce amoebic dysentery in 2 out of 3 volunteers; cysts obtained from one of these dysenteric patients (subject 1) parasitised but failed to produce dysentery in a further 3 volunteers. Another two volunteers fed with cysts from the non-dysenteric subject (subject 3) became parasitised and when cysts from one of these (subject 7) were fed again to 4 other persons it produced dysentery in one and parasitised another two.

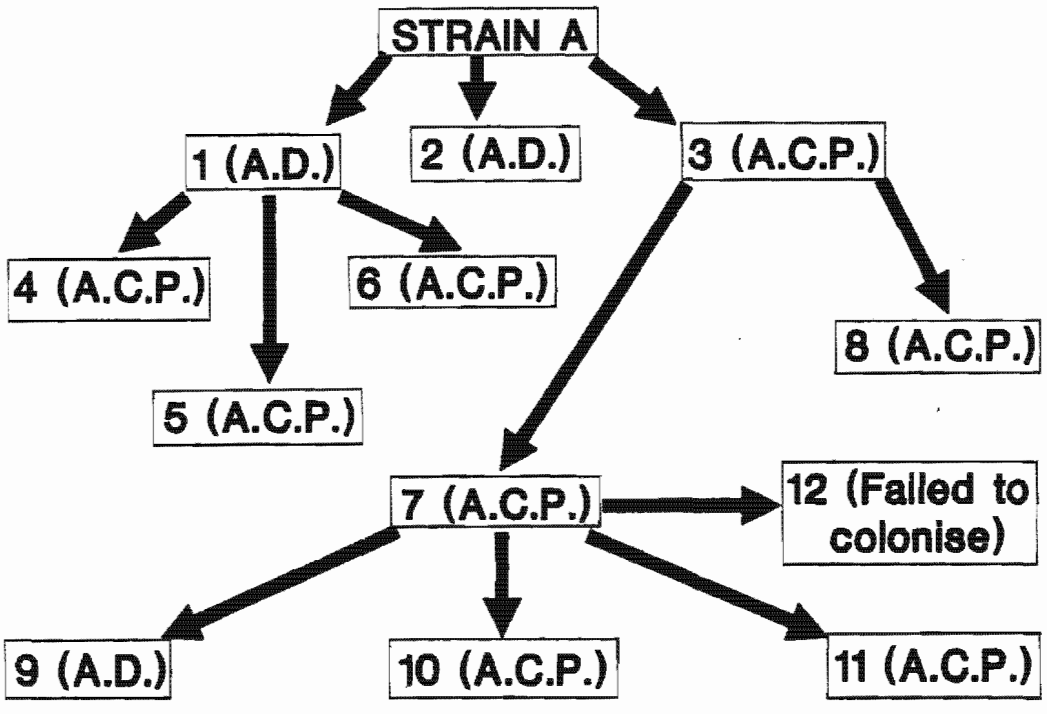

Figure 1.2 Feeding experiments of Walker & Sellards

Volunteers fed cysts (Strain A) from a convalescent carrier

AD = Amoebic dysentery

ACP = Asymptomatic cyst passer

Amoebae isolated from a liver abscess and from dysenteric stools failed to produce symptoms in 3 out of 4 volunteers. Furthermore, they succeeded in producing dysentery in one of 4 volunteers fed cysts obtained from an apparently healthy subject. They point out that the "small percentage of dysenteries resulting from these findings has no bearing on the aetiology but is evidence of the frequent latency of the disease" and "to what extent this latency, which is characteristic of entamoebic dysentery, is due to the chronicity of the ulcerative process, or whether or not the inability of Entamoeba histolytica to penetrate the healthy epithelium has any part in it, cannot at present be definitely answered".

Once it was appreciated that the quadrinucleate cysts of tetragena type were those of Entamoeba histolytica it was possible to determine the prevalence of the parasite and it soon became apparent that there was a gross discrepancy between the prevalence of the parasite and that of active disease. Wenyon & O'Connor, (1917) amongst the first to determine the prevalence of E. histolytica, point out that "the presence of such a large number of carriers amongst healthy men was not suspected and caused a somewhat startling revelation"; furthermore they were unable to predict the appearance of dysentery in these asymptomatic individuals. Dobell (1916), also described healthy carriers of E. histolytica. An attempt to explain the poor correlation between the prevalence of cyst passers and that of disease thus led to the

development of three broad schools of thought. The "Promethean" school whose chief protagonist was Dobell (1919), and the Swellengrebel (Kuenen & Swellengrebel, 1913) school which has been termed "commensal" recognised only a single species of E. histolytica while the third hypothesis, that of Brumpt (1928), separated the parasite into two morphologically indistinguishable species and will be called the "two species theory". These hypotheses differed basically in the status of the cyst passer and because of their profound influence on thoughts regarding the pathogenesis of amoebiasis and treatment of cyst passers, will be discussed in more detail below.

At this stage, it is appropriate to define four important concepts

1 PATHOGENICITY - is defined by Maegraith (1963) as the ability of an organism to produce disease in a host.

2 A STRAIN is an isolate of an organism from a patient.

3 VIRULENCE is defined by Kagan (1974) as the ability of the parasite to cause an overt clinical response in the host.

4 ZYMODEME is a strain of E. histolytica distinguished by isoenzyme electrophoresis.

1.3 PROMETHEAN THEORY

This school of thought maintains that all strains of E. histolytica are pathogenic. In its simplest form, as pointed out by Walker & Sellards (1913), "the entamoebae might be conceived to live as commensals in the intestine of their host and only when there occurred some depression of the natural resistance of the host or of its tissues ..... were the amoebae able to penetrate the intestinal epithelium, become tissue parasites and produce the characteristic lesions of entamoebic dysentery". Wenyon and O'Connor (1917) also regarded E. histolytica as an obligatory tissue parasite; they maintained that the efficacy of emetine (which is only administered parentally) in clearing cyst passers of E. histolytica depends on the ability of the parasite to invade the host's tissues and thus come into contact with the circulating drug; the inability of emetine to cure cysts passers of E. coli, they explained occurred since the latter parasite was strictly saprophytic and because it did not penetrate the intestinal wall it could not come into contact with the amoebicide.

Dobell (1919) commenting on the study of Wenyon & O'Connor (1917), which referred to the appearance of E. histolytica cysts in apparently healthy individuals, explains that "E. histolytica is, unlike most parasitic amoebae, a tissue parasite. It lives in and upon the living tissues of its hosts and it can exist in no other way..... This ideal condition for host and parasite alike is a state of equilibrium like that between Prometheus and the eagle -

the former regenerating sufficient tissue each day to compensate for the ravages of the latter. A human being in this 'Promethean' state of equilibrium is now called a 'carrier'". He (Dobell 1919) goes on to explain the development of disease by hypothesizing that "some individuals are unable to tolerate the parasite and react to their presence by developing acute dysentery". Dobell (1919) found it inconceivable that E. histolytica could actually lead a commensal existence and could find no reason why "an amoeba which is generally dependent upon living tissues for its nourishment should at times completely change its habits and become a feeder on bacteria". He (Dobell, 1919) also claimed that E. histolytica in the amoeboid state in freshly passed stools, normally never contained bacteria.

Flaws in the theory soon began to appear. Reinchenow (1926) estimated that the number of non-haematophagous amoebae and cysts discharged by symptomless carriers may reach 350000 per gram of faeces or 35 million daily; he found it implausible that enormous masses of amoebae could live in the gut wall without producing any intestinal disorder. Andrews and Atchley (1932) employing the benzidine test for occult blood found no traces of blood in the stools of healthy carriers of E. histolytica; they concluded from this evidence that it is unlikely that the parasite is an obligatory tissue parasite.

With the advent of Boeck and Drbohlav culture medium Dobell (1931), contrary to his earlier speculation, was able to demonstrate that

E. histolytica was able to ingest bacteria and starch granules in culture. He (Dobell, 1931) also demonstrated that on continued cultivation E. histolytica could lose its power to infect kittens and also to ingest erythrocytes. Later, Hoare (1952) conclusively demonstrated that the pathogenic amoebae can feed on bacteria and he concluded that E. histolytica, like E. coli, can lead a commensal existence.

According to Elsdon-Dew (1968), Dobell's earlier views (Dobell, 1919) were adopted by American workers. Craig (1927) claimed to have recorded symptoms in 50% of carriers which he ascribed to the presence of E. histolytica in the large intestine; he believed that the amoeba is a true tissue parasite and therefore there is "no such thing as a perfectly healthy carrier" of this parasite. A similar view was held by Faust (1944). Their views were further substantiated by the demonstration of a complement fixing antibody in almost all carriers of E. histolytica (Craig, 1927). The latter experiment was repeated by Meleny & Frye (Meleney 1944) and yielded a low proportion of positive results in carriers.

More recently Bray & Harris (1977a), while "not wanting to be labelled Promethean" believe in the existence of a number of strains of E. histolytica which vary in their pathogenic potential. They postulate that "rapid transmission (of the parasite) due to overcrowding leads to higher populations of E. histolytica and possibly selection of invasive variants. This in turn leads to more lesions and more invasions".

The "Promethean" theory has thus changed remarkably over the years from the belief that all E. histolytica strains are always equally pathogenic to the acceptance of the existence of several strains of differing pathogenicity and virulence.

1.4 THE COMMENSAL THEORY

1.4.1 Introduction

This theory was first publicised by Keunen & Swellengrebel (1913). It views E. histolytica as a gut commensal; these amoebae which are usually smaller (minuta) produce quadrinucleate cysts which are responsible for propagation of the species; further, some as yet unidentified stimulus causes the amoeba to mutate into a tissue invading form which is larger (magna); this mutation apparently deprives the tissue invasive forms of the capability of producing cysts. They considered the tissue or histolytica phase to be an offshoot of the minuta forms; these tissue forms, it was postulated, did not produce cysts. It is therefore assumed that once the mutation occurred and the parasite invaded tissues it effectively committed suicide in that it could not thereafter be transmitted to another host. The life-cycle is summarised in Fig. 1.1.

At the time of its proposal, this concept best explained the disproportion that existed between the prevalence of E. histolytica carriers and the prevalence of cases of invasive amoebiasis and was thus adopted by such eminent authors as Bos (1975), Elsdon-Dew (1968, 1978), Neal (1958) and Singh (1973). The "trigger" which was responsible for this hypothetical mutation has since captured the imagination of many researchers but has to date, escaped scientific resolution. A brief summary of some of these follow.

1.4.2 Nature of Gut Flora

The nature of the gut flora was one of the first to be investigated mainly because of the documented observation that bacteria were essential to initiate and maintain cultures of E. histolytica. Phillips et al (1972) however, were unable to infect germ-free and 'conventional' guinea pigs with pathogenic strains of E. histolytica (200:NIH, HK9 and ABRM) that had their virulence attenuated by prolonged in vitro culture. Phillips and Bartgis (1954) and Wittner and Rosenbaum (1970) were able to restore virulence to pathogenic strains of E. histolytica (that had lost their virulence during prolonged culture) by associating it with bacteria. Similarly Bos and Hage (1975) were able to demonstrate that the virulence of pathogenic strains could be further increased by associating them with "non-pathogenic" bacteria.

Neal (1956) demonstrated that the addition of bacterial flora from a pathogenic strain of E. histolytica modified the virulence of

another pathogenic strain whose virulence had became attenuated during prolonged *in vitro* culture; however, such a strain of bacteria did not affect the pathogenicity of a non-pathogenic strain (isolated from an asymptomatic carrier and shown not to cause caecal ulceration in mice). It is well recognised that *Clostridium perfringens* supports the growth of *E. histolytica in vitro*; however, the administration of the bacterium to rats that were already infected with *E. histolytica* had no effect on the capability of ulcer production by that strain of *Entamoeba* (Okamoto, 1954). Okamoto (1954) concluded that the "rate of infectivity and ulcer production was mainly determined by the pathogenicity of the amoeba inoculated".

1.4.3 Dietary Implications

The observation by Elsdon-Dew (1946) that amoebiasis in Durban occurred more frequently in the African population than in the White or Indian led him to postulate that diet may be a predisposing factor; the White and Indian population apparently consumed a diet with adequate amounts of protein while the African had a largely carbohydrate ("bun and lemonade") diet. He concluded that diet may manifest its effect by modifying the gut flora; however, his experiments with rats in this direction proved inconclusive (Elsdon-Dew 1956). Diamond (1982a) extensively reviewed the nutritional implications in amoebiasis but was not able to make any concluding remarks on the role of diet in the pathogenesis of amoebiasis.

Clinical studies (Lewis and Anita (1969), and Simjee (unpublished data - pers. com.)) mention malnutrition in association with amoebiasis. The malnutrition may be a result of the disease itself since many of these patients have anorexia on admission (personal observations). Furthermore, patients with invasive disease improved rapidly following drug treatment with a gain in weight and elevation of the serum albumin which remained elevated two months following discharge and return to their normal diet at home (Simjee unpublished data - pers. com.). Whether or not the cachexia seen in patients with amoebiasis is due to the effects thereof or to malnutrition has not been answered. The fact that the nutritional status of the patients improve following treatment and remain good for at least two months thereafter indicates that the disease may be responsible for the malnutrition and not vice versa.

Latour and Reeves (1965) provided evidence that iron was necessary for the growth of E. histolytica. From animal experiments Diamond et al (1978a) concluded that "introduction of relatively large amounts of iron, either parenterally or by feeding enhances the virulence of a virulent strain of axenically cultivated E. histolytica in an experimental animal". Bothwell et al (1984) could not demonstrate an elevated iron content in liver tissue obtained from autopsy cases of amoebic liver abscess compared to that of a control population sample.

Singh (1959) was able to demonstrate that the addition of cholesterol to culture media could make a previously non-pathogenic

strain of E. histolytica pathogenic to laboratory mice. His experiments however, could not be duplicated by Neal (1960). Bos and van de Griend (1977) demonstrated that the virulence of a pathogenic strain of E. histolytica could be restored by culture of the amoeba in an axenic medium rich in cholesterol.

1.4.4 Rapid Passage

The positive correlation between high prevalence rates of E. histolytica and overcrowding has been well described (Munoz, 1986). In addition, many researchers (Healy & Gleason, 1966; Neal & Vincent, 1956; Neal, 1951; Bos & Hage, 1975) have shown that the attenuated virulence of a pathogenic strain (by prolonged axenic culture) could be restored by passage through hamster liver. Powell et al (1966) noting these facts, postulated that rapid faecal transfer of the parasite, as would occur in a densely populated area, might in fact enhance the virulence of E. histolytica. Elsdon-Dew (1978) went on further to state that rapid transfer of faecal material from one human to another "implies either enhancement of virulence or some infective agent. As invasive amoebae do not produce cysts and are themselves too fragile to withstand the exterior (environment), we can rule out enhancement of virulence. Thus, some infective agent is indicated". Having ruled out bacterial and helminth infections he (Elsdon-Dew, 1978) hypothesised a viral agent as the trigger mechanism. Elsdon-Dew (1978) argued thus: "for the sake of argument envisage long external exposure, the cysts of E. histolytica would not survive

long enough to reach a new host and there would be neither commensal nor invasive amoebiasis. Such cysts might be able to survive days and at this transfer rate there would be commensal amoebiasis but no invasion. The virus could only survive a matter of hours and only where there was extremely rapid transfer would there be both commensal and invasive amoebiasis". While Diamond et al (1972) have demonstrated the presence of viruses in axenic pathogenic strains of E. histolytica the viruses would not influence the virulence of amoebae maintained in culture. A South African group recently described identical viruses in both pathogenic and non-pathogenic strains of E. histolytica (Olivier et al, 1984).

1.4.5 Conclusion

Thus, apart from the work of Singh (1959) which in fact could not be duplicated by Neal (1960) , no researcher has come up with a plausible trigger mechanism in spite of vigorous research activity in this area. The idea of a mutation has however been maintained from textbook to textbook - based merely on an hypothesis and not on firm scientific foundations. "Meanwhile the parasite continues to bite, as it were, on Mondays but not on Tuesdays - a habit distressing to orderly minds" (Editorial, 1979).

1.5 THE TWO SPECIES THEORY

1.5.1 Introduction

Brumpt (1928), noting the difference in prevalence of invasive amoebiasis in tropical climates and temperate areas put forward the concept that E. histolytica comprised two morphologically identical species. The one, which he termed E. dispar, was proposed to be non-pathogenic and confined to temperate zones, where in spite of a relatively high prevalence of asymptomatic carriers, the prevalence of disease was low. The other, for which he retained the name E. dysenteriae, he postulated was the pathogenic species (as its name implies) responsible for invasive amoebiasis; in addition, he claimed that this species was more prevalent in tropical zones where the prevalence of disease was considerably higher. In his opinion (Brumpt, 1928) it was possible to have healthy carriers of E. dysenteriae in whom there was mild tissue invasion, the amoebae in such cases feeding predominantly on bacteria; if for some reason the host's defences were compromised, E. dysenteriae then invaded into deeper tissues giving rise to symptoms. Such changes in host defence mechanisms however, did not have any effect on the invasive capability of E. dispar. E. dispar and E. dysenteriae in Brumpt's opinion had independent life cycles, both of them producing quadrinucleate cysts which were morphologically identical. From animal experiments he (Brumpt, 1928) was able to show that

infection with E. dispar did not confer immunity to infection with E. dysenteriae and vice versa (Brumpt, 1928).

1.5.2 Objections to Brumpt's Hypothesis

Even though this hypothesis best described the epidemiology of the parasite it was not widely accepted mainly (in the opinion of this author) because of such vociferous promotion of the Promethean and Commensalist theories. Dobell (1919) totally rejected the possibility of commensal non-pathogenic E. histolytica. Hoare (1950), one of the first to prove the existence of a commensal phase of E. histolytica commenting on Brumpt's hypothesis says "It is difficult to admit the existence of a non-pathogenic race of this amoeba when it is known to be potentially pathogenic even when its virulence for man is not manifested. Thus, it has been demonstrated that strains from diverse sources - including carriers from countries with a temperate climate - are capable of producing ulceration of the gut in experimentally infected animals".

Singh (1973), points out firstly, that amoebae from carrier cases do on occasions produce disease in experimental animals; secondly, that the virulence of amoebae vary in culture and thirdly, that a non-pathogenic strain can be made pathogenic by the addition of cholesterol. He (Singh, 1973) concludes that these findings "do not support the hypothesis that the large race of E. histolytica consists of two stable races differing only in virulence" and that "cholesterol is one of the triggers which can change the erstwhile

commensal amoeba into an invasive form". Elsdon-Dew (1968, 1978), rejected the two species hypothesis because he believed that invasive amoebae did not produce cysts and were thus unable to complete their life-cycle. This is surprising since Elsdon-Dew and co-workers (Geekie et al, 1958) did show that amoebae derived from amoebic liver abscess and grown in Locke-egg medium together with a bacterial complex "M" did produce cysts in vitro!

1.5.3 Strain Variation in E. histolytica

Neal (1951, 1957), Neal and Vincent (1955) and Bird & Neal (1962) concluded that there is a marked difference between strains isolated in culture from asymptomatic carriers and those isolated from acute cases of amoebic dysentery. The strains isolated from acute cases of amoebiasis produced ulceration in the caeca of laboratory mice while those from symptomless carriers, with a few exceptions were unable to invade the caecal wall. Such differences in strain variation were also demonstrated by Meleney (1944), Meleney & Frye (1937), Singh et al (1963), Vinayak et al (1977, 1981) and Guires (1982), amongst others.

Dobell and Laidlaw (1926), were amongst the first to demonstrate that prolonged culture could result in the attenuation of virulence of a pathogenic strain. Healy & Gleason (1966), Vincent & Neal (1956), Neal (1951, 1956, 1957) and Bos and Hage (1975) discovered that virulence could be restored to such attenuated strains by animal passage. Neal (1957) and Neal & Vincent (1956) however,

could not alter the virulence of a known non-pathogenic strain by passage through hamster liver; on the basis of these results they (Neal & Vincent, 1956) proposed that E. histolytica comprised two groups of amoebae differing only in virulence. Neal (1965) also demonstrated that cyst production occurred in a pathogenic strain of E. histolytica maintained in in vitro culture; trophozoites cultured from these cysts were still capable of producing caecal ulceration in laboratory animals. He (Neal, 1965) therefore concluded that no loss of virulence occurred during the process of encystation. The pathogenic strain of E. histolytica could therefore independently complete its life-cycle. However, in spite of having abundant evidence indicating the existence of two species of E. histolytica, Neal (1958) later stated that this concept was contradicted by epidemiological evidence. Neal (1958) argued that "...if there were two races or varieties of E. histolytica differing only in virulence, the geographical distribution of amoebic dysentery would suggest that the virulent race was confined to the tropics" and that "with the world-wide movement of troops during 1914-1918 and 1939-1945 the virulent race should have been introduced into many new localities; however, these fears proved groundless and dysentery due to E. histolytica is still rare". Neal (1958) went on to propose a change in virulence as shown diagrammatically in Figure 1.3.

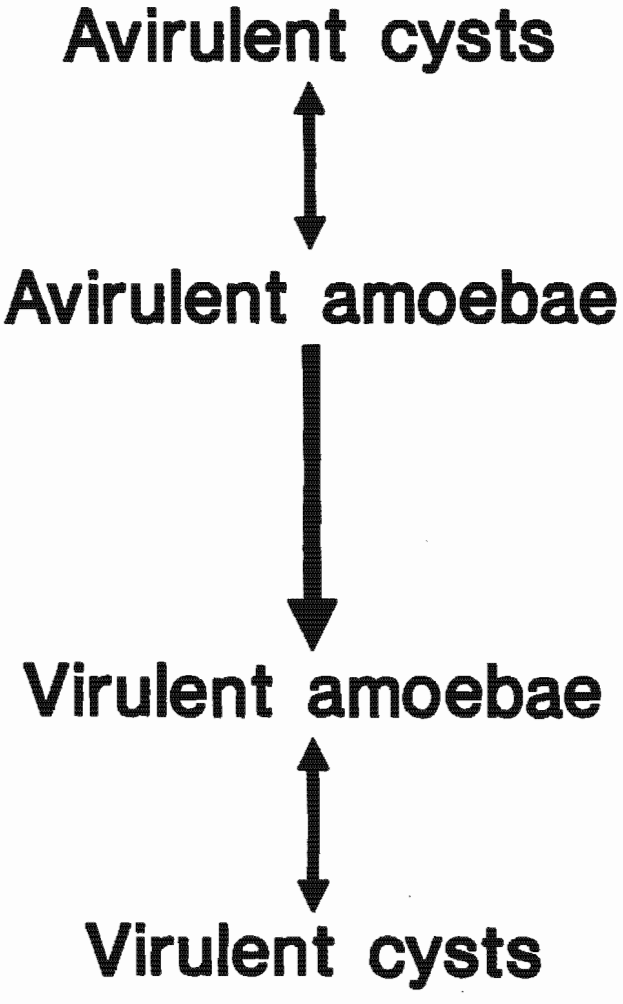

Figure 1.3 : Neal's representation of the life-cycle of E. histolytica (Neal, 1958).

However, it is interesting to note that Hoare (1958), in the light of evidence supporting the existence of strain variation, changed his earlier view (Hoare, 1950) (see page 21) asserting that "The human entamoebae with 4 nucleate cysts are represented by three types: the small non-pathogenic E. hartmanni and two strains of the large E. histolytica which differ in virulence, one being a harmless commensal that lives only in the lumen of the gut, while the other is a pathogen capable of invading the host's tissues with the production of pathological changes and clinical manifestations of amoebiasis". Unfortunately, at that time the only reliable method of determining whether or not a particular isolate of E. histolytica was a pathogen was by animal inoculation which made it completely impracticable for epidemiological studies.

During the years between 1958 and 1978 various other differences in the physiological properties between pathogenic and non-pathogenic E. histolytica were observed.

Trissl et al (1978) demonstrated that E. histolytica isolated from asymptomatic carriers phagocytosed erythrocytes more slowly than those isolated from patients with dysentery. Another group also under the leadership of Trissl (Trissl et al, 1977) found that concanavalin A could agglutinate pathogenic strains of E. histolytica to a greater extent than isolates of carrier strains of the amoeba.

Bos (1975) discovered that pathogenic strains of E. histolytica could be monaxenised with Crithridia spp. and even axenised while the same was impossible with strains from asymptomatic individuals.

It was not until 1978 that a reliable, reproducible biochemical assay was developed by Sargeaunt and co-workers (Sargeaunt et al, 1978) which could distinguish between pathogenic and non-pathogenic strains of E. histolytica on the basis of the electrophoretic isoenzyme patterns of four selected enzyme systems. As this piece of work is pivotal to the present thesis it will be discussed more fully.

1.5.4 Isoenzyme homogeneity in pathogenic and non-pathogenic E. histolytica

The electrophoresis of E.C. 2.7.5.1 phosphoglucomutase (P.G.M.) into its separate isoenzymes was first performed by Spencer et al (1964). Using human red cell lysates and tissue extracts from different individuals they observed that several distinct isoenzymes appear in each individual and further, that there were often clear cut differences between individuals; they were also able to demonstrate that these differences were genetically related and came to the conclusion that the isoenzymes represent a new human polymorphism.

Reeves et al (1967) the first to examine the electrophoretic isoenzyme patterns of E.C. 2.7.1.1 hexokinase (HK) in amoebae,

found the same two hexokinase (glucokinase) isoenzymes from each of the nine strains of E. histolytica of diverse geographical origin that they examined; all except one of these strains were isolated from naturally infected diseased humans. Furthermore they noted that the isoenzymes of H.K. found in E. histolytica were different to those of the low temperature strains of E. histolytica-like amoebae. The HK isoenzymes of the latter were identical to those of E. moshkovskii but different to those of E. invadens. They (Reeves et al, 1967) concluded that the electrophoretic behaviour of hexokinase provides a means of characterising typical E. histolytica and may help in distinghishing it from closely related organisms which were not human pathogens.

Montalvo & Reeves (1968) subsequently investigated eight cultures of E. histolytica by means of isoenzyme electrophoresis and found a single E.C. 5.3.1.9 glucose phosphate isomerase (G.P.I.) isoenzyme common to all; the isoenzymes of G.P.I. in E. histolytica were in addition distinct from those in the room temperature strains of E. histolytica-like amoebae, E. moshkovskii, E. terrapinae and E. invadens.

Reeves and Bischoff (1968) investigated three additional enzymes, ie P.G.M., E.C.1.1.1.40 L-malate:NADP dehydrogenase (decarboxylating) (M.E.) and an unclassified enzyme called NADP diaphorase as well as H.K. and G.P.I. described above. They observed that there were differences among the strains of E. histolytica regarding the isoenzymes of NADP diaphorase; and thus

for the first time polymorphism in E. histolytica had been recorded.

Ten years later Sargeaunt et al (1978) extended these observations even further: they studied the isoenzymes of P.G.M., G.P.I. and M.E. by means of electrophoresis and reported the following:

1 M.E. produced a single band and it appeared consistently in the same position for all the strains of E. histolytica studied.

2 G.P.I. produced three zones of activity termed alpha, beta and gamma; alpha being closest to the cathodal region and gamma towards the anode; they observed patterns of alpha, beta, alpha + beta and alpha + gamma in isolates of E. histolytica.

3 P.G.M. produced activity in four zones; alpha, beta, gamma and delta (delta being furthest from the cathode); alpha, beta, alpha + beta, alpha + gamma combinations were observed in various isolates of E. histolytica.

4 Notably, isolations of E. histolytica from asymptomatic carriers consistently showed P.G.M. isoenzymes in either alpha or combinations of alpha + beta or alpha + gamma positions while isolations from patients with invasive amoebiasis had a single P.G.M. isoenzyme in the beta position. For the very first time it had been shown that pathogenic and

non-pathogenic amoebae expressed different isoenzymes of P.G.M. and could thus be distinguished from each other.

Using the above three enzyme systems Sargeaunt and Williams (1978) observed isoenzymes differences in E. coli and E. histolytica. In 1979 Sargeaunt & Williams described the isoenzymes of the intestinal amoebae of man ie E. histolytica, E. hartmanni, E. coli, Endolimax nana and Iodamoeba butschlii and Dientamoeba fragilis; they pointed out that all the species were easily distinguishable by their characteristic isoenzyme patterns.

In another study by Sargeaunt et al (1980a) the isoenzymes of old well-documented strains of E. histolytica and the room temperature strains of E. histolytica-like amoebae as well as E. moshkovskii, E. invadens and E. chattoni were examined. The authors noted that "because of their very clear histories of symptomatic amoebiasis all the old well-documented strains of E. histolytica, such as NIH: 200, HK 9 etc, were as expected, found to belong to E. histolytica Group II"; these strains of E. histolytica have a "pathogenic" P.G.M. isoenzyme pattern with the isoenzymes showing strong activity in the beta position after electrophoresis. It is therefore apparent that isoenzyme polymorphism is a stable characteristic of E. histolytica since some of the E. histolytica strains had been in continuous in vitro culture for as long as 20 years. Furthermore, P.G.M. isoenzyme activity in the beta position was now established as a reliable marker for pathogenicity.

Farri et al (1980) thereafter described the HK of E. histolytica. They noted strong HK activity in two paired positions (Fig. 1.4) and pointed out that those strains which had previously shown a PGM isoenzyme band in the beta position always had advanced migration of the paired HK isoenzyme bands and could be clearly distinguished from strains with PGM activity in the alpha position. The differentiation of the pathogenic from the non-pathogenic strains of E. histolytica using the isoenzyme patterns of P.G.M. could thus be confirmed by the characteristic H.K. isoenzyme patterns.

In epidemiological studies in Mexico (Sargeaunt et al, 1980b; Sargeaunt et al, 1982a) and South Africa (Sargeaunt et al, 1982b,c; Jackson et al, 1982) the advanced HK isoenzymes as well as PGM isoenzyme activity in the beta position were verified as reliable and biochemically reproducible markers of pathogenic E. histolytica. The term "zymodeme" was introduced; it refers to a strain of E. histolytica determined by isoenzyme electrophoresis. By 1982 (Sargeaunt & Williams, 1982) a total of 18 zymodemes had been described (Fig. 1.5).

1.5.5 Conclusion

From the foregoing it has been shown that there is an abundance of data to support the existence of pathogenic and non-pathogenic strains of E. histolytica; some of these differences are summarised in Table 1.1. It is possible that the non-pathogenic strain (E.

dispar of Brumpt, 1928), is the more hardy and is therefore widely distributed in both tropical and temperate areas while the pathogenic strain (E. dysenteriae of Brumpt, 1928) has adapted to live in the tropics and sub-tropics where climatic and environmental conditions facilitate its transmission, producing a high prevalence of symptomatic amoebiasis in these areas.

TABLE 1.1 Differences between pathogenic and non-pathogenic strains of E. histolytica

Characteristic	Pathogenic	Non-pathogenic	Source
Caecal ulcers in mice	Yes	No	A
Resistance to lysis by complement	Yes	No	B
Agglutination in concanavalin A	Yes	No	C
Erythrophagocytosis	Fast	Slow	D
P.G.M. isoenzymes on electrophoresis	activity in beta position	activity in alpha position	E
H.K. isoenzymes on electrophoresis	Fast migrating	Slow migrating	F
Ability to be axenised	Yes	No	G
Passage through hamster liver	increases virulence	remains avirulent	H

Legend

A = Neal (1951, 1957); Neal and Vincent (1955); Bird and Neal (1962); Meleney (1944); Meleney and Frye (1937); Sing et al (1963); Vinayak et al (1977, 1981); Guires 1982).
B = Read et al, 1983
C = Trissl et al, 1977
D = Trissl et al, 1978
E = Sargeaunt & Williams (1979); Sargeaunt et al (1978).
F = Farri et al, 1980
G = Bos (1975
H = Neal (1957); Neal & Vincent (1956).

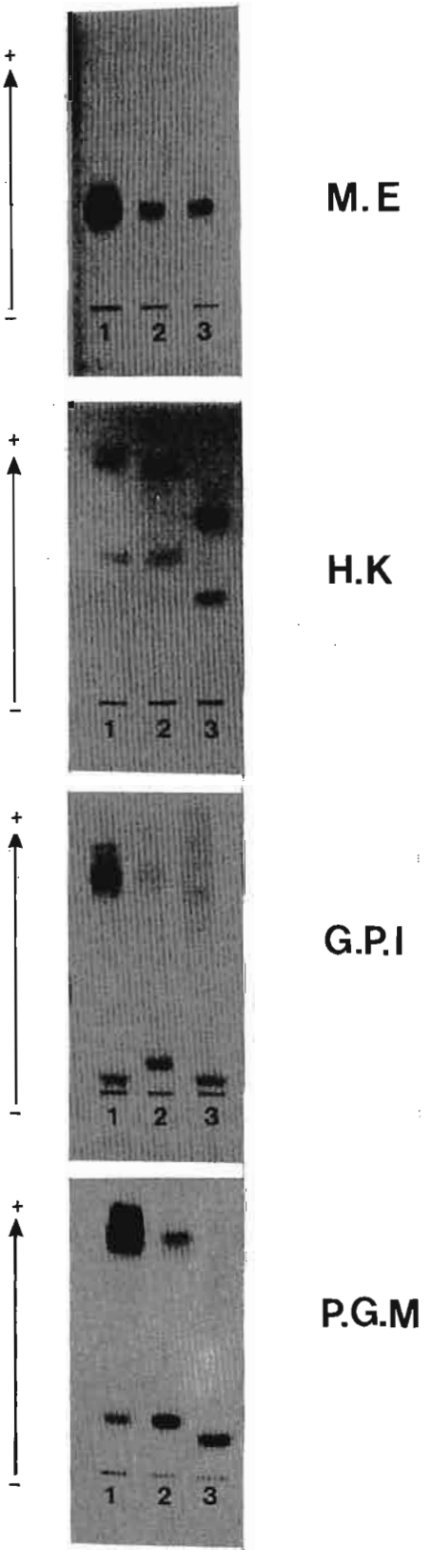

Figure 1.4 Zymodemes of pathogenic (lanes 1 & 2) and non-pathogenic E. histolytica (lane 3). NB: These are considered to be the "pure" forms of E. histolytica since they have single isoenzymes of PGM and GPI (Sargeaunt et al, 1984).

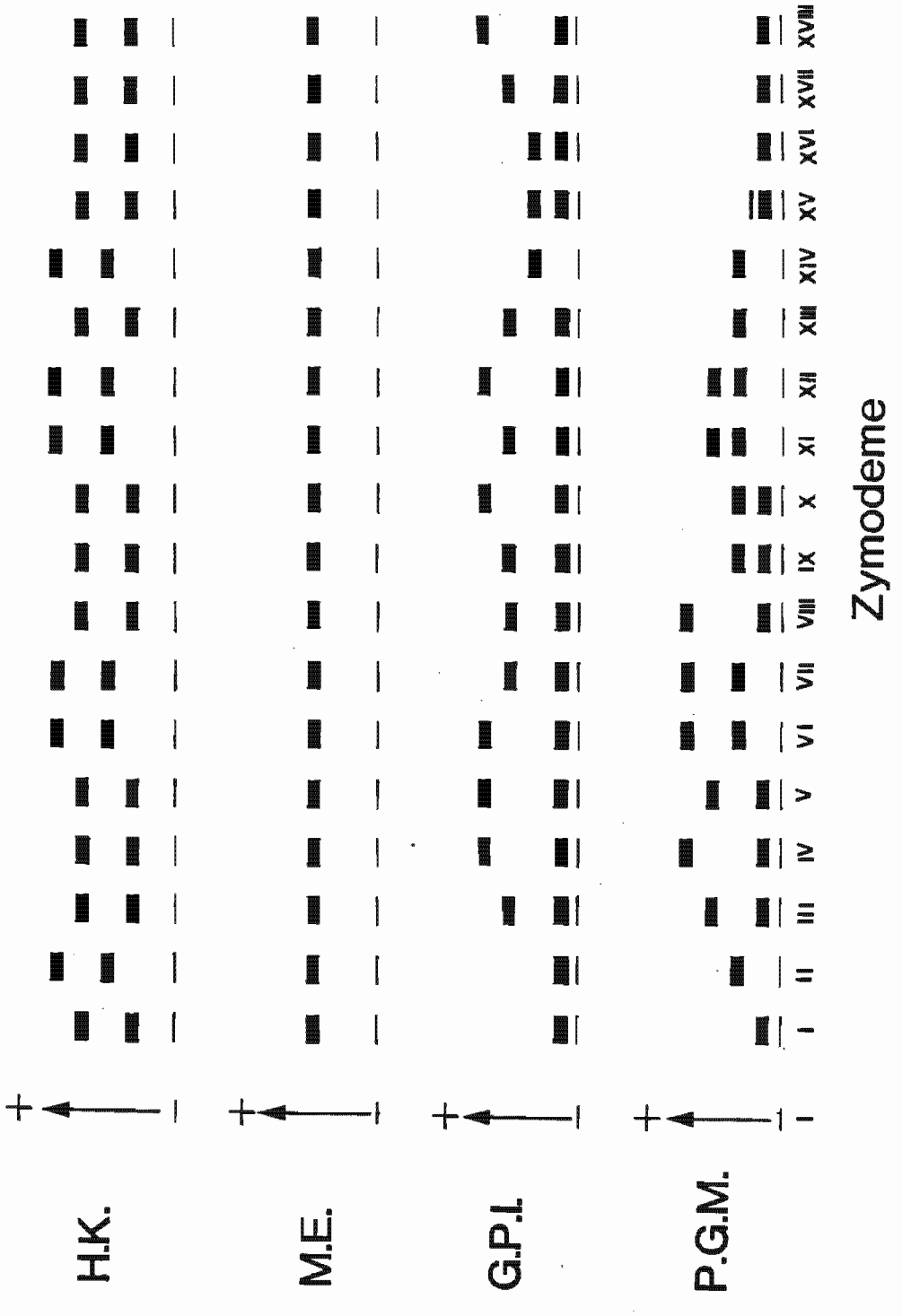

Figure 1.5 Zymodemes of E. histolytica (Sargeaunt & Williams, 1982)

1.6 AIMS AND OBJECTIVES OF THE STUDY

The methods of culturing of E. histolytica and performing isoenzyme electrophoresis had been established at the Research Institute for Diseases in a Tropical Environment at Durban by 1980. Preliminary surveys had shown the methods to have enormous potential for studying the unresolved mechanism of pathogenicity described earlier in this Chapter. A detailed epidemiological survey was therefore devised to comparatively investigate the relationships between the observed patho-physiological features of the organism and its host.

The following were the principal objectives of the study:

1 To determine the zymodemes of E. histolytica involved in the pathogenesis of amoebic liver abscess and amoebic dysentery.

2 To determine the prevalence of E. histolytica in a rural population and the frequency distribution of its zymodemes.

3 To attempt to determine the longevity of infection with the pathogenic as well as the non-pathogenic zymodemes and follow the natural history of infection by the pathogenic zymodemes.

4 To determine the seroepidemiology of amoebiasis in this population and to correlate the serological response to the zymodeme harboured by an individual.

5 To determine whether the pathogenic zymodemes are capable of producing cysts ie of completing their life-cycle.

CHAPTER 2

FREQUENCY DISTRIBUTION OF PATHOGENIC ZYMODEMES OF ENTAMOEBA HISTOLYTICA IN PATIENTS WITH INVASIVE AMOEBIASIS

2.1 INTRODUCTION

In epidemiological studies in Mexico, India and South Africa (Sargeaunt et al, 1978; 1980b; 1982a,b,c; Jackson et al, 1982) it was reported that pathogenic zymodemes II, VI, VII, XI and XII were always isolated from patients with amoebic liver abscess (ALA) or amoebic dysentery (AD). All the old, well-recorded, cultures of E. histolytica which had been isolated from diseased hosts and which had been shown to possess invasive properties in laboratory mice were found to belong to zymodeme II (Sargeaunt et al, 1980a). These pathogenic zymodemes can easily be distinguished from the non-pathogenic zymodemes by isoenzyme characterisation of amoebic lysates by the presence of a) phosphoglucomutase (PGM) isoenzyme in the beta position with the concurrent of the isoenzyme in the alpha position and b) more rapidly moving hexokinase (HK) isoenzymes (Fig. 1.4).

The study described in this chapter was primarily intended to determine:

1 The relative frequency of occurrence of the known pathogenic zymodemes in patients with ALA and AD in a South African population, and

2 To identify any new pathogenic zymodemes occurring in the study population.

During the course of the work culture methods were used to isolate E. histolytica from the liver pus; an opportunity thus presented itself to compare the effectiveness of microscopy and culture techniques in confirming the involvement of E. histolytica in liver abscesses in this environment. Patients with ALA or AD were treated with either metronidazole (Flagyl; Maybaker) or tinidazole (Fasigyn; Pfiser); for completeness the results of this treatment programme (Simjee et al, 1985a) will also be presented since certain pertinent epidemiological observations were made during the course of that work that have not previously been reported.

2.2 PATIENTS AND METHODS

The study was conducted at King Edward VIII Hospital in Durban during the period January to September 1981.

2.2.1 Amoebic Liver Abscess

Forty-eight Black patients (4 females and 44 males) with clinically diagnosed ALA were studied. Whenever possible the diagnosis of liver abscess was confirmed by ultrasound examination of the liver.

Sigmoidoscopy was performed on all patients. Where possible, a pre-treatment stool specimen was taken.

All abscesses were evacuated at the bed-side. Prior to aspiration the investigator scrubbed and donned gloves and mask. The area between the nipple-line and iliac fossa was swabbed first with 3% cetrimide and 0,3% chlorhexidine (Savlon; ICI Pharmaceuticals Ltd, South Africa) and then with 0,5% chlorhexidine gluconate in 70% industrial methylated spirits (Hibitane; ICI Pharmaceuticals Ltd, South Africa). Aspiration was performed under local anaesthesia (2% lignocaine) either at the point of maximal tenderness over the liver, or at the ideal point indicated by ultrasound examination of the liver. The first 10mℓ of pus obtained was immediately inoculated into Port-a-cult (Becton Dickinson & Co., Cockeysville, Maryland, USA) transport medium for bacteriological examination. The remaining pus was collected in 50mℓ aliquots; the terminal 50mℓ of pus was used for parasitological examination.

Patients were randomly allocated to treatment with either metronidazole (Flagyl, Maybaker) or tinidazole (Fasigyn, Pfizer) at a dose of 2g per day for 5 consecutive days; treatment was commenced immediately after aspiration. Abscesses were reaspirated again in those cases in which this was indicated on clinical grounds; the procedures described above were followed. If the patient did not show any clinical improvement after 5 days of therapy or if symptoms recurred thereafter, a second course of the same drug was administered. Post-treatment stool specimens were taken from

patients prior to discharge from hospital. Patients were discharged from hospital 5 days after completion of treatment.

Subsequently the patients were seen in the outpatient department 2 and 6 weeks following discharge and monthly thereafter. Ultrasound examination of the liver was performed monthly until complete resolution of the abscess occurred.

2.2.2 Amoebic dysentery

Nineteen Black subjects (two females and 17 males) were studied. The diagnosis of amoebic dysentery was made by finding haematophagus amoebae in the stools of patients presenting with dysentery. All patients were subjected to a sigmoidoscopic examination using a rigid sigmoidoscope with a standard cold light source; ulcer healing was monitored by sigmoidoscopic examination on days one and five of treatment and five days following completion of treatment. Where possible material for culture was obtained directly from ulcers in the sigmoid colon or rectum. Patients were randomly allocated to the two treatment groups viz metronidazole or tinidazole at a dose of 2g per day for 5 consecutive days. Retreatment conditions and patient follow-up were identical to those described above with ALA.

2.2.3 Bacteriological examination of pus

1 A gram stain was prepared for microscopy.

2 Pus was inoculated onto the following solid-plate media:

Two blood agar plates, 1 Mac Conkey's agar plate and 1 neomycin-blood agar plate. One blood agar plate and the neomycin-blood agar plate were anaerobically incubated in a Gas-Pak MD21030 (BBL microbiological Systems; Becton Dickinson & Co., Cockeysville, Maryland, USA) anaerobic jar with a Gas-Pak envelope for 48 hours. The MacConkey's agar plate and the other blood agar plate were aerobically incubated; the latter also contained 5% CO_2.

3 A small volume of pus was inoculated into thioglycollate liquid medium and aerobically incubated; it was sub-cultured after 24-48 hours on solid plates as described above. All isolates were identified by conventional methods.

2.2.4 Parasitological examination of pus

Specimens were processed as follows:

1 A wet smear was prepared for direct microscopy.

2 Amoebae were isolated in culture using:

a Locke egg medium according to the method of Freedman et al (1958)

b Robinson's medium (Robinson, 1968)

Sub-cultures were performed every 48 hours.

Smears of isolates were prepared by means of a modified Gomori stain according to previously described methods. (Sargeaunt & Williams, 1982).

2.2.5 Parasitological examination of stools

Specimens were processed as follows:

1 A wet smear was prepared for direct microscopy.

2 Amoebae were isolated in culture using Robinson's medium (Robinson, 1968). Smears of isolates were prepared as described above.

2.2.6 Isoenzyme electrophoresis of amoebic lysates

Water soluble lysates were prepared from all E. histolytica isolations according to the method of Sargeaunt & Williams (1979). These were stored as frozen beads in numbered vials in liquid nitrogen. Isoenzyme electrophoresis was performed on these in batches of 10 according to previously described methods (Sargeaunt & Williams, 1979). The enzymes studied were PGM and HK in addition to L-malate NADP oxidoreductase (decarboxylating) (ME) and glucose phosphate isomerase (GPI). A standard control (Strain 1604, zymodeme II) was included on each occasion the electrophoresis was performed. Permanent copies of electrophoretograms were prepared using a conventional photocopy machine - these were filed for future reference.

2.2.7 Serology

Serological observations on these patients are reported in Chapter 5; both the amoebic gel diffusion (AGDT) and the indirect immunofluorescent antibody (IFAT) tests were used (a strongly positive serological response was noted in all the sera tested).

2.3 RESULTS

2.3.1 Microbiology of the liver pus

E. histolytica was identified in a total of 42 cases (87%). In 10 specimens (21%) the parasite was seen on direct microscopy of the wet preparation. Using culture techniques the pathogen was isolated in 38 specimens (79%). Ten of the cultures died before lysates could be prepared. In all cases the liver pus obtained at the time of the initial aspiration was bacteriologically sterile (Gathiram et al, 1984). Secondary infection of an ALA with Escherichia coli occurred in a single case; the organism was sensitive to cotrimoxazole; cure was obtained on conservative management with this antibiotic.

2.3.2 Frequency distribution of zymodemes in ALA and AD.

Isoenzyme electrophoresis was performed on the lysates prepared from the isolates from both ALA and AD patients. All these isolates proved to be pathogenic zymodemes.

2.3.2.1 ALA

The zymodeme distribution of *E. histolytica* isolated from the ALA patients is shown in Table 2.1. Zymodemes XX was isolated for the first time during this study and has been described elsewhere (Gathiram and Jackson, 1985). Fig. 2.1 diagrammatically illustrates its isoenzyme bands in comparison to other more commonly occurring zymodemes.

TABLE 2.1 Frequency distribution of zymodemes in ALA patients

Zymodeme	Frequency
II	23/28 = 82,1%
XI	4/28 = 14,3%
XX	1/28 = 3,6%

2.3.2.2 Amoebic Dysentery

E. histolytica was successfully isolated in culture in only 10 (52,6%) patients. Table 2.2 depicts the frequency distribution of the zymodemes identified.

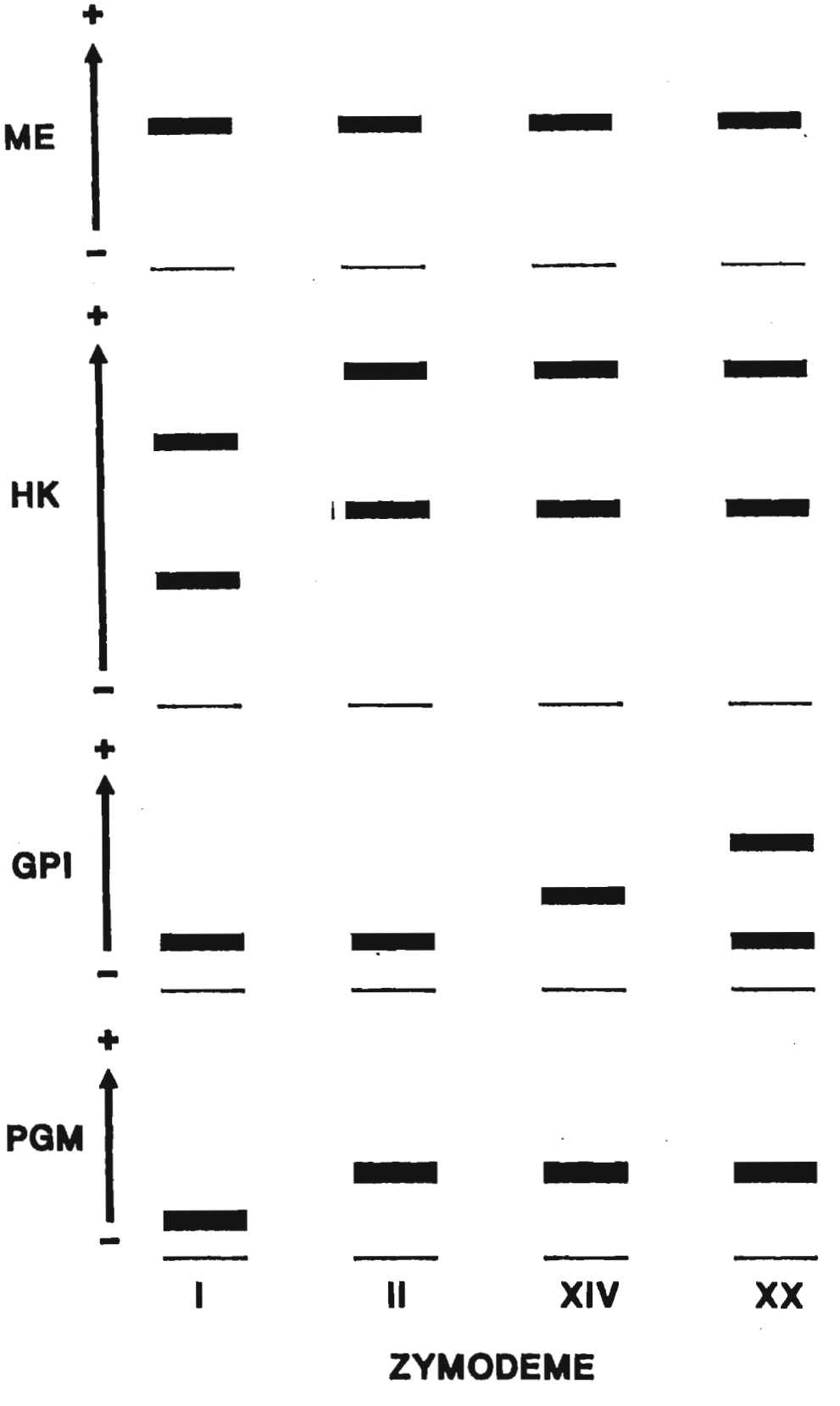

FIGURE 2.1 : Graphic illustration of the isoenzyme bands of zymodemes I, II, XIV and XX

TABLE 2.2 Frequency distribution of zymodemes in AD patients

Zymodeme	Frequency
II	8/10 = 80%
XI	2/10 = 20%

2.3.3 Response to Drug Treatment

2.3.3.1 ALA

Twenty seven patients received metronidazole and 21 patients received tinidazole. The two treatment groups were comparable in terms of age, haemoglobin and white cell count, volume of pus aspirated and the clinical response to treatment (Table 2.3) (Simjee et al, 1985).

It was noticed that serial ultrasound examination of the liver revealed that the abscess cavity became increasingly hypoechoic and assumed a smoother margin as healing progressed (Simjee et al, 1985b).

TABLE 2.3 Comparison of the two ALA treatment groups

	Metronidazole n=27	Tinidazole n=21
Age (years) +/-S.D.	35,4 +/- 9,2	35,3 +/- 11
Haemoglobin (g/dℓ) +/-S.D	12,2 +/- 2,1	11,6 +/- 1,8
White cell count ($X10^9/\ell$) +/- S.D	14,0 +/- 7,1	13,9 +/- 4,7
Pus aspirated (mℓ) +/- S.D.	432 +/- 583	477 +/- 390
Time for pain to disappear (days) +/- S.D.	4,24 +/- 3,80	5,24 +/- 2,31
Time for temperature to settle (days) +/- S.D.	5,20 +/- 3,80	5,23 +/- 2,17
Time for tenderness to disappear (days) +/- S.D.	7,96 +/- 4,23	7,94 +/- 3,19

2.3.3.2 Amoebic dysentery

Eight patients received metronidazole and 11 patients received tinidazole. The two groups were comparable in respect of age, sex, haemoglobin, white cell count and response to treatment. The results are summarised in Table 2.4.

TABLE 2.4 : Comparison of the two A.D. treatment groups

	Metronidazole n=8	Tinidazole n=11
Mean age (years +/- SD)	38,8 +/- 5,1	35,5 +/- 8,2
Sex ratio (M : F)	7 : 1	10 : 1
Mean white cell count ($x10^9/\ell$)	10,1 +/- 4,2	8,6 +/- 4,7
Mean haemoglobin (g/dℓ) +/- SD	13,8 +/- 1,1	14,6 +/- 1,4
Number with ulcers on sigmoidoscopy		
Day 1	6	11
Day 2	2	5
day 10	0	0
Mean time for pain to disappear (days) +/- SD	1,6 +/- 0,3	1,4 +/- 0,6
Mean time for diarrhoea to stop (days) +/- SD	1,4 +/- 0,2	1,4 +/- 0,3

In two subjects in the metronidazole treatment group ulcers were not observed on sigmoidoscopic examination; both patients had typical symptoms of AD and had haematophagous trophozoites in their stools and positive serological results. In one of these there was a poor response to metronidazole; the patient continued to have

colicky abdominal pain and mucoid stools. He was subjected to a barium enema examination and was shown to have a stricture in the mid transverse colon which was confirmed by colonoscopic examination. Histology of biopsy specimens showed intense inflammatory reaction with no evidence of E. histolytica or malignancy. Repeated stool cultures failed to isolate E. histolytica. The stricture resolved after a 10 day course of metronidazole.

2.3.4 E. histolytica zymodemes isolated from stools of patients with ALA before and after treatment

Pre-treatment stools were only obtained from 18 ALA patients. Difficulty in obtaining specimens was due either to the patients being constipated on admission or not having had a meal in the preceding 24 hours because of anorexia. Two patients had concomitant AD; E. histolytica could not be cultured from either of them. Stool cultures failed to isolate E. histolytica in a further 8 cases. The parasite grew in one culture but died out before electrophoresis could be done. The zymodemes of the 7 successful E. histolytica isolations as well as that obtained from the abscess pus of these patients are shown in Table 2.5.

TABLE 2.5 *E. histolytica* zymodemes in the liver pus and stools of ALA patients prior to treatment

Patient Number	Zymodeme pus	Zymodeme stool
24	no growth	XI
33	culture died	XI
46	XI	II
50	culture died	II
56	II	II
69	XX	XX
83	No growth	II

The following points are of significance

i All *E. histolytica* isolations were pathogenic zymodemes.

ii Pathogenic zymodemes were isolated from approximately 40% of stools of ALA patients in the absence of dysentery

iii In one case, zymodeme XI was isolated from the pus while zymodeme II was cultured in the stools.

Post-treatment stools were obtained from all ALA patients. *E. histolytica* was successfully cultured from a total of 8 patients (14,6%); of these lysates were successfully prepared from only three. The results are depicted in Table 2.6.

TABLE 2.6 *E. histolytica* zymodemes in post-treatment stools compared to zymodemes prior to treatment

Patient Number	Zymodemes (pus)	Pre-treatment zymodeme (stool)	Post-treatment zymodeme (stool)	Treated with
7	II	No stool	died	M
9	II	died	died	T
19	II	no stool	II	T
21	II	no stool	died	M
22	II	no stool	died	M
28	died	no stool	II	T
33	died	XI	II	T
56	II	II	died	T

Legend : M = metronidazole
T = tinidazole
Died = culture died before lysate could be prepared
No stool = no stool obtained on admission

Only pathogenic zymodemes were characterised in the 3 post-treatment cases where lysates were successfully prepared. In one of these a pathogenic zymodeme was isolated from the stool prior to treatment. Five of the patients from whom *E. histolytica* was isolated post-treatment had been treated with tinidazole. Notably in one case zymodeme XI was isolated from the stool before treatment while zymodeme II was identified after treatment.

2.3.5 Post treatment relapses of invasive disease in ALA Patients

The observations on the 6 patients who required a second or subsequent course of nitroimidazole are summarised in Table 2.7.

TABLE 2.7 Data on subjects who suffered a relapse and/or recurrence of ALA following treatment

Patient Number	Relapse at	EH Zymodeme in post treatment stool	Recurrence at	EH zymodeme in pus	Drug
22	3 weeks	Culture died	N/A	N/A	M
52	16 weeks	No EH	N/A	N/A	M
78	1 week	No EH	4 years	pus not cultured	T
76	1 week	No EH	N/A	N/A	T
19	16 weeks	II	7 months	No EH	
			3 Years	II	T
28	12 weeks	II	3 years	II	T

Legend : No EH = *E. histolytica* not isolated in culture

N/A = Not applicable

M = metronidazole

T = tinidazole

NB : Post-treatment stool was taken 5 days after completion of initial treatment

Two of the six had been treated with metronidazole (Table 2.7); one of them suffered a relapse after 3 weeks (relapse is defined as the recurrence of symptoms and signs of ALA; the abscess position being unchanged on ultrasound examination). The other patient (number 52 in Table 2.7) remained asymptomatic following his initial hospital stay, however, the size and position of his liver abscess did not change in 16 weeks; thereafter complete aspiration of the ALA and a second 5 day course of metronidazole resulted in rapid and complete resolution in the following 6 weeks.

Four patients requiring retreatment had initially received tinidazole; two of them did not respond adequately to treatment and required a further 5 days of the amoebicide; in one of these, recurrence of ALA occurred after four years (recurrence is the development of a new ALA after the initial one had been shown to be completely healed). The other two patients in the tinidazole group relapsed after being discharged - one of them after 12 weeks and the other after 16 weeks; the former patient, in addition, presented with recurrence of ALA after 3 years; the latter (patient number 19, Table 2.7) presented with recurrence of ALA on two occasions and because significant epidemiological observations were made this patient will be discussed in detail in Chapter 6.5.

In those cases where relapse occurred aspiration of the ALA was not possible in one instance. In the 5 cases where pus was successfully aspirated E. histolytica could be cultured in only one; the isolate proved to be zymodeme II. In all instances the pus was bacteriologically sterile.

Of the three cases where recurrence occurred the liver aspirate was not cultured in one; in the two where E. histolytica was successfully cultured both the organisms isolated were found to be zymodeme II (Table 2.7).

Notably three patients who relapsed after being discharged had incomplete clearance of E. histolytica from their stools following the initial course of treatment (Table 2.7).

2.3.6 E. histolytica zymodemes in post-treatment stool cultures from AD patients

Post-treatment stool samples were obtained from all patients. In two cases (10,5%) zymodeme II was isolated post-treatment (Table 2.8). The zymodemes were identical to that obtained prior to treatment.

TABLE 2.8 E. histolytica zymodemes in post-treatment stools compared to zymodemes before treatment in patients with AD

Patient number	Pre-treatment zymodemes	post-treatment zymodemes	treated with	relapse at
32	II	II	M	no relapse
58	II	II	T	one month

M = metronidazole
T = tinidazole

2.3.7 Post-treatment relapses in AD

One patient (number 58, Table 2.8) suffered a relapse one month after discharge; his stool was found to be teeming with haematophagous *E. histolytica*; the organism was successfully isolated in culture and proved to be zymodeme II. Complete cure was obtained following a second 5-day course of tinidazole. Of significance is the fact that this patient still had a pathogenic zymodeme in his stool following initial resolution of dysentery (Table 2.8).

2.4 DISCUSSION

As noted previously by others (Wilmot, 1962; Maddison *et al*, 1959) bacteria are not involved in the pathogenesis of ALA as the pus is bacteriologically sterile. Culture methods considerably increase the accuracy of diagnosis of ALA by finding the causative organism; however the technology and facilities are not readily available in all centres. Furthermore this study has shown that microscopic examination of freshly aspirated terminal pus will ensure a diagnosis in 21% of cases; in the remainder a reasonable diagnosis can be made on the basis of the nature of the pus in addition to confirmation of its bacteriological sterility, positive serological finding (see Chapter 5) and the response of the patient to anti-amoebic therapy.

From the zymodeme analysis it is apparent that the presence of a PGM isoenzyme band in the beta position (in the absence of one in the alpha position) supported by finding advanced hexokinase isoenzymes bands are reliable markers of pathogenic strains of E. histolytica; this reaffirms the results of Sargeaunt and coworkers (Sargeaunt et al, 1978; 1980a,b; 1982a,b,c; Jackson et al, 1982).

In agreement with the findings in Mexico (Sargeaunt et al, 1980b, 1982a) and Durban (Sargeaunt 1982b; Jackson et al, 1982) zymodeme II occurred most frequently; however zymodemes VI, VII and XII described previously from Mexico (Sargeaunt et al, 1980b, 1982a) have not been observed in the present study. Sargeaunt et al (1984) recently reported that all isolates of pathogenic E. histolytica from India belong to zymodeme XIV; included in this study were stocks from his earlier work in India which he initially identified as zymodeme II (Sargeaunt et al, 1978). The fact that zymodeme XIV has not been found in any of the Durban isolates is surprising since a large proportion of Durban's population are Indian. Until the introduction of statutory segregation of the population groups the Indian and African population groups lived "cheek by jowel" in the amoebiasis hyper-endemic area of Cato Manor (Elsdon-Dew, 1946) and therefore transmission of the parasite from one population group to the other must have occurred.

Zymodeme XX, a new pathogenic zymodeme has been described for the first time in this geographical area (Gathiram and Jackson 1985). It is similar to zymodeme II but has an additional isoenzyme band in the gamma position in GPI (Fig. 2.1).

The results indicate that metronidazole and tinidazole are equally effective in the treatment of ALA using a single oral dose of 2g daily for 5 days (Simjee et al, 1985a). The observations reported here are in agreement with those reported by Powell and co-workers (Powell et al, 1967; 1969; Powell and Elsdon-Dew, 1972) during the past two decades in this geographical area; it may therefore be concluded that there has not been development of any resistance to metronidazole since 1967. However, other investigators (Islam and Hassan, 1978; Bakshi et al, 1978 and Kokhani et al, 1978) in India and Bangladesh have reported greater cure rates with tinidazole than with metronidazole.

The poorer results obtained with metronidazole in India and Bangladesh cannot be readily explained. Having excluded the possibility of the dosages of drugs used, Simjee et al (1985a) speculate that the difference in results may be due to the fact that evacuation of all abscesses as part of the therapeutic regimen in this study may have played a role. Additionally, Sargeaunt et al (1984) have shown that there are zymodeme differences between pathogenic E. histolytica found in India and those found elsewhere; pathogenic zymodeme XIV (Fig 2.1) is found exclusively in India while the pathogenic zymodemes II, VI, VII, XI, XII, and XX have been found in other parts of the world. They (Sargeaunt et al, 1984) consider zymodeme XIV to be genotypically different to the other pathogenic zymodemes. It is possible that the variable response to the nitroimidazoles may in part be due to the variable susceptibility of the different strains of E. histolytica to the drugs; this possibility needs further elucidation in order to identify the ideal amoebicide for different geographical areas.

The post-treatment stool results have not been reported previously and only became apparent when they were analysed at the time of writing of this thesis. It will be seen from Table 2.6 that E. histolytica isolations occurred in 14,6% of ALA patients following treatment. Additionally, it is apparent from Table 2.5 that pre-treatment isolations of E. histolytica from the stools of patients with ALA are pathogenic zymodemes. It can therefore be presumed that post-treatment isolations should also be pathogenic zymodemes, since mixed infections with E. histolytica have not been recorded in the Durban area (Jackson et al, 1982). It is thus highly probable that those E. histolytica cultures that died before zymodeme determination could be done (Table 2.6) were also pathogenic zymodemes. By extrapolation therefore, incomplete cure probably occurred in a considerable number of patients, an observation observed slightly more frequently with tinidazole than with metronidazole (Table 2.6) in this study. This observation is of epidemiological importance since treatment renders the patients symptom-free very rapidly. These individuals would normally be discharged from hospital and become asymptomatic carriers of pathogenic zymodemes with the potential of transmitting the infection to others.

It is notable that patients 19 and 28, (Table 2.7) who experienced relapses after treatment and subsequently also developed recurrence of ALA still had infections with pathogenic zymodemes following what appeared to be adequate treatment of the initial ALA. A similar outcome was noted in patient number 58 in the AD group (Table 2.8) and patient 22 (Table 2.7) who also probably had

pathogenic E. histolytica in his stools after treatment. Thus if relapses and recurrences are to be prevented complete eradication of the parasite from the bowel should be mandatory; such therapeutic intervention will also curtail further transmission of the parasite and thus logically aid in decreasing the prevalence of ALA and AD. A prolonged course of metronidazole or tinidazole may effect such a cure by interrupting the life-cycle (by killing the trophozoites) while allowing excretion of the cysts. This still needs to be evaluated. Furthermore concomitant use of luminal amoebicides like diloxanide furoate or diiodo-hydroxyquin might prove valuable. Neither of the latter are presently available in South Africa and require re-evaluation.

CONCLUSIONS

The following noteworthy observations were made:

1 In the Durban area zymodemes II, XI and less commonly zymodeme XX were the only zymodemes isolated from pathological lesions where E. histolytica is obviously invasive

2 In the cases where it was possible to isolate E. histolytica from pre-treatment stools pathogenic zymodemes occurred concurrently in the faeces of patients with ALA often in the absence of dysentery.

3 Zymodeme XIV which has been reported to be the only pathogenic zymodeme occurring in India has not been isolated from patients with invasive disease

4 Both metronidazole and tinidazole are highly effective in eliciting a clinical cure in ALA and AD

5 After treatment a number of patients continued to pass cysts with pathogenic zymodemes and thus had become asymptomatic carriers

6 Clinical relapse was observed and is believed to be associated with incomplete clearance of E. histolytica from the gut

CHAPTER 3

A PARASITOLOGICAL SURVEY IN A PERI-URBAN POPULATION NEAR DURBAN (MALAGASY) WITH PARTICULAR REFERENCE TO THE ZYMODEME DISTRIBUTION OF ENTAMOEBA HISTOLYTICA

3.1 INTRODUCTION

Parasitological surveys conducted along the Natal coastal area have yielded important information regarding the intensity of parasitic infestation in this geographical area. The prevalence of *E. histolytica* from these studies has been estimated at between 4% (Schutte *et al*, 1981) and 27% (Elsdon-Dew, 1946). A notable limitation of these studies has been that they have either been hospital-based (Elsdon-Dew, 1946) or have been confined to the examination of schoolchildren (Schutte *et al*, 1981). Furthermore, in these surveys the prevalence figures for *E. histolytica* have been estimated by finding the cystic stage of the parasite in a single stool examination by direct microscopy of the specimen coupled with examination of cyst concentrates obtained by either formalin-ether sedimentation or zinc sulphate flotation methods. It has been shown that by the use of culturing techniques, especially Robinson's culture medium (Robinson, 1968) which selects for *E. histolytica*, a more accurate assessment of the carrier population can be made; culturing techniques are preferred to a combination of the above methods when it is important to determine the proportion of carriers in a population (Walsh, 1986; Bray & Harris, 1977b).

Previous studies conducted in Durban (Sargeaunt et al, 1982b,c; Jackson et al, 1982, and the study described in Chapter 2) provided a sound background on the zymodemes of E. histolytica occurring in this area. The following observations were made:

A The more commonly occurring non-pathogenic zymodemes (I and III), had a similar distribution pattern to that found by Sargeaunt et al (1980b, 1982a, 1978, 1984) in Mexico and India.

B The pathogenic zymodemes (II and XI) had comparable frequencies of occurrence in Mexico and South Africa, while zymodeme XIV (Fig 2.1) is the only pathogenic zymodeme isolated in India (Sargeaunt et al, 1984).

C All the non-pathogenic zymodemes were isolated from individuals in whom symptoms of amoebic disease was absent whereas all E. histolytica isolates from cases of proven invasive amoebiasis were pathogenic zymodemes (Sargeaunt et al, 1982b; Jackson et al, 1982; Chapter 2).

D Of significant epidemiological importance was the discovery that three E. histolytica isolations from a random hospital out-patient population proved to be pathogenic zymodemes (Sargeaunt et al, 1982b). However, since no clinical history was recorded, it was impossible to ascertain whether or not these subjects had dysentery or were genuine symptomless carriers of pathogenic zymodemes of E. histolytica. Since the

patients described in this study were visiting the hospital for such diverse illnesses as hypertension, diabetes and orthopaedic problems and not for reasons that would suggest that they had invasive amoebiasis it was concluded that they were symptomless carriers of pathogenic zymodemes.

E *E. histolytica* with the anomalous zymodeme XIII, first described from Mexico, (Sargeaunt *et al*, 1980b) was also identified in a subject from the Durban area (Jackson *et al*, 1982). This zymodeme has a pathogenic pattern in phosphoglucomutase (PGM) with a non-pathogenic pattern in hexokinase (HK); (Fig. 3.1). For lack of clinical information it was not possible to determine whether or not this "hybrid" or "mixed" zymodeme is truly pathogenic.

F Three new zymodemes ie XVI, XVII and XVIII (Fig. 1.5) were characterised for the first time (Jackson *et al*, 1982). These zymodemes have not been isolated from subjects elsewhere in the world and may therefore be unique to South Africa.

G These investigations also emphasised that culture and electrophoresis yields more precise epidemiological information than conventional microscopy (Jackson, *et al* 1982).

If the pathogenic zymodemes are in fact a distinct species (as the introduction to this thesis implies and as suggested by Sargeant (1987)), which is morphologically indistinguishable from the more

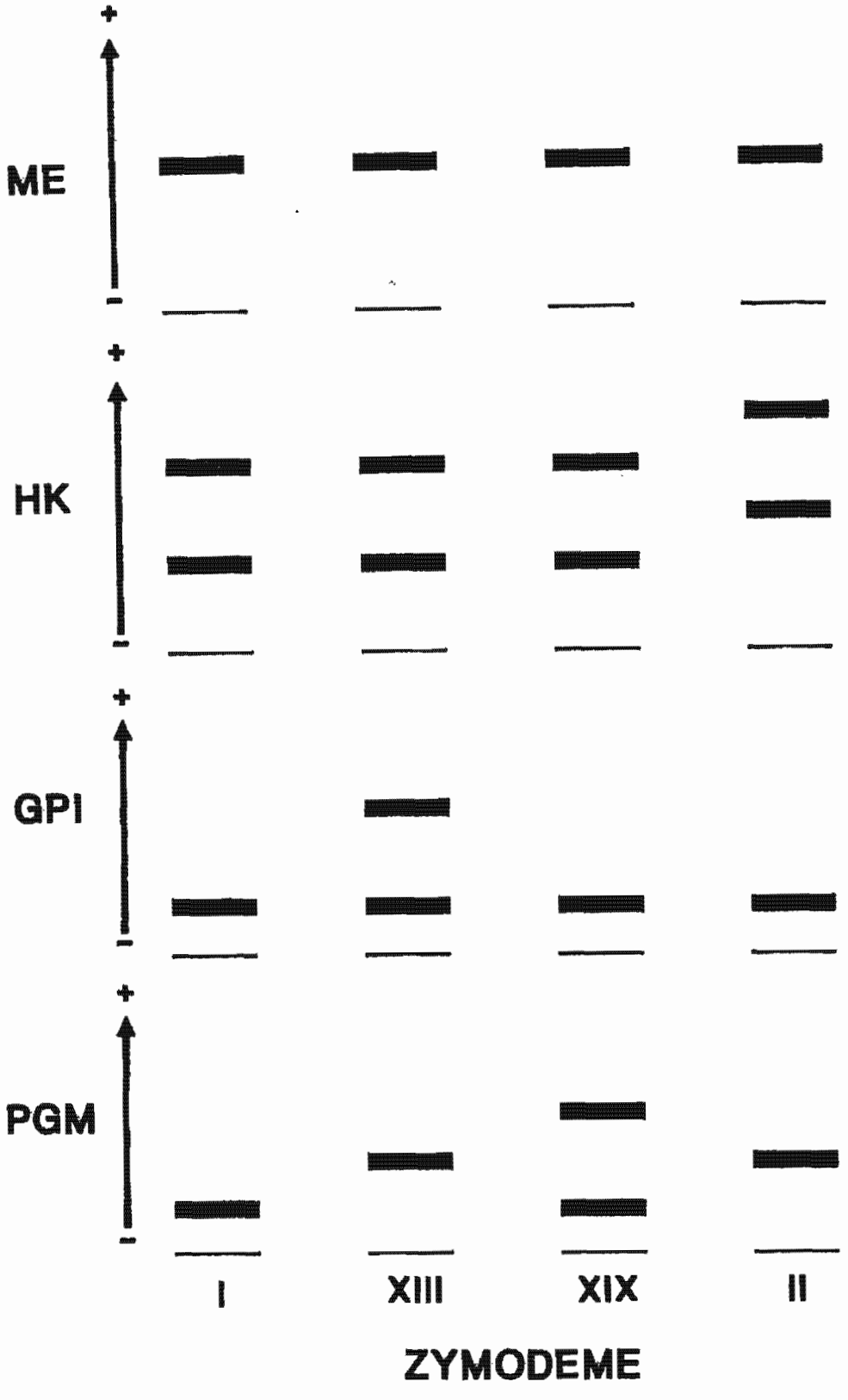

FIGURE 3.1 : Graphic illustration of the isoenzyme bands of zymodemes I, XIII, XIX and II.

commonly occurring non-pathogenic zymodemes it would be reasonable to assume that they should occur at a measurable prevalence rate in a population where amoebiasis is endemic. The results of Sargeaunt et al (1982b,c) although not conclusive, did seem to imply this.

Considering the foregoing, an extensive community-based epidemiological investigation was indicated, so that critical questions could be answered. These unresolved issues included:

1 What is the prevalence of E. histolytica in this endemic amoebiasis area?
2 The range of zymodemes and the frequencies with which they occur
3 The prevalence of E. histolytica in the different age and sex groups
4 What proportion of E. histolytica isolations are pathogenic zymodemes and how are they distributed in the population?
5 Is zymodeme XIII pathogenic or non-pathogenic?

3.2 MATERIALS AND METHODS

3.2.1 Study area

Malagasy, a moderately populated peri-urban village and its surrounds on the southern boundary of Durban was chosen as the study area. There is no piped water but water is readily available from two nearby streams and their tributaries. Many families use

rain water or buy tap water for drinking; however all families use river water for washing and may even drink it when water from another source is not available. It was therefore difficult to divide the population into users of chlorinated and non-chlorinated water. The dwellings have mud walls and floors with a roof of either thatch or corrugated iron and consist of one to five rooms; sewerage disposal facilities are absent in approximately 1/4 of the dwellings while the remainder have shallow pit latrines. The study area was chosen because of

1 its proximity to the research laboratory

2 its easy accessibility by motor car

3 it falls within the magisterial district of Umbumbulu which is an endemic amoebiasis area.

The survey was conducted between February and June of 1982.

3.2.2 Study population and collection of specimens

The total population of the village studied consisted of approximately 2500 Black people; there are approximately 240 families. Only those family units where consent was obtained from a senior family member were considered for inclusion in the study. As a result therefore, 2045 individuals from 224 families comprised the study population.

A brief clinical history with particular reference to symptoms of intestinal amoebiasis was obtained from each individual; their age, sex and occupations were also recorded. Each family unit's water source and sewage disposal facilities were also noted.

Collection of stools was made on a single occasion from all individuals in the study population; a stool jar appropriately labelled was left for each member of the family with instructions to retrieve a portion of a freshly passed stool. These were collected the following morning. A total of 1390 stool specimens were thus obtained for examination giving a compliance of about 68%. Non compliance was attributed mainly to strong traditional taboos associating stools with witch-craft; although no accurate figures are available, this tended to affect adult males more than any other section of the population. The age/sex distribution of the subjects studied is shown in Table 3.1.

3.2.3 Stool parasitology

The stool specimens were processed as follows:

1 A portion of each specimen was inoculated into Robinson's culture medium (Robinson, 1968).

2 In those cases where amoebae were successfully cultured, water soluble lysates were prepared from the harvested amoebae; these were stored as beads in liquid nitrogen.

TABLE 3.1 Age-Sex distribution of study population

AGE	MALE	FEMALE	TOTAL
0-1	45	43	88
2-5	88	122	210
6-10	141	115	256
11-15	89	100	189
16-20	49	85	134
21-25	31	62	93
26-30	34	63	97
31-35	20	43	63
36-40	21	49	70
41-45	20	23	43
46-50	15	24	39
Over 50	46	62	108
TOTAL	599	791	1390

3 A fixed stained smear of the amoebae isolated was prepared according to the modified Gomori method (Sargeaunt & Williams, 1982).

4 The zymodemes of the organisms isolated were determined according to the method of Sargeaunt and Williams (1979); the enzymes studied were glucose-phosphate isomerase (GPI);

L-Malate NADP oxidoreductase (decarboxylating) (ME); phosphoglucomutase (PGM) and hexokinase (HK). On each occasion electrophoresis was performed a standard control was included; it was a known pathogenic zymodeme II (strain 1604) which was maintained in axenic culture at the RIDTE.

5 The formol-ether concentration method was used for the first 852 consecutive specimens and these were examined microscopically for the presence of parasites. This was done to determine the level of parasitic infestation in the study population as well as to compare the prevalence of E. histolytica obtained by this method to that obtained by culture and isoenzyme electrophoresis.

3.2.4 Statistical analysis

After consultation with a statistician of the South African Medical Research Council Institute for Biostatistics, the chi-squared test was employed to test for statistical differences in parasite prevalence for the various sub-populations.

3.3 RESULTS

3.3.1 Zymodeme Distribution

In this population the prevalence of E. histolytica, inclusive of both pathogenic and non-pathogenic zymodemes, was 10% (141/1390); pathogenic zymodemes were found in apparently healthy asymptomatic

individuals at a prevalence of 1% (14/1390) (Gathiram & Jackson, 1985).

Figure 3.2 depicts the zymodeme distribution in the study population. The following observations are considered noteworthy (Gathiram & Jackson, 1981):

1 Most of the zymodemes described previously (Sargeaunt et al, 1982b,c; Jackson et al, 1982) were also isolated in this study area.

2 Non-pathogenic zymodemes I and III were the most common with zymodeme I occurring more frequently than zymodeme III;

3 Non-pathogenic zymodeme VIII was not isolated.

4 Non-pathogenic zymodemes, XVI, XVII and XVIII, which were reported for the first time by Jackson et al (1982) in this geographical area have continued to occur.

5 A new non-pathogenic zymodeme, tentatively designated zymodeme XIX (Gathiram & Jackson, 1985), has been observed for the first time. Its isoenzyme banding pattern is depicted in Figure 3.1.

6 Pathogenic zymodemes II, XI and XX described in Chapter 2, were observed infrequently with pathogenic zymodemes II and XI being the most common and occurring with the same frequency

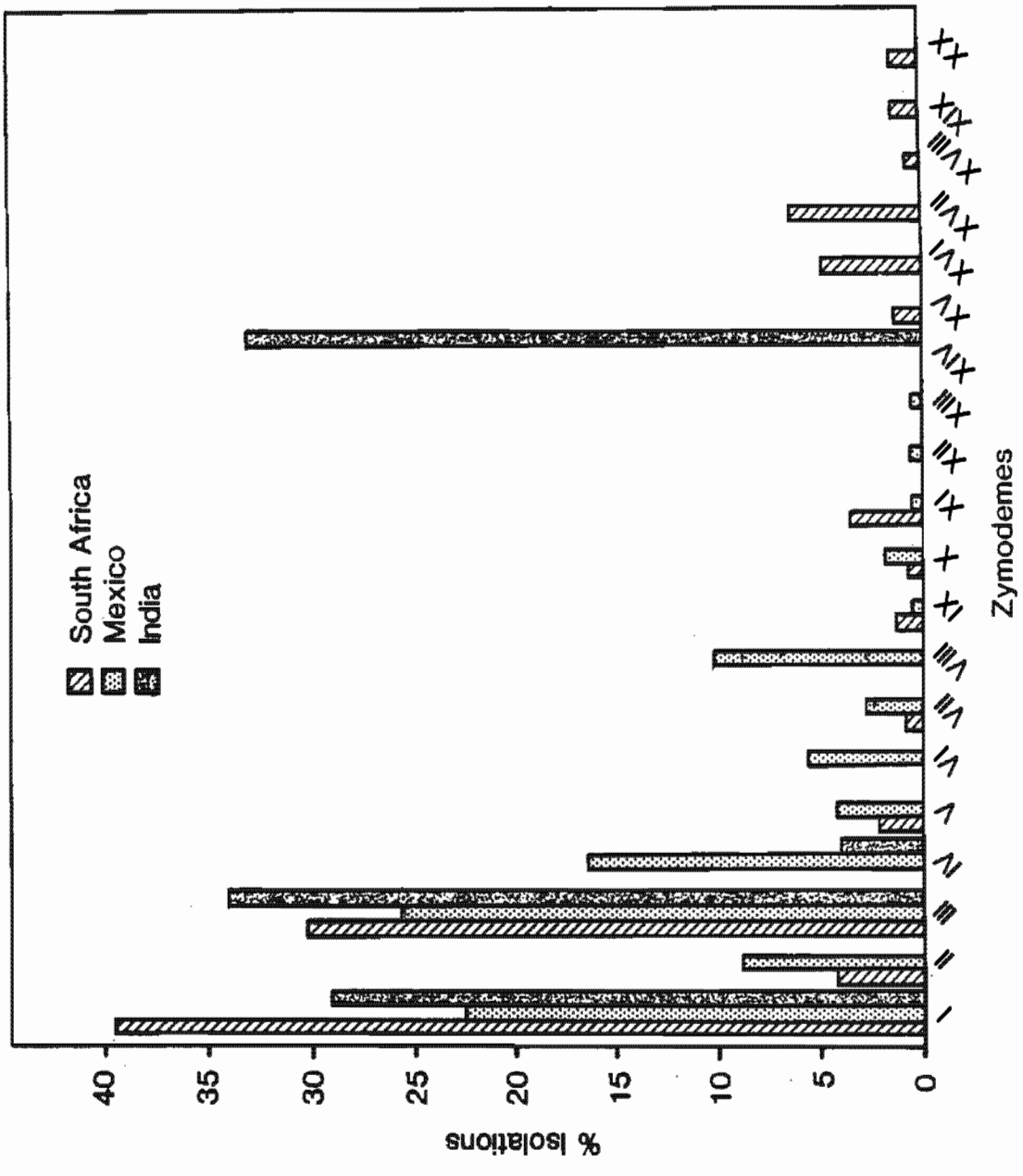

FIGURE 3.2 : Frequency distribution of zymodemes of E. histolytica in the study population (South Africa), Mexico and India

7 Zymodeme VII, a pathogenic zymodeme, described for the first time from Mexico (Sargeaunt et al, 1980a) was isolated for the first time.

8 Pathogenic zymodemes VI, XII and XIV were not isolated in this study.

3.3.2 Gastrointestinal symptoms in carriers of E. histolytica

The frequency of occurrence of diarrhoea and abdominal pain in the E. histolytica-infected and uninfected groups is summarised in Table 3.2.

TABLE 3.2 Gastrointestinal symptoms in carriers of E. histolytica.

	E. histolytica infected n=139	E. histolytica free n=1251		
	n	n	χ^2	p =
Abdominal pain	10 (7,2%)	38 (3,0%)	5,29	0,025
Diarrhoea	4 (2,9%)	20 (1,6%)	1,60	N.S.

Key : N.S. = Not significant

It is interesting that abdominal pain occurred more frequently (p=0,025) in the group infected with E. histolytica all of whom were carriers of non-pathogenic zymodemes; 24 subjects also had mild diarrhoea (between 4 to 6 loose stools per day); in most

subjects the symptoms subsided within 2-3 days. Two subjects developed severe diarrhoea and when they were admitted to hospital a diagnosis of cholera was made; they were treated conservatively and the symptoms spontaneously regressed.

Only two of the individuals from whom a pathogenic zymodeme was isolated subsequently developed mild symptoms of dysentery and abdominal pain. On sigmoidoscopic examination one of these had ulcers in the rectum and furthermore, microscopy revealed haematophagous trophozoites of E. histolytica in his stool. Both these patients were treated with metronidazole with subsequent improvement of symptoms. A more detailed account of the follow-up of these carriers of pathogenic zymodemes will be presented in Chapter 6.

3.3.3 Prevalence of E. histolytica with regard to age and sex

Figure 3.3 depicts the age-sex prevalence of E. histolytica in the study area. This was prepared from all available data and includes both pathogenic and non-pathogenic zymodemes. As described in a previous study (Oyerinde et al, 1979) infection with E. histolytica steadily increases in frequency from infancy to adult life and then tends to reach a plateau at 21-25 years. The overall prevalence of E. histolytica was greater in females (98/791 = 12,39%) than in

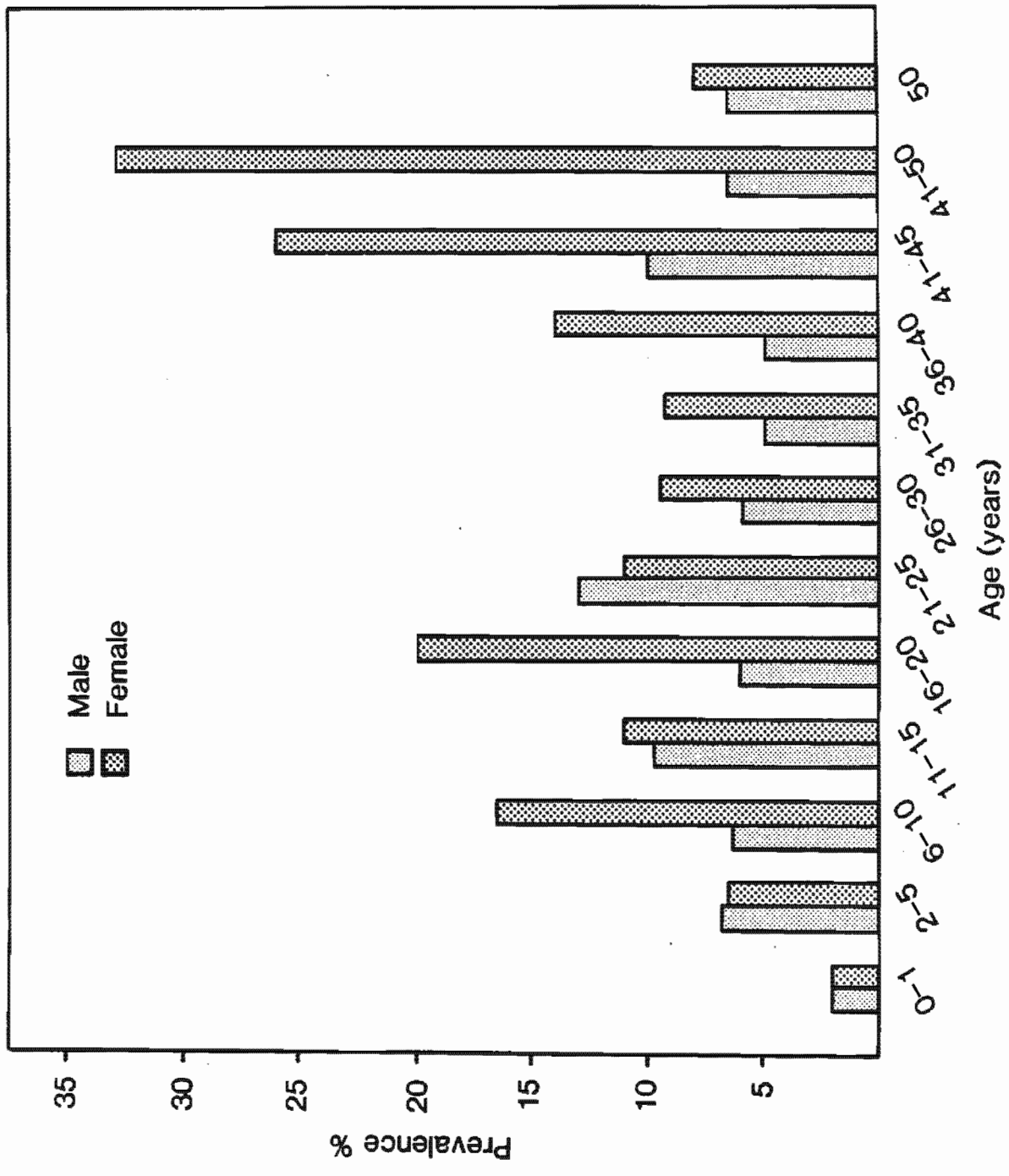

FIGURE 3.3 Age-Sex prevalence of E. histolytica

males (43/599=7,17%); the difference was statistically significant (χ^2 = 11.49, p=0.0001). The zymodeme frequency in relation to the sexes of infected individuals is shown in Table 3.3 and has been graphically represented in Figure 3.4; it is apparent that the non-pathogenic zymodemes (especially I and III) occurred more frequently in females than in males; pathogenic zymodemes, on the other hand, seemed to be equally common in both sexes.

3.3.4 Mixtures of zymodemes

As reported previously (Sargeaunt et al, 1982b,c; Jackson et al, 1982), infections with more than one zymodeme per individual are rarely seen in Durban. In fact, such infections were observed in three subjects only. These proved difficult to clone and each of the zymodemes in the mixture (all non-pathogenic) were not determined.

3.3.5 Prevalence relationships between intestinal parasites and E. histolytica.

Table 3.4 lists the prevalence of intestinal parasitic infections found in the 852 subjects whose stools were examined microscopically. For comparative purposes the prevalence levels of these parasites have been divided into an E. histolytica-infected group and an E. histolytica-free group. The level of parasitic infestation in this community was rather high with most individuals harbouring two or more parasites. Using microscopy of formal-ether concentrates the prevalence of E. histolytica in this population was approximately four times lower than that obtained by the

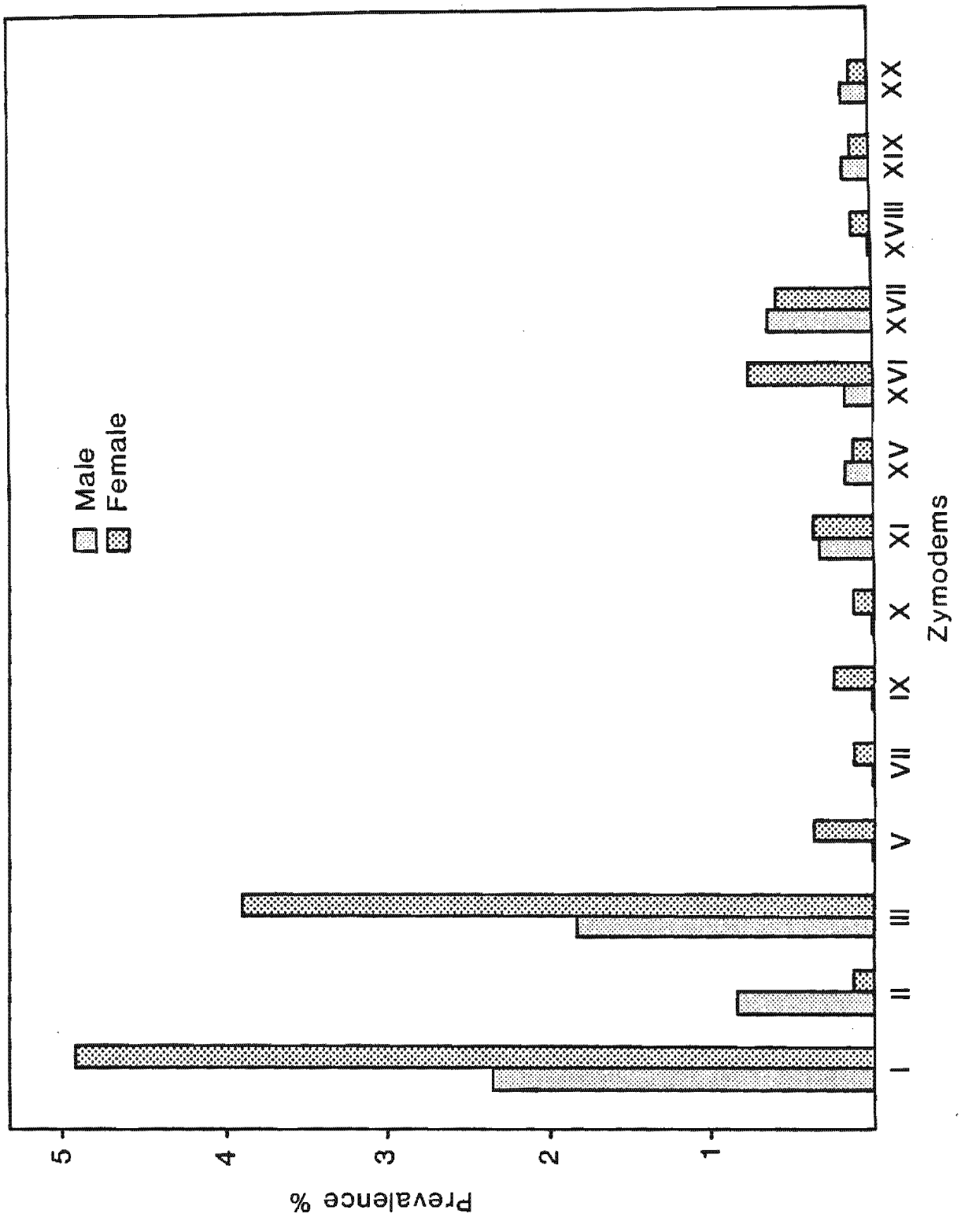

FIGURE 3.4 Comparative distribution of zymodemes of E. histolytica in males and females

TABLE 3. 3 Zymodeme distribution in males and females

ZYMODEME	Males n=599 Isolations (n)	Males n=599 Prevalence %	Females n=791 Isolations (n)	Females n=791 Prevalence %	Total n=1390 Isolations (n)	Total n=1390 Prevalence %
I	16	2,67	39	4,93	55	3,96
II	5	0,84	1	0,13	6	0,43
III	11	1,84	31	3,91	42	3,02
V.	-	-	3	0,38	3	0,22
VII	-	-	1	0,13	1	0,07
IX	-	-	2	0,25	2	0,14
X	-	-	1	0,13	1	0,07
XI	2	0,33	3	0,38	5	0,36
XV	1	0,17	1	0,13	2	0,14
XVI	1	0,17	6	0,78	7	0,50
XVII	4	0,66	5	0,63	9	0,65
XVIII	-	-	1	0,13	1	0,07
XIX	1	0,17	1	0,13	2	0,14
XX	1	0,17	1	0,13	2	0,14
Mixture	1	0,17	2	0,25	3	0,22

TABLE 3.4 Parasite prevalence in subjects with E. histolytica in comparison to those without E. histolytica infection

	E. histolytica free n=760		E. histolytica infected n=92		χ^2	p ≤
	Isolations (n)	Prevalence %	Isolations (n)	Prevalence %		
E. hartmanni	156	20,5	37	40,2	17,0	0,001
E. coli	315	41,4	82	89,1	76,8	0,001
E. nana	154	20,3	43	46,7	21,2	0,001
I. butchlii	71	9,3	31	33,7	43,9	0,001
G. lamblia	55	7,2	6	6,5	3	N.S
T. trichuria	659	86,7	88	96	5,3	0,025
A. lumbricoides	411	54,0	75	81,5	24,1	0,001
Hookworm sp	129	17,0	40	43,4	40,5	0,001
S. mansoni	120	15,7	28	30,4	12,0	0,001
Strongyloides	12	1.6	1	1	2,3	N.S.

Legend : N.S. = not significant

culturing method (2,8% vs 10%). Of note is that the prevalence of the majority of the gut parasites is significantly greater (see Table 3.4) in those individuals who had concomitant E. histolytica infections.

Figures 3.5 and 3.6 depict the age prevalence of the following parasites

Entamoeba hartmanni	)
E. coli	)
Endolimax nana	) cystic forms only
Iodamoeba butchlii	)
Trichuris trichiura	)
Ascaris lumbricoides	) ova only
Schistosoma mansoni	)

E. histolytica - culture and zymodeme determined.

It is immediately apparent that the prevalence curves of the intestinal protozoa closely parallel each other and are distinctly different from the pathogenic helminths T. trichiura, A. lumbricoides and S. mansoni.

3.3.6 Hygiene standards and E. histolytica prevalence levels

Apparently the prevalence of E. histolytica in this community was found to be directly affected by the sanitary disposal facilities

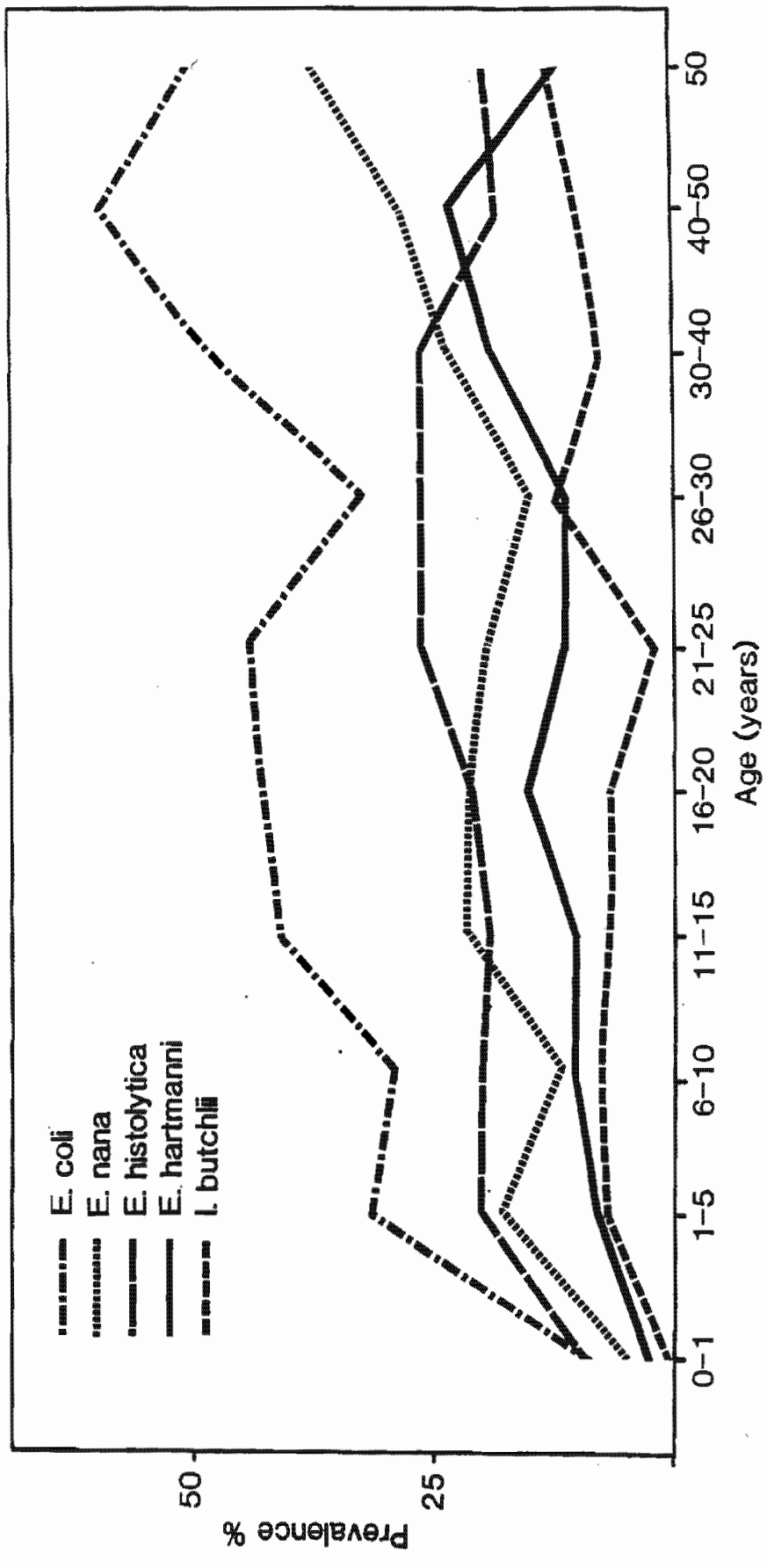

FIGURE 3.5 Age prevalence of intestinal protozoa

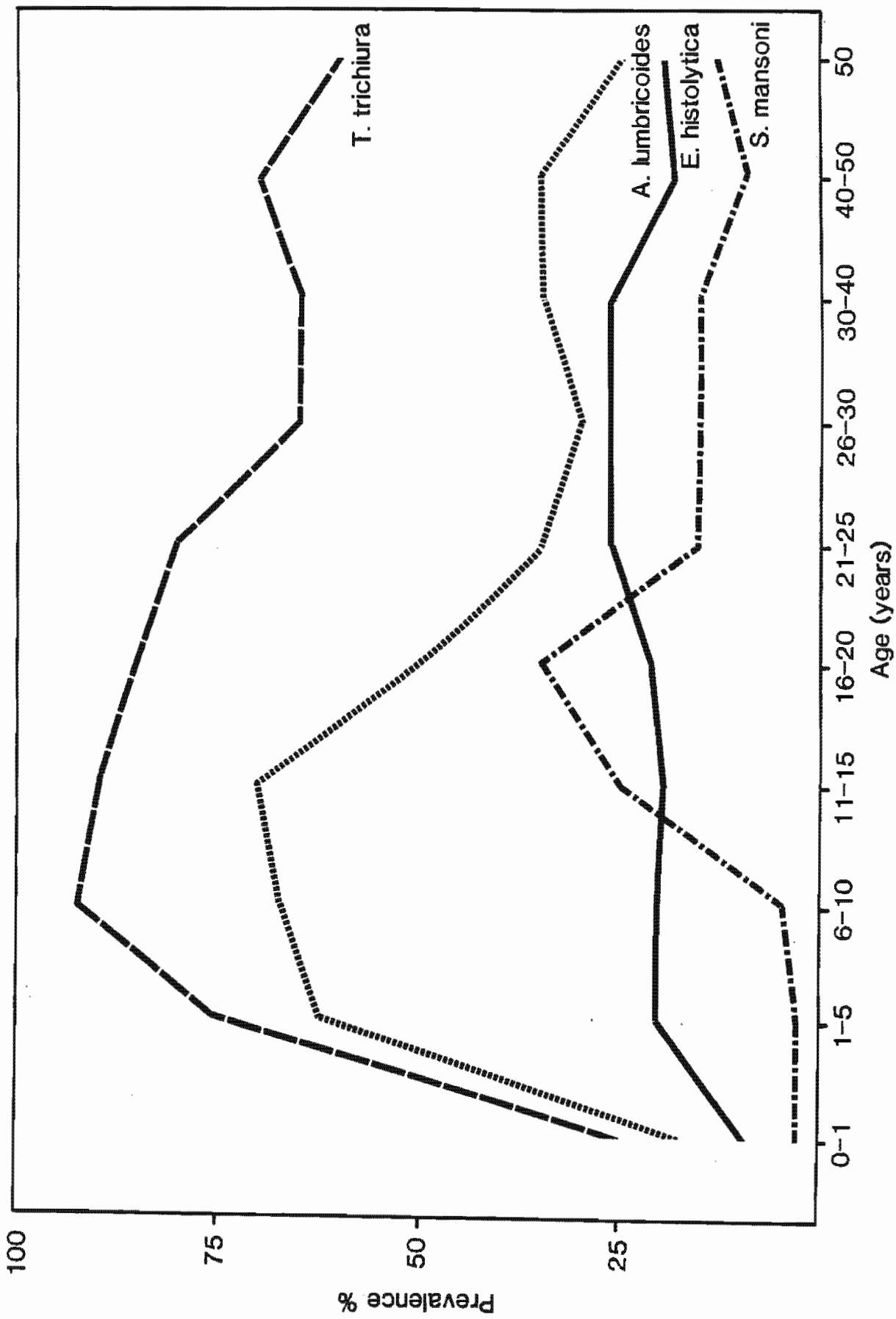

FIGURE 3.6 Age prevalence of intestinal helminths and E. histolytica

available but not by the source of drinking water - these results are summarised in Table 3.5.

It will be noted that availability of sewage disposal facilities (pit-toilets) was associated with a significantly lower prevalence of E. histolytica. Although the prevalence of the parasite was marginally higher in users of river water this was not statistically different from those who used tap water predominantly for drinking purposes; however, it must be stressed that it was difficult to accurately divide the population into users of river water and tap water since use of the latter was exclusively for drinking purposes and not for the washing of hands and foodstuffs; in addition, when tap water was not available, river water was also used for drinking. In fact, all available treated water was used for drinking or cooking of meals and virtually none was used for washing of hands or vegetables. Washing of clothes and bathing occurred directly in the rivers that run through the village.

TABLE 3.5 E. histolytica prevalence rates related to sanitary facilities and type of drinking water

	No of Isolates	Prevalence %	
Latrine n=1010	57	5,64	$\chi^2 = 38,66$ p=0,01
No latrine n=380	68	17,89	
Tap water n=59	5	8,47	$\chi^2 = 0,002$ p=N.S.
River water n=1331	134	10,06	

Legend : N.S. = not significant

3.3.7 Relationship of employment status with prevalence of E. histolytica

The prevalence of E. histolytica in employed adults and unemployed adults greater than 15 years old is depicted in Table 3.6. There was no significant difference in the prevalence of E. histolytica in adults who were employed in comparison to those who were out of work.

TABLE 3.6 Prevalence of E. histolytica in employed and unemployed adults

	n	No of isolations	Prevalence	
Males employed	n=119	9	7,6)	χ^2 =0,22 p=N.S.
" unemployed	n=117	4	3,3)	
Females employed	n=65	6	9,2)	χ^2 =0,93 p=N.S.
" unemployed	n=346	41	11,8)	

Legend : N.S. = Not Significant

3.4 DISCUSSION

For purposes of comparison the distribution frequency of zymodemes reported by Sargeaunt et al (1980b, 1982a, 1984) for Mexico and India are represented graphically in figure 3.2. The wide range of isoenzyme polymorphism observed in both pathogenic and

non-pathogenic strains of E. histolytica that have been isolated in Durban and Mexico, in contrast to the limited number of zymodemes (a total of 4 ie I, III, IV and XIV) identified in India is of interest. With reference to the pathogenic zymodemes; Sargeaunt et al (1984) are of the opinion that zymodeme XIV (which expresses a single GPI isoenzyme in the beta position) is genotypically different from zymodemes II, XI, VII, XII and XX; the latter all express a slower migrating GPI isoenzyme band (alpha position) (Figs. 1.5 & 2.1) and are therefore considered to be genotypically related. They (Sargeaunt et al, 1984) postulate that "after mutation, genetic drift originally separated zymodemes II and XIV"; they go on further to predict, on this assumption, that zymodeme XIV will be found on the African continent due to recent immigration to Africa from India. In this (Gathiram & Jackson 1985) and previous studies (Sargeaunt et al, 1982 b, c; Jackson et al, 1982) in this geographical area zymodeme XIV has not been isolated. However, a single case was identified during a study of schoolchildren in the same geographical locality in 1984 (Chapter 8); study of family contacts of this patient did not reveal any additional infections with zymodeme XIV; whether or not this case represents a point mutation is presently unknown. In the opinion of Sargeaunt et al (1984) those zymodemes expressing single bands in PGM and GPI ie I, II and XIV are considered to be "pure" strains while those with more than one isoenzyme band are thought to be phenotypes derived from these strains. Why such a large number of different phenotypes of both pathogenic and non-pathogenic E. histolytica occur both here and in Mexico is not known; perhaps the internal gut environment (see Chapter 6) or the transfer of genetic

information between either pathogenic or non-pathogenic strains of E. histolytica (Sargeaunt, 1985) plays a part and this will undoubtedly provide much scope for future research.

It was shown previously (Chapter 2, Sargeaunt et al, 1982b)) that all cases of invasive amoebiasis were caused by pathogenic zymodemes. The observation in the current study that pathogenic zymodemes occur in 1% in of the population studied (Gathiram & Jackson, 1985), is of epidemiological importance for three reasons: firstly, these carriers are clinically healthy and they could therefore be responsible for disease transmission; secondly, the occurrence of pathogenic zymodemes at a measurable prevalence level implies that the pathogenic E. histolytica may well be a distinct species in its own right and thirdly it implies that 1% of the population are at risk of developing invasive amoebiasis at any time.

It must be stressed that although the prevalence of E. histolytica determined in the area studied was 10%, this is not representative of the entire Black population of South Africa. The prevalence can be expected to vary throughout the country and thus sampling limited groups in a population can be misleading; for instance, using culturing methods and isoenzyme electrophoresis in the Durban area - the parasite was found to have a prevalence of 19% in a hospital out-patient population (Sargeaunt et al, 1982b) and 33% in school-going children (Sargeaunt et al, 1982c) in an area approximately 10 km south of the study area described in this thesis. Furthermore, the present prevalence was obtained by

culturing a single stool specimen from each subject; undoubtedly a more accurate figure can be obtained by repeating cultures on 3 or 4 consecutive days (Bray and Harris, 1977b). The prevalence data presented in this study cannot be compared directly with that of other workers (Elsdon-Dew 1946, Schutte et al, 1981) in this geographical area because, firstly, during this study E. histolytica was isolated by culture methods whereas they only employed microscopy and secondly, these earlier studies were hospital-based or confined to schoolchildren rather than community-based surveys. One cannot therefore comment accurately on changes in prevalence levels over the last 40 years.

Abdominal pain occurred more frequently in the E. histolytica infected group and surprisingly this was most obvious in those carriers of non-pathogenic zymodemes; these latter subjects also had a higher prevalence of other intestinal parasites such as Ascaris lumbricoides, Trichuris trichiura and Schistosoma mansoni (Table 3.4) and therefore it is not possible to attribute this symptom directly to the presence of E. histolytica. Nanda et al (1984) reported a higher prevalence of diarrhoea in culture negative subjects; after further investigation all these subjects were diagnosed to have irritable bowel syndrome. They (Nanda et al 1984) found no difference in the frequency of abdominal pain in infected (approximately 60% of these had non-pathogenic zymodemes (Sargeaunt et al, 1984)) and non-infected subjects. Goldmeier et al (1986 & Allason-Jones et al, 1986) found no difference in the frequency of gastrointestinal symptoms in homosexual carriers of non-pathogenic zymodemes compared to E. histolytica-free

homosexuals. Therefore it is considered improbable that infection with non-pathogenic zymodemes of E. histolytica is directly associated with the presence of gastrointestinal symptoms.

The significantly higher prevalence of E. histolytica in households that do not have latrines is of importance since defaecation in these cases usually occurs in close proximity to the dwelling. Bray and Harris (1977b) showed a positive correlation between prevalence and distance between place of defaecation and living quarters. Chandler (1954) and Spencer et al (1976) found a positive correlation between the prevalence of E. histolytica and lack of sanitary facilities. It has also been shown that the prevalence of E. histolytica can be correlated with the type of water supply (Munoz, 1986) and indicates that future studies comparing the prevalence rates in rural and urban populations could be of value.

The higher prevalence of A. lumbricoides and T. trichiura in subjects infected with E. histolytica also highlights the fact that transmission of the amoeba is by the faecal-oral route and stresses the importance of the food handler in parasite transmission.

A surprising finding was the higher prevalence of E. histolytica in females since both ALA and AD occur more commonly in males (Wilmot 1962, 1973 and Chapter 2). It must however be stressed that the majority of these were non-pathogenic zymodemes; the pathogenic zymodemes occurred in males and females with equal frequency. Elsdon-Dew (1946) on the other hand found a higher prevalence of cyst passers in males. It may be argued that women are more likely

to come into contact with the parasite because they are responsible for cleaning up the house and maintaining the vegetable patches where defaecation commonly takes place.

The prevalence of the non-pathogenic zymodemes increases sharply from infancy to the age of 5 years followed by a gradual increase in prevalence between 15 to 25 years at which stage it passes through a plateau rising again in later adult life. The prevalence curves for the other intestinal protozoa are similar. This implies that there is an absence of development of protective immunity (Knight, 1975). On the other hand the prevalence of the helminths which are obligatory invaders of the hosts' tissues such as A. lumbricoides, T. trichiura and S. mansoni reach a peak and then fall off steadily to reach a plateau at 20 years suggesting either the development of immunity or different levels of exposure. Unfortunately due to the small numbers of carriers of pathogenic zymodemes available in each age group it has not been possible to draw an age-prevalence curve but it is believed that the curve might well be comparable to that of the other tissue invasive parasites.

Elsdon-Dew (1946) found that the higher prevalence of E. histolytica was among the lower and no-income groups; he suggested that diet and housing may therefore play a part in the aetiology of amoebiasis. No significant difference in the prevalence in employed and unemployed adults could be demonstrated suggesting that the home environment is probably more important in disease transmission.

Culture and electrophoresis are useful tools for measuring the prevalence of E. histolytica. Culturing techniques are four times more efficient than microscopy and give the total prevalence of E. histolytica. With electrophoresis the prevalence of pathogenic and non-pathogenic zymodemes can be determined. A combination of the two methods can thus allow one to draw precise conclusions on the endemicity of amoebiasis in an area.

CHAPTER 4

A FOLLOW-UP STUDY OF THE ZYMODEME DISTRIBUTION IN THE MALAGAZY AREA WITH PARTICULAR REFERENCE TO VARIATIONS IN ISOENZYME POLYMORPHISM AND INCIDENCE OF E. HISTOLYTICA INFECTIONS.

4.1 INTRODUCTION

During the course of the survey described in Chapter 3, the following unexpected conclusions were reached:

a *E. histolytica* infections (non-pathogenic zymodemes) were more common in females than in males (Gathiram & Jackson, 1985); this was surprising since invasive amoebiasis is more common in males (Wilmot, 1962, 1973 and Chapter 2).

b Infections with non-pathogenic zymodemes were associated with occurrence of abdominal pain (see Chapter 3). It was however, argued that this symptom could be attributed to the higher prevalence of helminth infections occurring concomitantly with *E. histolytica* (see Chapter 3).

A second, repeat survey of the same population was therefore initiated to confirm these observations. In addition, an attempt was made to answer the following: (i) the outcome of infection in subjects previously shown to harbour non-pathogenic *E. histolytica*, after a one year period; (ii) the incidence of new infections and (iii) identification of new carriers of pathogenic *E. histolytica*.

4.2 MATERIALS AND METHODS

Subjects: A total of 795 subjects from the same study area described in Chapter 3 were studied during the period February to April 1983. Of these a cohort of 451 subjects had a stool examination in both the 1982 (Chapter 3) and 1983 surveys; 344 subjects were examined for the first time. As described previously in Chapter 3, a brief history of gastrointestinal symptoms was obtained for each subject.

The distribution of the population is summarised in Table 4.1 in terms of their age and sex.

TABLE 4.1 Study population in 1983 divided into age and sex groups.

AGE	MALE	FEMALE	TOTAL
0-1	24	26	50
2-5	55	54	109
6-10	83	78	161
11-15	51	52	103
16-20	36	42	78
21-25	23	35	58
26-30	21	36	57
31-35	11	27	38
36-40	12	23	35
41-45	5	13	18
45-50	12	15	27
Over 50	19	42	61
TOTAL	352	443	795

The stool specimen was processed as before (Chapter 3) but excluded microscopical examination. The chi-squared test was used to test for statistically significant differences between populations.

4.3 RESULTS

Eighty-one isolations inclusive of both pathogenic and non-pathogenic zymodemes were made, giving a prevalence of 10,2%. Seventy-six (9,5%) of the isolates were non-pathogenic zymodemes; in 3 cases, the zymodeme of the E. histolytica culture could not be determined because the bands in GPI and PGM were very faint; all of them had characteristic non-pathogenic HK patterns and were classed as non-pathogenic zymodemes. Five isolates (0,64%) were pathogenic zymodemes.

4.3.1 Zymodeme distribution

Figure 4.1 indicates the distribution of E. histolytica zymodemes in the population and compares it to that found in 1982. The following noteworthy observations were made:

1 It was evident that the most frequently isolated zymodemes were III, I and XVII with zymodeme III occurring far more frequently.

2 Non-pathogenic zymodeme VIII was isolated for the first time in this study area while non-pathogenic zymodemes IX, X, XV

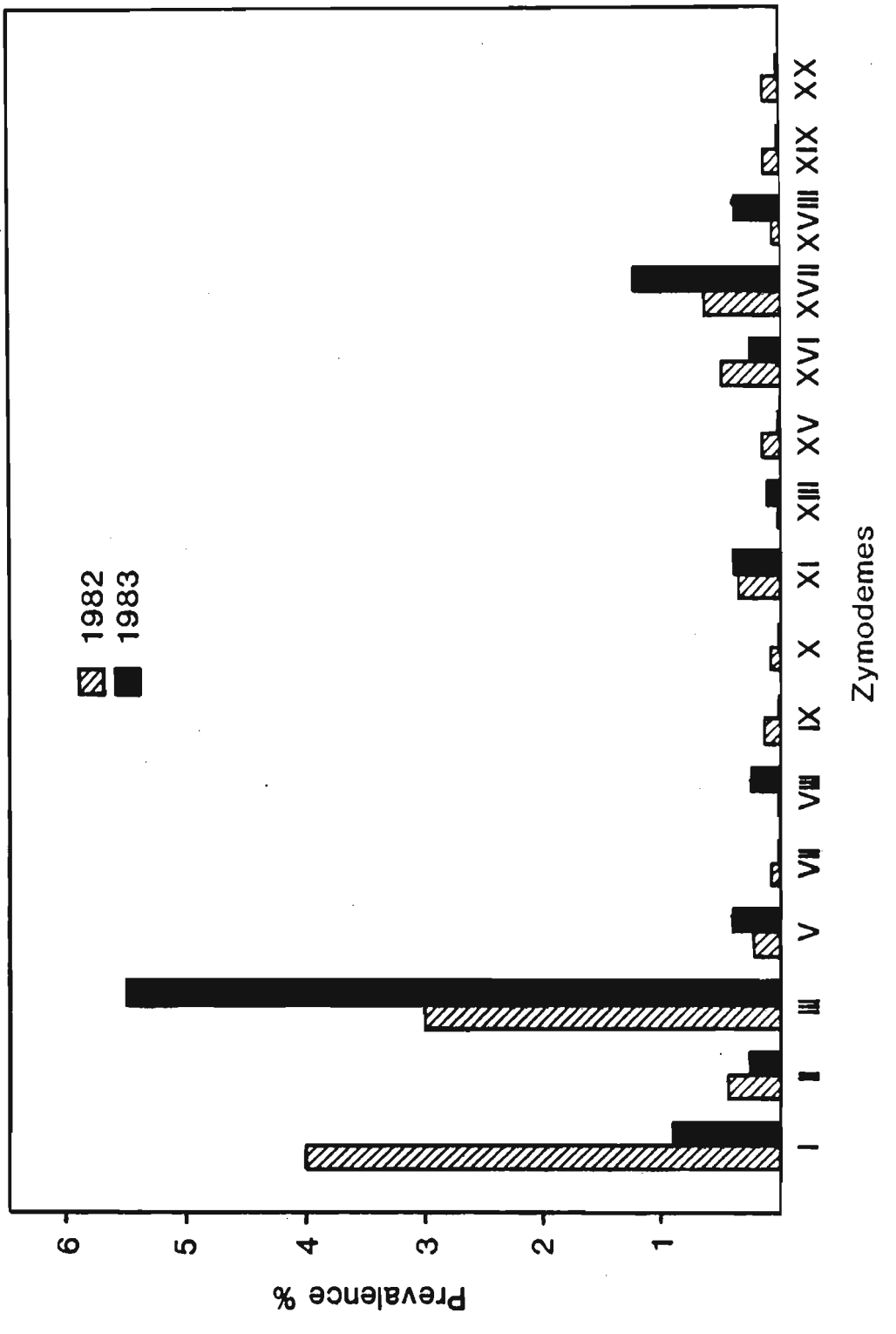

FIGURE 4.1 Zymodeme Distribution in 1982 and 1983

and XIX which were isolated in 1982 (Chapter 3) were not observed again.

3 Non-pathogenic zymodeme IV has never been isolated in this geographical area.

4 Pathogenic zymodemes II and XI continued to occur; zymodemes VII and XX were not isolated in 1983.

5 Zymodeme XIII was observed for the first time in this study area; it is an anomalous zymodeme as it displays a typical pathogenic pattern in PGM and a non-pathogenic pattern in HK.

4.3.2 Incidence of new infections

For the estimation of incidence only the cohort of 451 subjects who had been examined in 1982 as well as in 1983 could be used. The results are summarised in Figure 4.2

From the Venn diagram in Figure 4.2 it will be noted that:

1 Prevalence in 1982 = 61/451 = 13,5%

2 Prevalence in 1983 = 48/451 = 10,6% ie there was a slight fall in prevalence from 1982 to 1983

3 The number of new infections occurring in 1983 = 38/451 = 8,4%

4 51 subjects who were found to be infected in 1982 had apparently spontaneously lost their infections when reexamined in 1983

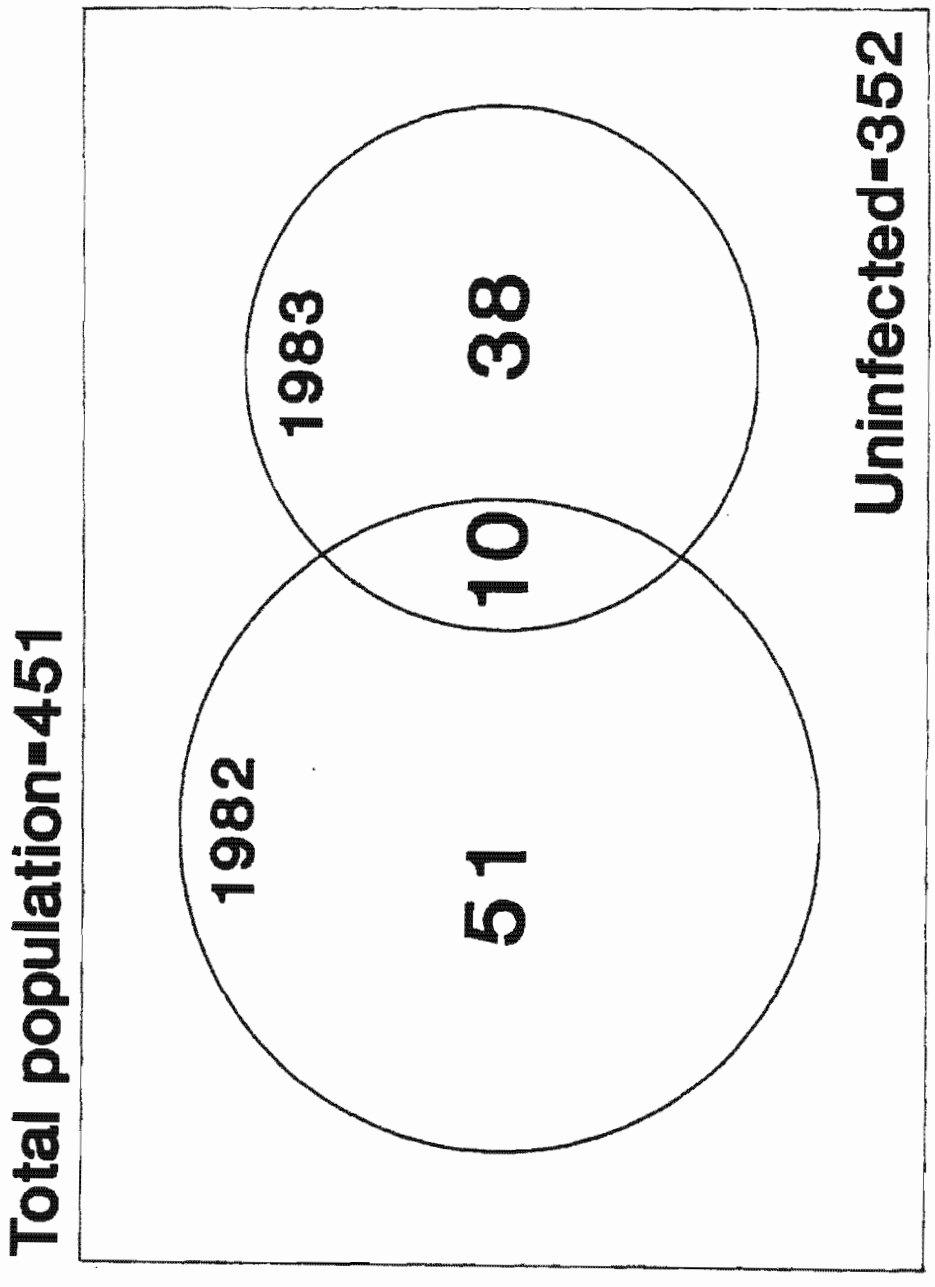

FIGURE 4.2 Prevalence of E. histolytica infection in 1982 and 1983

5 Only 10 subjects had E. histolytica on both occasions. Of interest is the fact that 4 of these harboured the same zymodeme while 6 were infected with E. histolytica of a different zymodeme (Table 4.1).

Table 4.1 provides a summary of all E. histolytica infections recorded over the two year period. The following observations were considered important:

1 The tendency for infections to occur in family clusters. Of a total number of 171 infections, 120 occurred in clusters of 2 to 5 in 42 family units.

2 All pathogenic infections in 1983 seemed to have occurred de novo ie they were not preceeded by infections with non-pathogenic zymodemes.

4.3.3 Relationship of prevalence to sex and availability of sanitary disposal facilities.

The overall prevalence was again higher in females (53/443=12%) than in males (28/352=7,9%); however this difference was not statistically different. As described in Chapter 3, a lower prevalence of E. histolytica infections was recorded in subjects who used pit latrines (30/363=19,1%) than in those who did not (49/157=7,8%); the difference was again statistically significant (χ^2=123; $p \leq 0{,}001$).

TABLE 4.2 (Page 1 of 5). E. histolytica isolations for 1982 and 1983

1982		1983	
Specimen No.	Zymodeme	Specimen No.	Zymodeme
* 8		2075	XI
* 6		2003	XVII
-	-	2026	III
* 27		2048	II
* 26		2047	II
29	I	-	-
* 12		2021	XI
* 10		2020	III
* 17		2024	VIII
* 94		2077	XVII
-	-	2008	XVII
-	-	2011	XVII
** 198	III	2063	III
* 193	I	2094	
* 192	XVI	2097	
* 201	I	2031	
-	-	2061	I
** 221	III	2070	III
* 222		2039	VIII
* 220		2038	III
* 256		2056	XVII
-	-	2072	III
-	-	2088	XVII

NOTES :
1 Isolates grouped by paraentheses denote a family cluster
2 * denotes subjects who were examined in 1982 and 1983
3 ** denotes subjects from whom E. histolytica was isolated in 1982 and 1983
4 -- denotes that examination was not done
5 A blank space under zymodeme signifies absent growth for E. histolytica
6 E.H. = E. histolytica of possible mixed zymodemes

TABLE 4.2 (Page 2 of 5) E. histolytica isolations for 1982 and 1983

1982		1983	
Specimen No.	Zymodeme	Specimen No.	Zymodeme
-	-	2142	XVII
-	-	2141	III
* 76		2242	III
* 55		2203	III
* 275	XVII	2171	
* 301		2176	III
* 281	I	2186	
335	III	-	-
274	III	-	-
267	I	-	-
276	I	-	-
-	-	2259	III
-	-	2254	III
* 62		2212	III
* 59		2209	III
* 63		2218	III
-	-	2260	III
* 86		2232	III
* 82		2233	III
* 112		2250	III
-	-	2282	III
-	-	2269	V
-	-	2268	III
163	XVI	-	-
176	I	-	-
* 248		2103	I
* 250		2107	XVI
* 205	XIX	2102	
* 245	I	2128	
** 242	I	2219	III
240	I	-	-
* 230	III	2413	
* 265		2133	III
263	III	-	-
* 261		2137	III
264	XVII	-	-
-	-	2123	III
-	-	2124	III
259	I	-	-
-	-	2157	I
457	III	-	-
-	-	2411	XVII
* 448	III	2385	
* 415	V	2382	
-	-	2435	I
-	-	2436	V

TABLE 4.2 (Page 3 of 5) *E. histolytica* isolations for 1982 and 1982

1982		1983	
Specimen No.	Zymodeme	Specimen No.	Zymodeme
-	-	2300	III
-	-	2298	EH
-	-	2294	III
** 326	I	2274	XVII
-	-	2275	EH
351	I	-	-
362	I	-	-
422	I	-	-
475	V	-	-
** 460	I	2402	I
* 478		2404	V
* 477	III	2403	
* 459	I	2399	III
* 97		2415	XVII
-	-	2416	III
* 438	I	2374	
* 449		2391	XVII
* 304	I	3311	
360	I	-	-
407	I	-	-
414	III	-	-
423	I	-	-
425	XIX	-	-
* 347	-	2466	XVII
* 497	I	2496	
* 492	I	2497	
* 499		2453	III
500	I	-	-
* 556	III	2494	
** 534	III	2500	III
* 543		2472	XVI
518	XVII	-	-
* 529		2451	XVII
* 589		2471	I
623	I	-	-
* 625	III	2563	
586	III	-	-
* 609	III	2501	
* 627	II	2531	
* 622	V	2532	
* 596	XI	2520	
* 595	XX	2523	
* 597	II	2521	
* 591	I	2522	
* 615	I	2507	
599	I	-	

TABLE 4.2 (Page 4 of 5) E. histolytica isolations for 1982 and 1983

1982		1983	
Specimen No.	Zymodeme	Specimen No.	Zymodeme
430	I	-	-
457	III	-	-
• 631	III	2581	
643	III	-	-
648	III	-	-
649	III	-	-
651	XV	-	-
672	III	-	-
676	III	-	-
704	III	-	-
705	III	-	-
706	I	-	-
707	III	-	-
• 709	VIII	2572	
• 745		2648	III
• 738	I	2710	
• 739	X	2645	
• 743	XVII	2647	
• 746	I	2712	
• 749	I	2701	
758	XVI	-	-
784	III	-	-
• 785	IX	2704	
• 722		2533	EH
• 608	III	2615	
-	-	2641	XVII
•• 811	I	2677	III
-	-	2637	XI
-	-	2636	I
• 812	II	2638	
• 846		2681	III
•• 844	I	2722	XIII
-	-	2717	III
-	-	2737	III
•• 878	I	2704	III
• 882	III	2707	
• 879	I	2703	
• 877	III	2706	
• 945	I	2787	
• 929	XVII	2780	
• 931		2779	III
• 933		2782	III
• 930	III	2776	
• 934	XVI	2775	
• 954		2757	III
• 955	I	2755	

TABLE 4.2 (Page 5 of 5) *E. histolytica* isolations for 1982 and 1983

1982		1983	
Specimen No.	Zymodeme	Specimen No.	Zymodeme
791	I	-	-
• 799	XX	2711	
• 801	VII	2696	
• 803	XI	2715	
• 834	I	2741	
819	I	-	-
830	XVII	-	-
842	I	-	-
845	I	-	-
• 864	I	2743	
• 778		2750	III
1352	I	-	-
1355	I	-	-
1365	I	-	-
1366	I	-	-
1384	III	-	-
1406	XVI	-	-
1413	XVI	-	-
1418	XI	-	-
1421	III	-	-
1424	III	-	-
1455	III	-	-
1460	III	-	-
• 24	I	2076	
-	-	2026	III
• 778		2750	III
-	-	2803	III
985	I	-	-
1058	XVII	-	-
1062	I	-	-
1187	XV	-	-
1189	III	-	-
1190	III	-	-
1195	I	-	-
1209	I	-	-
• 1043	XI	2887	
1072	I	-	-
• 1219	XI	2889	
1221	III	-	-
1230	XVI	-	-
1234	III	-	-
1236	III	-	-
• 1250	II	2891	
• 1271	II	2894	
1338	III	-	-
1350	IX	-	-

4.3.4 Gastrointestinal symptoms

All carriers of both pathogenic and non-pathogenic zymodemes were asymptomatic at the time of the initial stool collection. The four carriers of pathogenic zymodemes were followed-up for periods of 6 months to one year and will be discussed in Chapter 6. Of those subjects who were free of E. histolytica infection one had diarrhoea and eight complained of abdominal pain.

4.4 DISCUSSION

The most surprising finding in this study was the shift in prevalence of the non-pathogenic zymodemes. Figure 4.1 depicts the zymodeme distribution in the period February to April 1983 and for comparison those for the period February to June in 1982 (Chapter 3). It is possible, of course, that infections with zymodeme III may obscure a concomitant infection with zymodeme I; due to technical difficulties the E. histolytica isolations could not be cloned on a regular basis. However, both Sargeaunt et al (1982b,c) and Jackson et al (1982) reported that mixed infections with two zymodemes is rare in Durban. If, in fact, the results do reflect the true state of affairs there must have been a strong selection pressure for zymodeme III and to a lesser extent zymodeme XVII and I infections. Alternatively since all the non-pathogenic zymodemes have an alpha band in both PGM and GPI Sargeaunt et al (1984) have proposed that they are all genotypically similar to zymodeme I, and the isoenzyme polymorphism noted is phenotypic. It is believed that environmental changes may consequently result in the switching on

or off of certain isoenzymes resulting in the observed banding patterns (Gathiram and Jackson 1987 and Chapter 6). The fact that this country suffered its most severe drought in 14 years during 1983 may have influenced these resultant zymodemes. Water was in such short supply that rationing had to be instituted in the city of Durban. In the area in question the streams were reduced to a trickle; this must have affected the standard of hygiene and since food was also in short supply, the diet of the study population must have been affected. These factors may have acted on the gut flora which might in turn have affected the amoebae. A further argument in support of this hypothesis is that in instances where infections occurred in family clusters, two to three different zymodemes were often characterised within the family unit (Table 4.1). It can be argued that if these zymodemes are an absolutely stable property of the amoeba then one might expect all family members to be infected by the same zymodeme.

The fact that a change in zymodeme from pathogenic to non-pathogenic and vice versa was never recorded is very important as this lends support to the hypothesis that the pathogenic and non-pathogenic zymodemes are two distinct species and that a change from commensal (non-pathogenic) to pathogenic forms does not occur. It is proposed that those genes coding for pathogenicity are stable characteristics of the parasite and that the isoenzyme pattern that can be demonstrated from pathogenic zymodemes are reliable markers of pathogenicity. This will be discussed in more detail later in this thesis.

The results therefore support the arguments expressed in a recent editorial in The Lancet (1986) as well as those of Sargeaunt (1987). The apparent change in zymodeme from pathogenic to non-pathogenic patterns produced under laboratory conditions by Mirelman et al, (1986a,b) has not been observed in this in vivo study.

E. histolytica cysts are vulnerable to dessication and therefore lower prevalence figures are usually expected in the drier months (Elsdon-Dew, 1946, Bray & Harris, 1977b). No change in prevalence was noted from 1982 (10%) to 1983 (10,2%) even though this area experienced its most severe drought during 1983.

Spontaneous eradication of non-pathogenic zymodemes occurred in approximately 84% (51/61) of infected subjects. This phenomenon has also been reported by Nanda et al (1984); they observed that apparent self-cure occurred in 15 subjects with E. histolytica infections within a period ranging from 1 to 19 months. Beaver et al (1956) experimentally infected 81 human volunteers with cysts obtained from an asymptomatic carrier; 7 of the 23 subjects apparently spontaneously lost their infections within a period ranging from 21 days to 8 months.

Considering the abovementioned gastrointestinal symptoms that occurred in the E. histolytica infected subjects, the observations recorded concur with that of Nanda et al, (1984) and indicate that

non-pathogenic E. histolytica cannot be implicated as the causative agent of many of the bowel complaints attributed to it.

The occurrence of clustering of E. histolytica infections in families is important. Such family clustering of infections is a recognised phenomenon (Munoz, 1986; Spencer et al, 1977) and implies direct person-to-person transmission and also emphasises the role of the food-handler in parasite transmission. It is therefore prudent to assume that overcrowding will result in very high prevalence figures especially if this is combined with poor facilities for the disposal of human waste. Conversely, provision of good housing with proper sanitary facilities and the availability of sufficient water for the washing of hands and food must have high priority in the prevention and control of amoebiasis.

CHAPTER 5

SEROEPIDEMIOLOGY OF AMOEBIASIS : SEROLOGICAL RESPONSES TO THE ZYMODEMES OF ENTAMOEBA HISTOLYTICA

5.1 INTRODUCTION

Sero-epidemiological studies on amoebiasis have been conducted by workers in several countries (Maddison et al, 1965 - South Africa; Healy & Gleason, 1972 - USA; Bos et al, 1980 - Surinam: Spencer et al, 1977 - USA); apparently they all concluded that the presence of antibodies implies contact between E. histolytica and its host's tissues and therefore provides an index of the role of E. histolytica as a pathogen in any population studied. However, none of these workers was in a position to compare the serological responses of subjects harbouring different zymodemes. Sensitivities of 95 to 100% for the serologic testing for amoebic liver abscess (ALA) and 85 to 95% for amoebic dysentery (AD) have been recorded (Healy, 1986); on the other hand, sensitivity of these tests for asymptomatic cyst passers was much lower and displayed a variable prevalence related to the occurrence of the parasite and disease; an adequate explanation for the latter was not forthcoming. These earlier studies however, indicated that the pathophysiological properties of the parasite influenced the serological response of the host.

During the course of the surveys described in Chapters 2, 3 and 4, the serological responses of individuals harbouring pathogenic and

non-pathogenic zymodemes were investigated in an attempt to determine whether the biochemical marker for pathogenicity (ie isoenzyme electrophoresis) could elucidate the abovementioned anomalous observations. Serological results observed during an outbreak of invasive amoebiasis in a previously non-endemic area as well as those from an area where only non-pathogenic zymodemes could be isolated are presented for comparison.

5.2 MATERIALS AND METHODS

5.2.1 Study population

A total of 1939 subjects from three localities within the Republic of South Africa were studied , viz:

a the Durban area (Natal)

b the magisterial distric of Philippi (Cape Peninsula)

c the magisterial district of Gazankulu (Transvaal)

5.2.1.1 Durban

Both healthy subjects and hospital patients with amoebic liver abscess (ALA) and amoebic dysentery (AD) were studied.

a Healthy subjects: A total of 303 subjects from the study area (Malagasy) described in Chapters 3 and 4 were investigated. One hundred and eighty-eight of them were examined during the period February to April 1983 described in Chapter 4. Twelve were carriers of pathogenic zymodemes who had been identified

in 1982 (Chapter 3). The remaining 103 were from a school in the same village. Blood specimens were collected from each subject.

b Patients with invasive amoebiasis: 38 patients with ALA and 16 with AD were studied; the clinical details as well as the zymodemes isolated from these subjects have already been presented in Chapter 2. Blood and stool specimens were collected from each patient.

5.2.2.2 Philippi

This is an agricultural district on the outskirts of Cape Town. The living conditions for the labourers employed here varied from excellent to squalid. The farms on which transmission of amoebiasis was found to occur all had poor hygienic standards; they were crowded and many did not have latrines; defaecation commonly occurred in the tilled fields. Water for irrigation, washing of hands, clothes, food and drinking was commonly obtained from ditches which collected "run-off" water from the fields. Much of the area is below sea-level and the water table is very close to the surface.

During the period March to August 1984, 10 cases of ALA and 4 cases of AD were diagnosed at the Victoria Hospital in Cape Town; this is an area where a previous seroepidemiological study showed that the endemicity of amoebiasis was low (Maddison et al, 1965). It was noted that 9 of the patients with ALA were resident on a single

farm (farm 40) in Philippi; further, the tenth patient came from an adjacent farm (farm 45) in this district (Fig 5.1). The local health authority was informed of the epidemic, and the Research Institute for Diseases in a Tropical Environment was approached for assistance in ascertaining the level of endemicity of amoebiasis. At the same time the local health authority undertook a mass treatment programme of cyst passers in an effort to eradicate carriers of E. histolytica; most residents in the area thus had been treated with metronidazole (Flagyl; Maybaker), 800mg three times daily for 8 days before the survey was started.

The study was carried out in collaboration with the Dept of Community Health of the University of Cape Town and the Cape Peninsula local health authority. After consultation with an epidemiologist a total of 199 stool and 441 blood samples were taken from 755 random subjects on 16 farms (Fig 5.2). This sample represented 58,4% of the total population of the district.

5.2.2.3 Gazankulu

This is a rural area situated adjacent to the North Eastern Transvaal; invasive amoebiasis is rare in the area; 1000 children were examined during an extensive screening programme for schistosomiasis and other intestinal parasites; 1000 serum and 800 stool samples were examined. Culture for E. histolytica and zymodeme determination as well as serological testing were done routinely as part of the investigation.

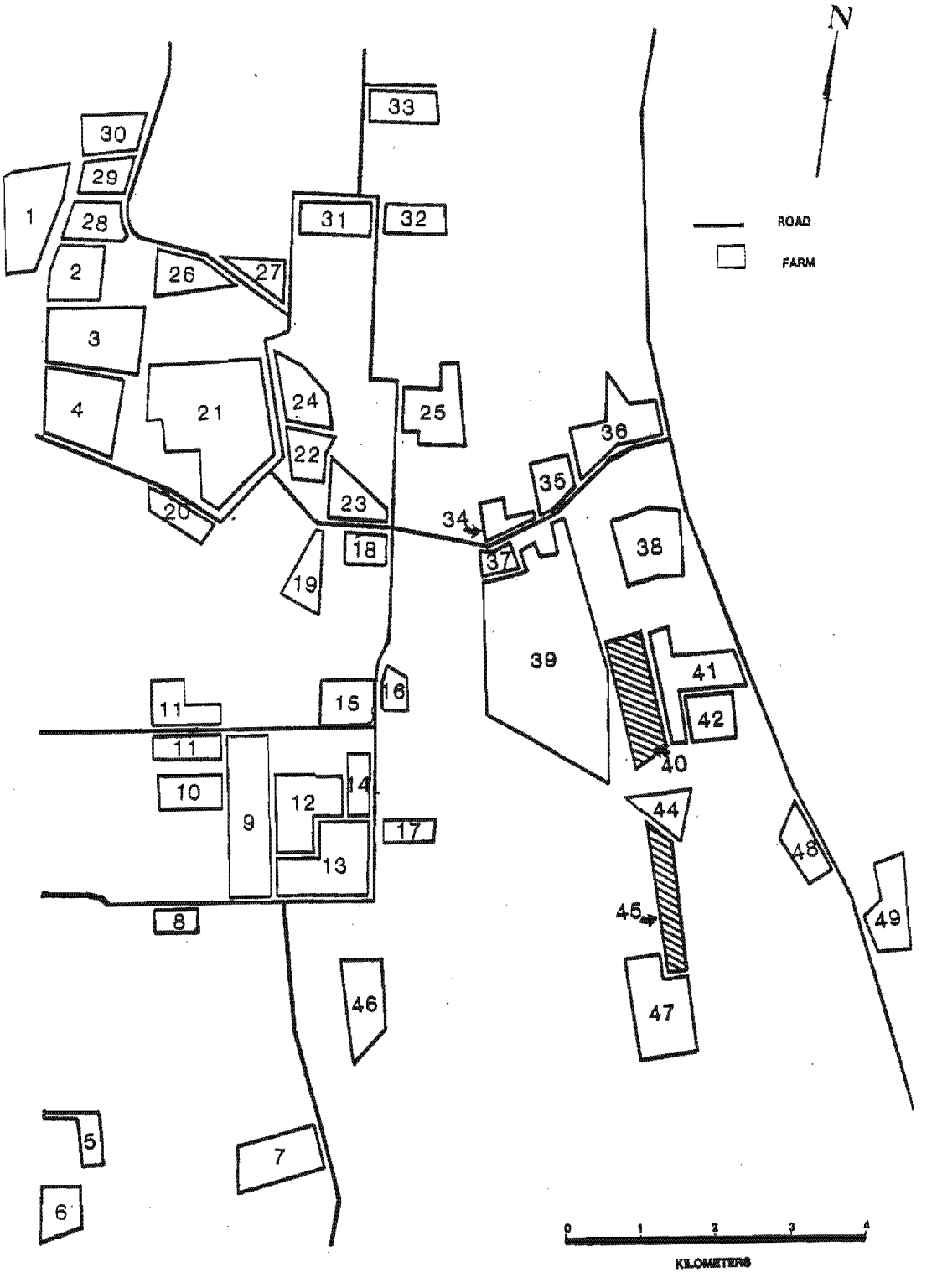

Figure 5.1 Diagrammatic representation of the location of farms in Philippi

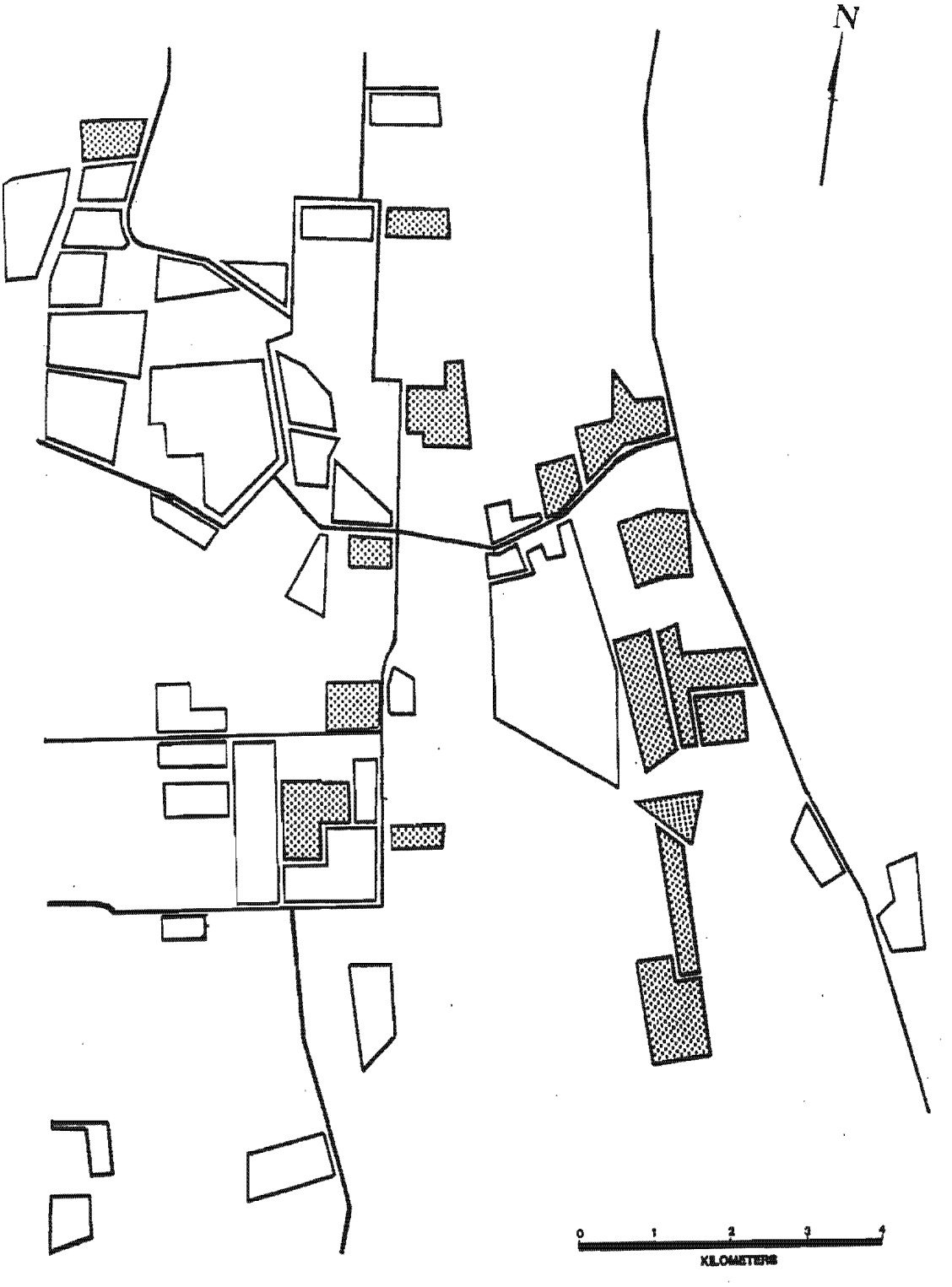

Figure 5.2 Farms chosen for the seroepidemiological study.

(Shown by the 'dotted' shading).

5.2.3 Serological methods

Serum was separated, numbered and stored in 0,5mℓ aliquots at -20°C. The amoebic gel diffusion test (AGDT) and indirect fluorescent antibody test (IFAT) were performed according to the methods described by Jackson et al (1983, 1984). In the case of the IFAT only the specific IgG was determined. An individual was considered to be serologically positive when one or both tests proved to be positive. A response in the AGDT was regarded as strongly positive when precipitins were seen at 20 hours and in the IFAT when the titre was greater than 1/500.

5.2.4 Isoenzyme electrophoresis

Stools were cultured and zymodeme determination was performed as described above (Chapter 2).

5.2.5 Statistical analysis

The chi-squared test was used as previously described.

5.3 RESULTS

5.3.1 Durban

In order to assess the serological responses to the zymodemes of E.

histolytica the study population was divided into 6 groups depending on whether or not E. histolytica was isolated from the stool and the zymodemes expressed by the isolates; these are described below:

Group 1: No stool obtained; in this group of 46 subjects only blood was taken for serological examination. They represented a random population sample and were used to assess the "background" positivity of the population.

Group 2: E. histolytica was not isolated from these 207 subjects using culture; this cohort can be considered to be "E. histolytica"-free.

Group 3: Non-pathogenic zymodemes were isolated using culture. There were 33 subjects in this group.

Group 4: Seventeen asymptomatic carriers of pathogenic zymodemes.

Group 5: Sixteen patients with AD.

Group 6: Thirty-eight patients with ALA.

The serological results are presented in Table 5.1. In Groups 1 and 2 zymodeme determination was not possible; the results of these are therefore listed under not applicable (N/A). Included in the study groups 5 and 6 are subjects from whom no amoebae were isolated in culture from stools and pus respectively; therefore in these cases zymodeme determination was not possible; these are also listed under N/A.

TABLE 5.1 : SEROLOGICAL RESPONSES TO E. HISTOLYTICA

STUDY GROUPS	NO.	ZYMODEME (NUMBER WITH POSITIVE SEROLOGY / TOTAL WITH THAT ZYMODEME)														SEROLOGY	
		I	II	III	V	VII	VIII	X	XI	XIII	XVII	XVIII	XX	Φ MIX	NA Ψ	NO. POS	%
1 No stool collected	46														9/46	9(19,6)	
2 No amoeba cultured	207														28/207	28(13,5)	
3 Carrier (non-pathogenic E. histolytica)	33	1/3		4/17	0/1		1/2	0/1		0/1	1/6	0/1		0/1		7(21)	
4 Carrier (pathogenic E. histolytica)	17		8/8			1/1			7/7				1/1			17(100)	
5 Amoebic dysentery	16		8/8						2/2						6/6	16(100)	
6 Amoebic liver abscess	38		23/23						4/4				1/1		10/10	38(100)	

Φ Mixture of zymodeme types isolated in one culture. This occurred once and proved to be a mixture of 2 non-pathogenic zymodemes.

Ψ Zymodemes for these cases not available since stools were not collected. They are included for comparative purposes and give an estimate of background antibody levels

The occurrence of seropositivity (Table 5.1) in the random population was 19,6% (Group 1). While in the E. histolytica culture negative Group 2 and those harbouring non-pathogenic zymodemes (Group 3) the seropositivity ranged from 13,5% (in Group 2) to 21% (in Group 3); these latter 2 groups were not significantly different from the control group 1 (χ^2=0,55; p = 0,5 and χ^2=0,01; p=0,9 respectively). All subjects with confirmed invasive amoebiasis proved to be seropositive, as were those asymptomatic subjects from whom pathogenic zymodemes were isolated.

The results also highlighted a quantitative component in the serological response and this is evident in Table 5.2. Subjects harbouring pathogenic zymodemes (groups 4 to 6) had a marked tendency to develop strongly positive serological reactions (94-100%). In contrast, subjects in groups 1 to 3 were usually sero-negative; only 2-4% were found to be strongly posititive.

There was no significant difference (χ^2=0,056; p= 0,5) in the seropositivity of males (8/47=17,02% seropositive) and females 20/141=14,18% seropositive). The occurrence of seropositive responses in subjects living in dwellings with toilets was similar to that observed from those that did not have toilets (15,2% vs 14,5%).

TABLE 5.2 Extent of seropositivity in study groups 1 to 6 from the Durban area

Study Group	No	Serology		
		Positive (%)	Strongly No	positive (%)
1	46	9	2	(4)
2	207	28	4	(2)
3	33	7	1	(3)
4	17	17	17	(100)
5	16	16	15	(94)
6	38	38	37	(97)

The frequency distribution of positive AGDT at 20 hrs and 40 hrs is depicted in Figure 5.3 and titres for the IFAT in Figure 5.4. It is apparent that while both strong and weak positive responses are recorded a higher proportion of the subjects have lower titres in comprison to subjects from the Philippi area (see 5.3.2 below).

5.3.2 Philippi

Of the 23 E. histolytica isolations 6 were pathogenic zymodemes (II, XI) and 17 were non-pathogenic zymodemes (I, III, VI, XVII).

All subjects harbouring pathogenic zymodemes were strongly positive. The frequency distribution of the seropositive results are displayed in Figures 5.5 (AGDT) and 5.6 (IFAT). Of note is that a higher proportion of subjects strong seropositive responses when compared to subjects from the Malagasy area near Durban (see 5.3 above). The overall prevalence of seropositive responses was 7,7%; however, the strongly seropositive responses were confined to the residents of only 5 of the 21 farms studied (Fig 5.7) and the frequency distribution of the AGDT results is shown in Fig 5.8.

5.3.3 Gazankulu

It is interesting that only non-pathogenic zymodemes (I, III, IV, V, VIII, XVII, XVIII) were isolated with zymodeme III occurring most frequently. There were 67 isolations of E. histolytica giving a prevalence of 8,4%. Zymodeme IV was isolated for the first time in the Republic of South Africa. All sera tested gave sero-negative responses with the AGDT.

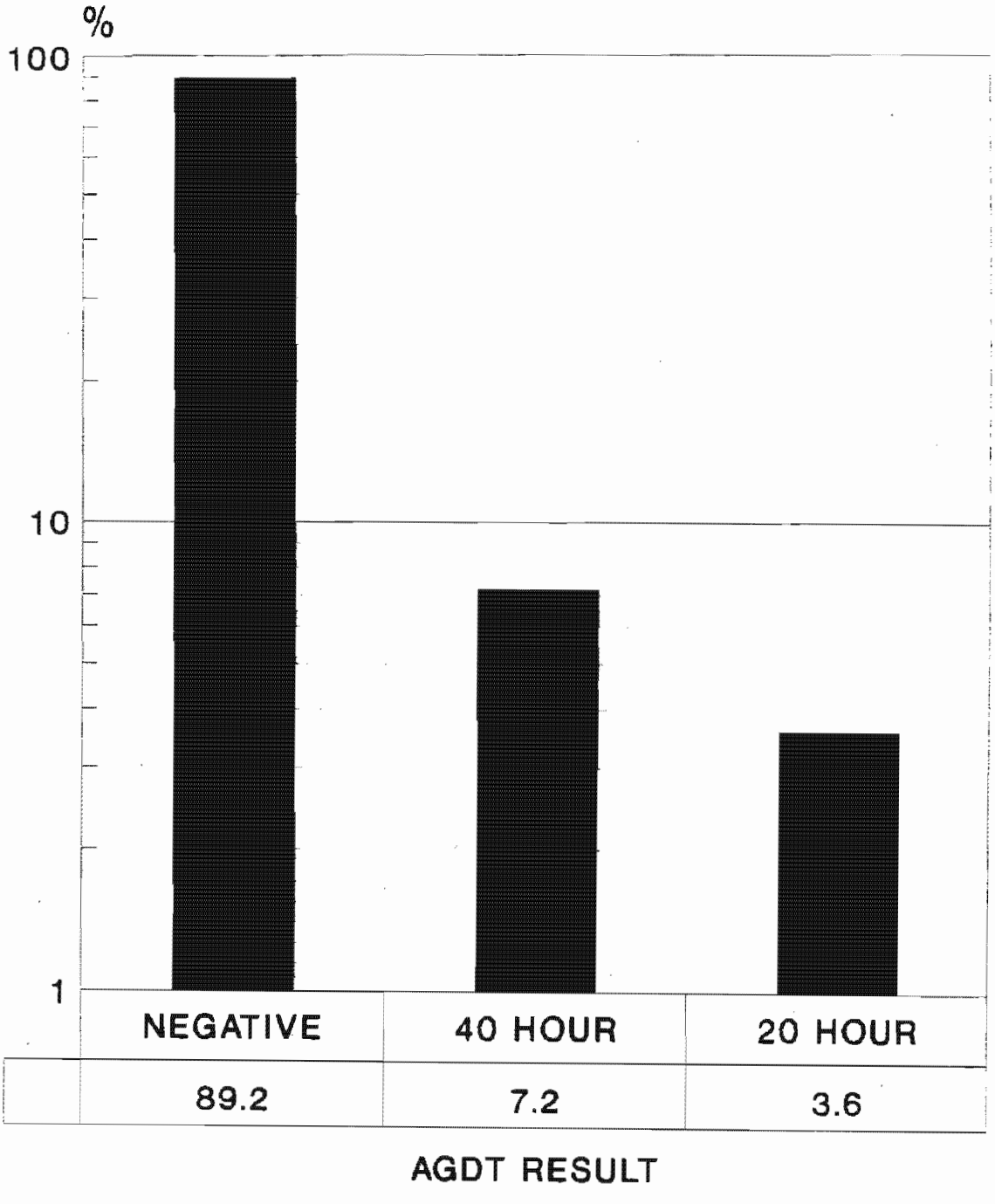

Figure 5.3 : AGDT Results in Malagasy - an endemic area south of Durban

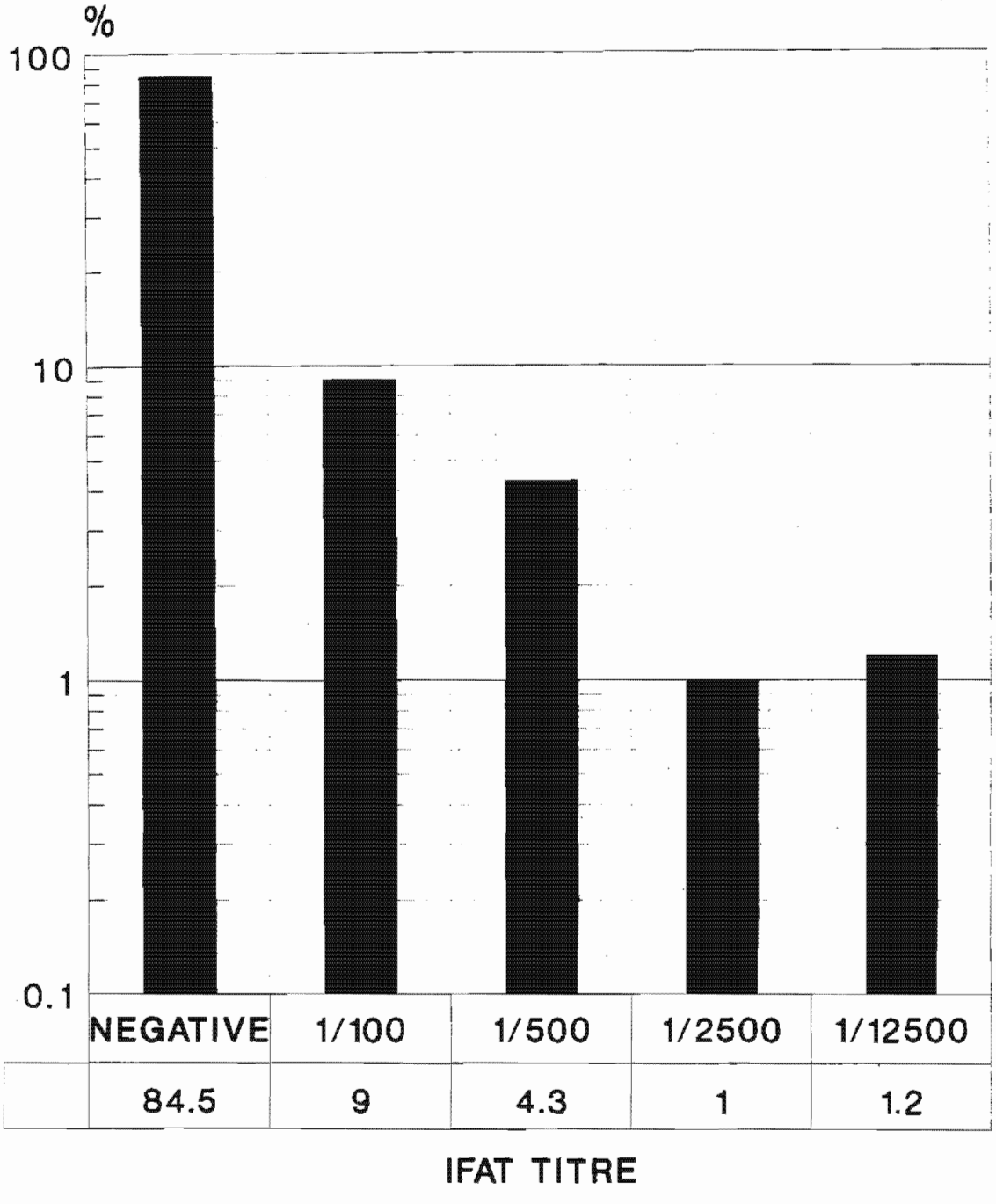

Figure 5.4 IFAT results in Malagasy an endemic area south of Durban

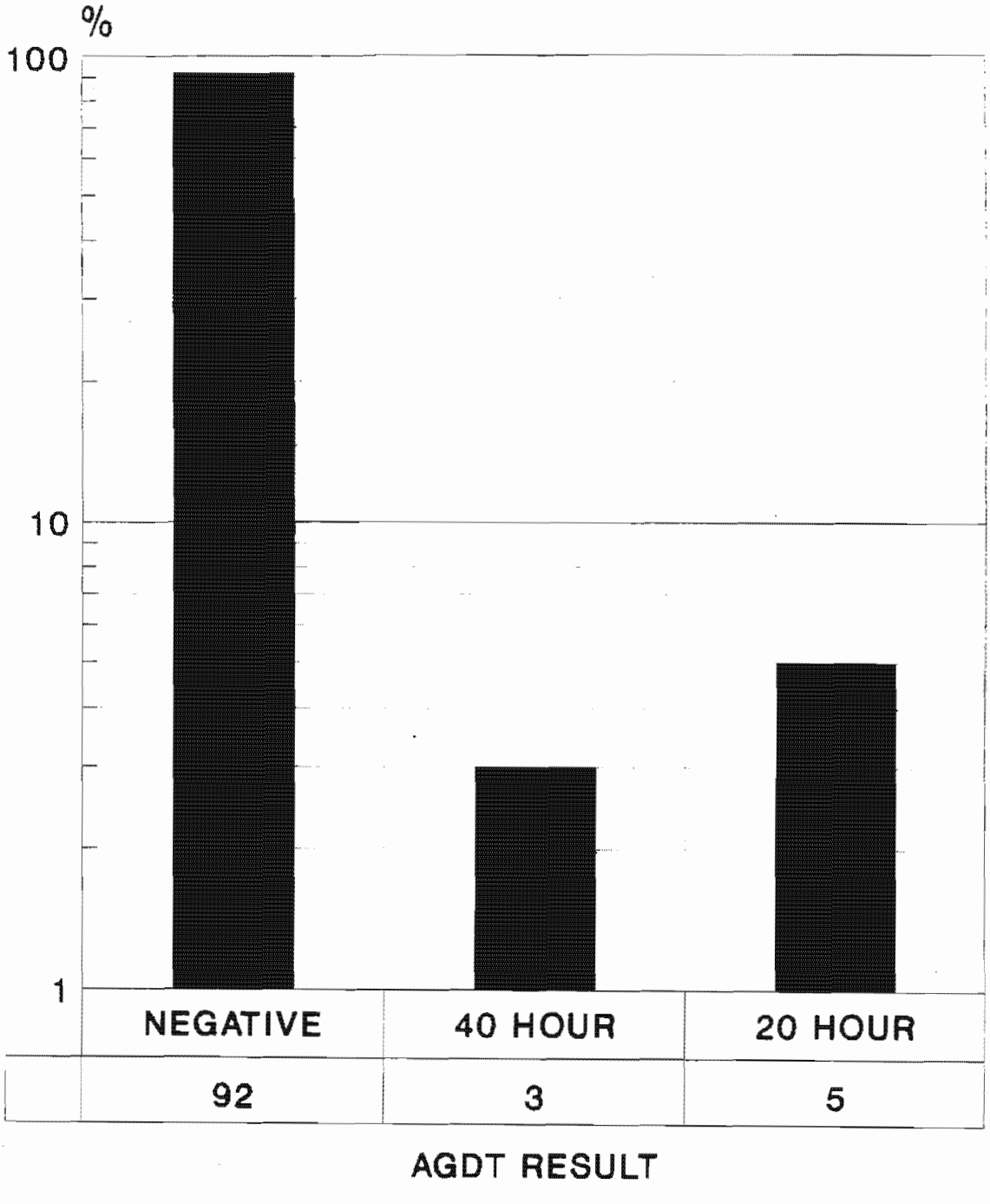

Figure 5.5 AGDT results in Philippi (Cape Peninsula) during an epidemic

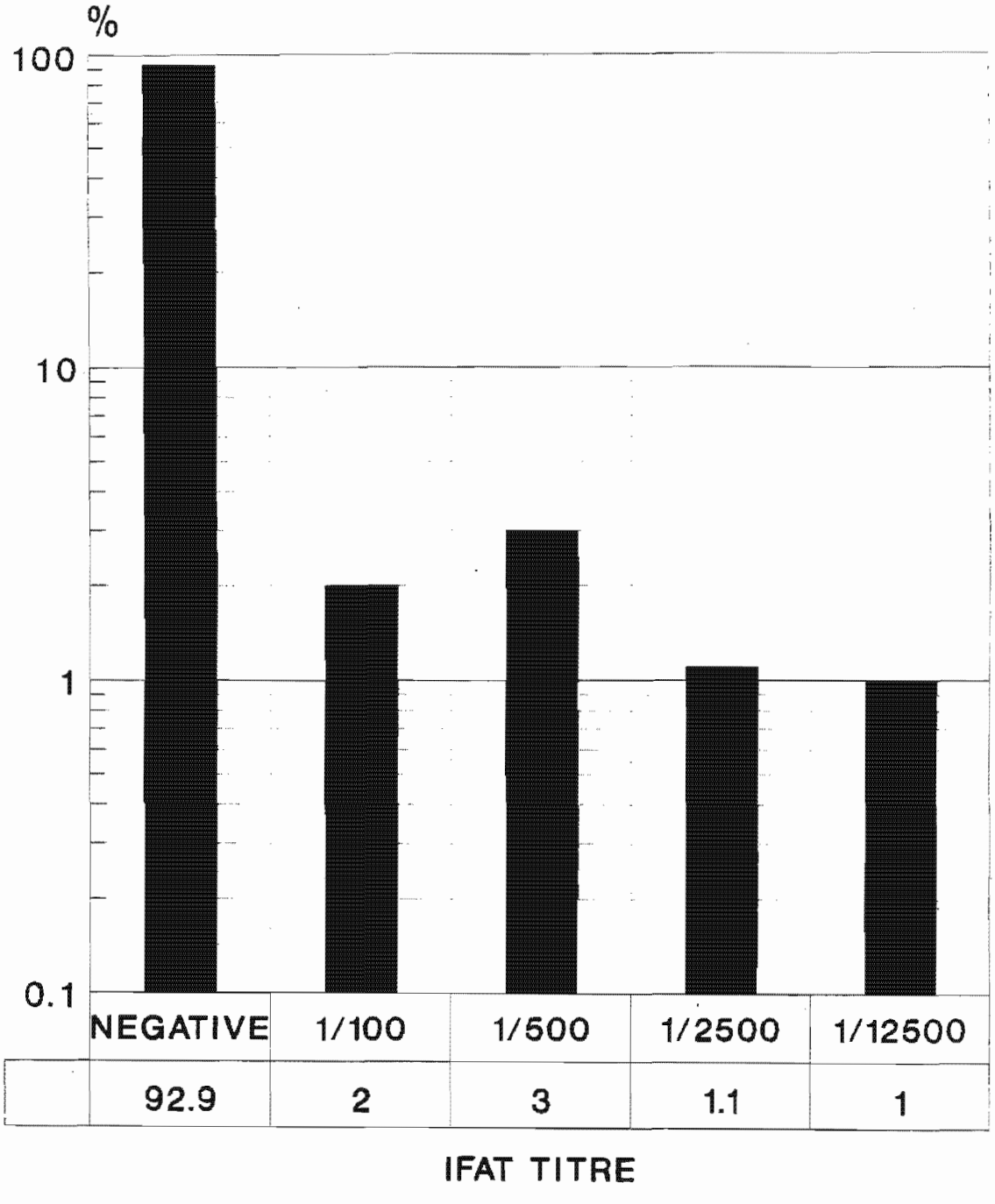

Figure 5.6 IFAT results in Philippi (Cape Peninsula) during an epidemic

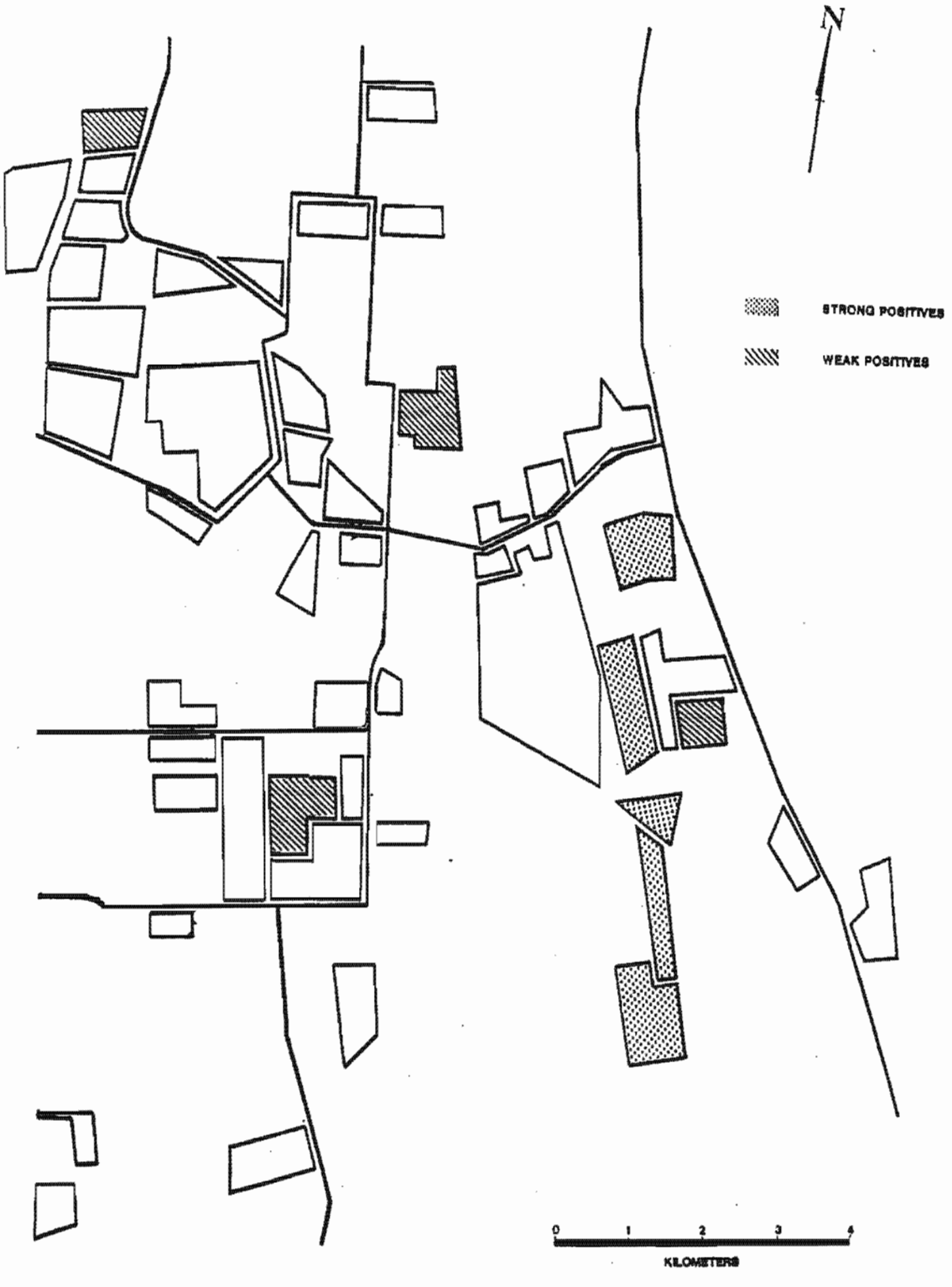

Figure 5.7 Location of seropositive subjects in Philippi

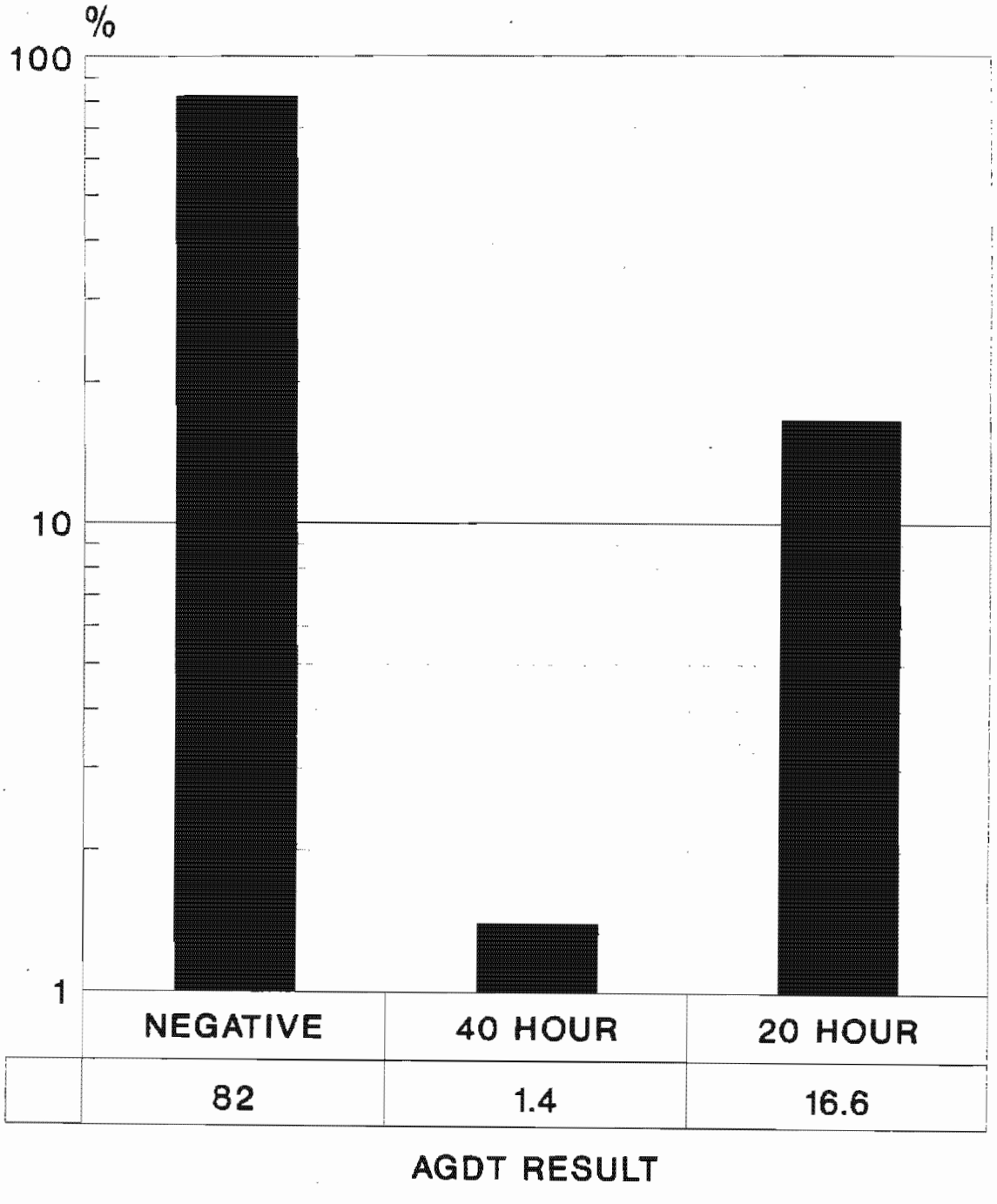

Figure 5.8 AGDT results in Philippi on the five farms on which the strongly seropositive subjects were resident

5.4 DISCUSSION

The most striking observation of this study was that the pathogenic zymodemes isolated from 17 apparently healthy subjects had induced their hosts to mount a marked antibody response. (Tables 5.1 and 5.2). Furthermore, the seropositivity of healthy asymptomatic subjects harbouring pathogenic E. histolytica was directly comparable to that of symptomatic subjects in whom clinical disease was proven. This demonstrated that pathogenic zymodemes of E. histolytica can elicit an antibody response in their hosts irrespective of whether or not they experience symptoms of amoebiasis (Jackson et al, 1985). Thus, it is apparent that whenever a pathogenic zymodeme is present in an individual, the parasite is in contact with the host's tissues and is therefore potentially capable of deeper tissue penetration which may result in the development of clinical disease. Furthermore, only 21% of subjects harbouring non-pathogenic zymodemes were seropositive; this latter level of seropositivity was not significantly different from that observed in the random population sample of this endemic area and implies that the non-pathogenic zymodemes do not invade the host's tissue. It is hypothesised that the absence of an antibody response in individuals harbouring infections with non-pathogenic zymodemes is due to its non-invasive behaviour.

The fact that the serological result has both a quantitative and a qualitative component can readily be appraised by comparing Tables 5.1 and 5.2. The frequency of strong seropositivity in subjects

infected with non-pathogenic E. histolytica (3%; Group 3) is comparable to those who are not infected at all (2%; Group 2); these are in turn similar to the general population (4%; Group 1). Furthermore, subjects with invasive disease and symptom-free carriers of pathogenic zymodemes were not only 100% seropositive but they also had a much higher frequency (94-100%) of strongly positive responses. This difference in stimulation of the humoral immune system of the host by pathogenic and non-pathogenic strains of E. histolytica indicates that these amoebae differ physiologically. Neal et al (1968) and Vinayak et al (1981) compared the serological titres of asymptomatic subjects infected with E. histolytica to the virulence of the strain of E. histolytica isolated from them by means of caecal scoring in rats; they found a positive correlation between the virulence of E. histolytica (as judged by the degree of caecal ulceration in rats) and positive serological responses in the hosts from whom these amoebae were isolated. Referring to these independent studies it can be argued that the presence of a strongly positive serological reaction is highly indicative of current infection by a pathogenic E. histolytica zymodeme with or without concomitant symptoms. These serological tests are therefore of considerable significance in screening populations for the presence of pathogenic E. histolytica (Jackson et al, 1985). Therefore, unlike Healy (1986) who stated that positive serology in seroepidemiological surveys provides an index of invasive disease in the population, it can now be stated that a strongly positive serological reaction (using the AGDT) is a marker of both active as well as potential disease.

The 100% seropositivity in subjects with invasive amoebiasis is higher than that recorded in other studies (Healy & Gleason, 1972; Patterson et al, 1980; Maddison et al, 1967) albeit a more logical result since the parasite is in fact invading the hosts' tissues. In the experience of this laboratory (unpublished results) the use of a single test such as the reliable AGDT occasionally does not record a positive result in patients with invasive amoebiasis, possibly due to inadequate production of precipitating antibodies at the time of testing. The high seropositivity recorded in the present study is attributed to the simultaneous use of two complementary tests the AGDT and IFAT. These tests are believed to be complementary because the IFAT utilises intact amoebae and therefore detects antibodies directed against the surface membrane of E. histolytica while the AGDT utilises homogenised amoebae and thus detects antibodies against aqueous soluble antigens. The use of complementary tests probably increases the chances of detecting seropositive cases in the other groups in which invasive disease is absent thereby providing more precise epidemiological data.

Although the serological responses of the subjects in Groups 1-3 (Table 5.I) were not statistically different, they probably reflect the environmental conditions experienced by these individuals. In Group 1, 19,5% of the subjects were seropositive with 4% of them displaying a strong positive reaction; since no parasitological information is available for the group it is reasonable to assume that this level of seropositivity is representative of the population in the area being studied and constitutes what Elsdon-Dew (1978) termed "the background noise". The individuals in

Group 2 had the lowest prevalence of seropositivity (13,5%) which is to be expected as they constitute a selected group of subjects who were proven by culture techniques to be free of infection with E. histolytica. The subjects in Group 3 could be expected to have a higher than normal chance of being in contact with pathogenic E. histolytica as the presence of non-pathogenic zymodemes in them at the time of the study indicates that their life-style and environment were suitable for transmission of the parasite (both pathogenic or non-pathogenic); this is probably reflected in the higher rate of seropositivity (21%) observed in them. It is therefore not surprising that in the community where only non-pathogenic zymodemes (Gazankulu) were isolated only seronegative results were obtained; this has also been the experience of Goldmeyer et al (1986) and Allason Jones et al (1986) in their study of male homosexuals. The variability of the serological response in asymptomatic carriers of E. histolytica (Healy, 1986) in different communities can therefore be directly ascribed to exposure to pathogenic E. histolytica.

Zymodeme XIII has not been classified as either pathogenic or non-pathogenic since it has a phosphoglucomutase (PGM) isoenzyme pattern that is typical of a pathogenic strain while its hexokinase (HK) isoenzyme pattern is that of a characteristic non-pathogenic zymodeme. This is a rare zymodeme but it has previously been detected in subjects in Durban (Sargeaunt et al, 1982b) as well as in Mexico (Sargeaunt et al, 1980b). Conclusive evidence is still needed to determine whether or not it is a pathogen. According to the findings of the present study the solution may lie in the

serological response elicited by this zymodeme which was found to be negative suggesting that it may well be non-pathogenic.

In a seroepidemiological study conducted in this geographical area, 20 years ago, Maddison et al (1965) stated that in "clinically negative" cases, the prevalence of seropositivity was 15,2%. In the study groups 1 to 3 above, the frequency of seropositivity was similar to that recorded by Maddison et al (1965). The background seropositivity recorded in the present study (19,5%) is however somewhat higher than that obtained by Maddison et al (1965); this is probably due to the use of complementary serological tests rather than to an increase in the prevalence of pathogenic zymodemes. It may therefore be concluded that the level of endemicity of pathogenic E. histolytica has remained the same during the past 20 years in the Durban area.

Kagan (1976), Elsdon-Dew (1978) and more recently Healy (1986) discuss the value of serological and microscopical evaluation of the prevalence of E. histolytica in a population; these workers conclude that because of the more objective nature of serological testing, this method is superior to stool microscopy in evaluating E. histolytica prevalence. During the course of the present study it has been demonstrated that the serological responses to the non-pathogenic zymodemes, which are by far the most prevalent form of E. histolytica, tend to be negative; therefore seroepidemiological surveys would not necessarily be an accurate method of determining the prevalence of these non-pathogenic zymodemes. The present study has confirmed that culturing methods

combined with isoenzyme electrophoresis are essential for establishing the true prevalence of non-pathogenic zymodemes (Chapters 3 and 4). On the other hand, as mentioned above, serology is invaluable for assessing the prevalence of pathogenic zymodemes. Optimisation of epidemiological data on E. histolytica may therefore be derived by using serological techniques in addition to zymodeme determination of the parasites particularly since the latter involves culturing them - a method already demonstrated to be superior to conventional microscopy for locating E. histolytica.

Kagan (1976) also pointed out that the levels of endemicity of amoebiasis may be ascertained by preparing antibody profiles based on the work of Healy & Gleason (1972), Juniper et al (1971) and Spencer et al (1976). However, the present study has shown that only pathogenic E. histolytica are capable of inducing a humoral response, therefore such antibody profiles would prove useful in determining the endemicity of pathogenic zymodemes only. In an endemic area a bimodal curve is to be expected; the subjects with higher titres are those with current infestations of pathogenic E. histolytica (mainly sub-clinical) and those with lower titres are subjects who were infected with pathogenic zymodemes in the past. When the antibody frequency curve becomes skewed toward the higher titres this would imply the presence of an epidemic and should be treated as a matter of urgency. An opportunity to test this hypothesis occurred during the course of this study. In the endemic amoebiasis area close to Durban where the proportion of pathogenic zymodemes was between 6 to 10% of all E. histolytica isolations, a bimodal curve was found (Figs 5.3 and 5.4). On the other hand the

population of Philippi in whom an amoebiasis epidemic had occurred, had a higher proportion of pathogenic zymodemes (6/23=26%). The antibody frequency distribution curve was again essentially bimodal but had became skewed towards the higher titres (Figs 5.5 and 5.6). This tendency towards a higher proportion of strongly seropositive responsiveness becomes more apparent when only those farms, where transmission of pathogenic zymodemes (farms 38, 40, 44, 45 & 46 in Fig 5.7) was shown to have occurred, are considered separately; this is depicted in Fig 5.8. In contrast, in Gazankulu where no pathogenic zymodemes were isolated, the population was 100% seronegative despite an E. histolytica prevalence level of 8,4%.

CHAPTER 6

A LONGITUDINAL STUDY OF CARRIERS INFECTED WITH PATHOGENIC ZYMODEMES OF ENTAMOEBA HISTOLYTICA

6.1 INTRODUCTION

Sargeaunt et al (1982b), while conducting an epidemiological survey in the Durban area, reported that pathogenic E. histolytica had been isolated from three individuals attending an out-patient clinic in Durban. Although it was presumed that these subjects did not present to the clinic with amoebiasis (since these were specialised clinics ie hypertension, diabetes mellitus, asthma, arthritis and orthopaedics) adequate clinical data were not available and therefore it was impossible to ascertain whether or not they had symptoms of invasive amoebiasis. Therefore it could not be established whether or not they were truly asymptomatic carriers of pathogenic E. histolytica.

Furthermore, during the extensive epidemiological study conducted in 1982 and 1983 (Chapters 3 & 4) it was found that pathogenic zymodemes of E. histolytica occurred in approximately 1% of apparently healthy individuals in the study area concerned (Gathiram and Jackson, 1985). The object of this study was to assess the outcome of carriers of pathogenic zymodemes during an extended follow-up period.

6.2 MATERIALS AND METHODS

A total of 21 subjects of all age groups and both sexes were identified. These carriers of pathogenic E. histolytica originated from a total population of 1837 individuals; 14 of them were derived from the epidemiological survey of 1390 individuals in 1982 (Chapter 3); pathogenic zymodemes were also isolated from a further 5 subjects during a survey of 795 subjects in 1983 (Chapter 4) and the remaining 2 carriers were identified during a survey of 103 schoolchildren in the same district (Chapter 5).

Subjects from whom pathogenic zymodemes of E. histolytica were isolated were admitted to the out-patient department of King Edward VIII Hospital (Durban) for clinical examination. Sigmoidoscopy was performed on all these carriers, except those who were under 3 years old. Where possible, 5 mℓ of blood was drawn from each subject. In addition, stool specimens were again taken from all patients and examined microscopically for the presence of parasites using the formol-ether concentration method. Because of the delay in establishing the zymodemes of the amoebae isolated in culture, clinical examinations were usually carried out 2 to 4 weeks after the initial stool collection.

Subjects who were free of clinical signs or symptoms of invasive amoebiasis were followed up at monthly intervals for one year; a history of symptoms of invasive amoebiasis was taken and a complete physical examination was performed on each occasion; stool culturing was repeated after 6 months when stools were collected

daily for 3 consecutive days. Individuals who developed clinical manifestations of invasive amoebiasis, were treated with metronidazole. They were also followed up in the same way as the abovementioned asymptomatic subjects who did not show any symptoms.

The sera of subjects were tested for the presence of amoebic antibodies by means of both the amoebic gel-diffusion (AGDT) and indirect immunofluorescent (IFAT) tests as described in Chapter 5.

6.3 RESULTS

The results obtained during this study are summarised in Table 6.1. Pathogenic zymodemes of E. histolytica were isolated from the stools of 21 subjects whose ages ranged from 1 to 54 years. There were equal numbers of males and females.

The most common pathogenic zymodemes were II (nine subjects) and XI (nine subjects). Pathogenic zymodemes VII (one subject) and XX (1 subjects) were also isolated (Gathiram & Jackson, 1987).

None of the subjects gave a history of symptoms relating to invasive amoebiasis at the time of the initial collection of stool samples. However, two patients (No 1 and 2 in Table 6.1) complained of having blood in their stools and cramp-like abdominal pain when examined two weeks later; at the same time haematophagous trophozoites of E. histolytica were observed in the stool of one of them; this patient also had small ulcers in his rectum and distal sigmoid colon compatible with amoebic colitis. The stool of the

TABLE 6.1 : DATA ON CARRIERS OF PATHOGENIC ZYMODEMES OF E. HISTOLYTICA

SUBJECT NO	AGE(YRS)	SEX	INITIAL ZYMODEMES	MICROSCOPY	SEROLOGICAL RESULTS	REPEAT ZYMODEME AT 6 MONTHS	STOOL CULTURE AT 1 YEAR
1	2	M	II	EHC	++	NO GROWTH	
2€	10	M	XI	HT	++	II	NO GROWTH
3€	8	M	XX		++	NO GROWTH	
4	2	M	II	EHC	++	NO GROWTH	
5	17	F	VII			NO GROWTH	
6	18	F	XI	EHC	++	NO GROWTH	
7ℵ	14	M	II	EHC	++	XI	NO GROWTH
8ℵ	20	F	XI		++	NO GROWTH	
9	48	F	XI		NO SERUM	NO GROWTH	
10 ➤	15	F	XI		++	II	NO GROWTH
11 ➤	8	F	II		++	XI	NO GROWTH
12 ➤	13	M	II	EHC	++	NO GROWTH	
13	17	M	XI	UPC	++	NO GROWTH	
14	7	M	II		++	XI	NO GROWTH
15 ◊	18	M	XI	EHC	NO SERUM	NO GROWTH	
16 ∝	3	F	II		++	NO GROWTH	
17 ∝	1	F	II		NO SERUM	NO GROWTH	
18 ∝	54	M	XI		++	NO GROWTH	
19 ◊	12	M	XI		++	NO GROWTH	
20	13	F	II		++	ND	
21	15	M	II		++	ND	

€, ℵ, ∝ } FAMILY MEMBERS LIVING IN SAME HUT

➤ EXTENDED FAMILY, LIVING AS NEIGHBOURS

◊ NEIGHBOURS

LEGEND: EHC = E. histolytica cysts; HT = haematophagous trophozoites; UPC = unidentified pre-cysts; ++ = strongly positive; ND = not done - lost to follow-up.

NOTES : (i) Subjects 1 and 2 both developed dysentery;
(ii) subject 2 had amoebic colitis on sigmoidoscopy;
(iii) subjects 1, 4, 16 and 17 were not subjected to sigmoidoscopy owing to their age;
(iv) no abnormalities were detected on sigmoidoscopy in the remainder of the study population;
(v) no E. histolytica cysts were detected by stool microscopy from any of the subjects at 6 months and 1 year.

other dysenteric patient revealed quadri-nucleate cysts of E. histolytica. Both subjects became asymptomatic within three days of treatment with the standard dosage of metronidazole (Flagyl; Maybaker) i.e. 50 mg/kg/day in 3 divided doses for 5 consecutive days. It is interesting to note that pathogenic E. histolytica (a different zymodeme (II) from the one isolated initially (XI)) was again identified from one of these subjects when stool cultures were repeated 6 months later; after a further six months repeat cultures showed that this patient had apparently spontaneously lost the infection without developing clinical signs or symptoms of amoebic colitis during the intervening period.

Nineteen of the subjects were asymptomatic throughout the study period; in addition, colonic or rectal ulceration was not observed in any of the individuals examined by sigmoidoscopy. E. histolytica cysts were detected in five of the 19 subjects. Two subjects subsequently left the study area and could not be followed up for the whole period; none of the remaining 17 complained of any symptoms of amoebic colitis during the 1 year follow-up period. When stool cultures were repeated six months after the initial isolation of pathogenic zymodemes, 13 individuals had apparently spontaneously lost their infections. Four subjects were still infected with pathogenic zymodemes of E. histolytica; however, surprisingly the zymodemes were different in all four cases from those isolated initially (Table 6.1). After a further six months all subjects were free of E. histolytica infections on the basis of stool culture and microscopy.

It was also observed that the pathogenic zymodemes tended to occur more frequently amongst individuals belonging to the same family unit as well as among those living in neighbouring huts.

As reported in Chapter 5, a strongly positive serological reaction was obtained from all sera tested.

6.4 DISCUSSION

The fact that 1% of apparently asymptomatic individuals harbour pathogenic zymodemes (Gathiram & Jackson, 1985) is important from a public health point of view. These subjects represent a public health hazard as they are potential reservoirs of infection. Asymptomatic carriers are more likely to spread the disease (thus maintaining endemicity) than symptomatic patients with invasive amoebiasis; the latter will seek medical advice and promptly obtain treatment. The fact that 2 subjects developed dysentery shortly after the initial examination emphasises the necessity to identify and treat this group in the population in order to avoid the morbidity and mortality caused by infection with the parasite. Therefore, unlike Nanda *et al* (1984) who believe that treatment of cyst passers is not necessary it is proposed that treatment should at least be based on the pathogenicity of the amoebae isolated in each case. Where facilities for culturing and isoenzyme electrophoresis are not available the medical practitioner could be guided by the serological results since carriers of pathogenic zymodemes have been shown to have strongly positive serological responses (Jackson *et al*, 1985, Chapter 5) which are directly

comparable to those recorded from subjects with invasive amoebiasis; on the other hand, carriers of non-pathogenic zymodemes tend either to be sero-negative or to have low antibody titres which in turn are directly comparable to those recorded from (non-infected) subjects dwelling in an endemic area. Where no reliable supportive tests are present the decision to treat the infected individual must rely on clinical judgement and becomes the responsibility of the consulting medical practitioner.

It is of interest to note that "self cure" apparently occurred in all individuals within 12 months after the original isolation of the parasite, while only two subjects developed amoebic colitis. This phenomenon of self cure has also been observed in carriers of non-pathogenic zymodemes (Chapter 4). Nanda et al (1984) followed up 15 of 34 culture positive subjects for periods ranging between 1 and 19 months and reported that spontaneous cure occurred in all cases and no patient developed invasive amoebasis; by extrapolation it can be concluded that zymodemes were determined on approximately 50% of the E. histolytica isolates as this was apparently reported in an earlier publication (Sargeaunt et al, 1984); although they apparently overlooked the fact that 41% of the isolates from New Delhi (Sargeaunt et al, 1984) proved to be pathogenic zymodemes it is highly likely that approximately half their (Nanda et al, 1984) subjects were carriers of pathogenic zymodemes.

The presence of high levels of E. histolytica antibodies in all the subjects tested is an indication of sub-clinical tissue invasion by the parasite. The pathogenesis of deeper tissue invasion is

therefore probably dependent on factors other than the pathogenicity of E. histolytica, for example, the nutritional (Singh, 1959; Diamond et al, 1978; Diamond, 1982) and immune status (Kagan, 1973, 1974; Salata and Ravdin, 1986) of the host.

The prevalence of pathogenic E. histolytica was the same in males and females. Furthermore, it was noted in Chapter 5 that there was no significant difference (X^2=0,056; p 0,5) in the prevalence of seropositive responses of males (8/47=17,02% seropositive) and females (20/141=14,18% seropositive). Therefore, it would appear that males and females have an equal chance of being infected with the potentially invasive form of E. histolytica. This is at variance with the markedly higher prevalence of invasive amoebiasis observed in males. To illustrate this male:female ratios of 17:2 for patients with amoebic dysentery (AD) and 44:4 for patients with amoebic liver abscess (ALA) were observed in Chapter 2; this supports the observations of Wilmot (1962, 1973) in the Durban area. He, (Wilmot 1962, 1973) reported male:female ratios of 2:1 for AD and 7:1 for ALA. It would appear that while males and females have an equal chance of being infected with pathogenic zymodemes including sub-clinical tissue invasion by the parasite, females are protected by some mechanism from acquiring the more severe form of the disease.

Quadrinucleate cysts of E. histolytica were found in the faeces of 6 individuals harbouring pathogenic zymodemes. Since isoenzyme electrophoresis revealed no evidence of mixed infections with pathogenic and non-pathogenic zymodemes it seems reasonable to

assume that pathogenic E. histolytica do produce cysts. This will obviously complicate the situation regarding drug treatment. Metronidazole (Flagyl; Maybaker) and the other nitroimidazole derivatives, although highly effective against the trophozoite, has no effect on E. histolytica cysts (Knight, 1980). However, clinical trials have shown that metronidazole, given over a prolonged period (7-8 days), cleared cysts from the colon presumably by killing off the trophozoite source thereby preventing both re-infections and recrudescences (Knight, 1980). Other effective luminal amoebicides for treating cyst passers such as diloxanide furoate and diodoquin also need to be tested for their efficacy in controlling the passing of cysts.

The clustering of infections with pathogenic strains of E. histolytica in family groups and closely associated neighbours is important and suggests direct person to person transmission. Such family clustering of infections with both pathogenic and non-pathogenic zymodemes was reported on a much larger scale in Chapter 4 and has been observed by others (Spencer et al, 1976, 1977; Munoz 1986, Dykes 1980). Hence, overcrowding can be expected to result in a greater prevalence of pathogenic E. histolytica. Furthermore the observation that some individuals can harbour these pathogenic zymodemes for periods of up to 6 months without developing invasive amoebiasis, is good evidence that a carrier state does exist. Considering the foregoing, follow-up of patients with invasive amoebiasis after treatment, (since approximately 15% of them became asymptomatic carriers of pathogenic zymodemes following treatment (Chapter 2)), as well as their contacts, should

also become mandatory in order to effectively control disease transmission. This could be a useful public health measure in the management of amoebiasis and the matter should receive urgent attention.

A surprising finding (Table 6.1) was that all subjects from whom E. histolytica was successfully cultured 6 months after the initial isolation, still had pathogenic zymodemes but, in all the cases they now belonged to different pathogenic zymodemes (from zymodeme II to XI and vice versa). Since laboratory conditions had remained consistent it is considered that these apparent zymodeme changes could be attributed to one of the following: (i) re-infections; (ii) infection with mixed pathogenic zymodemes with one zymodeme being predominant in the gut at different times or (iii) induction of the genes to 'turn' the isoenzymes 'on or off' in response to environmental pressures. The first suggestion ((i) above) seems unlikely since this relatively large number of re-infections with pathogenic E. histolytica would seem improbable in a population where the prevalence of pathogenic zymodemes is 1%. Considering that there was a seasonal change (summer to winter and vice versa) during the period in question it is likely that the diet of these rural people could have changed markedly. The change in diet could in turn have influenced the gut flora which may have affected the isoenzymes of the amoebae by either of the other two routes (ii & iii) proposed. Since the observations were made on freshly isolated uncloned cultures it is possible that there were mixtures of zymodemes II and XI and the shift in isoenzyme patterns could then plausibly be explained by the second suggestion (ii above);

however, in previous studies (Jackson et al 1982; Sargeaunt et al 1982) it was reported that infections with mixed zymodemes are uncommon in this geographical area. Alternatively, the observation in this study could indicate that the same organism can express both zymodeme II and XI patterns at different times depending on the environmental conditions prevailing in the gut at the time of isolation; the resulting variable banding pattern observed in phosphoglucomutase (PGM) and glucose phosphate isomerase (GPI) could thereby be due to genetically controlled turning on or off of isoenzymes.

The abovementioned suggestion that pathogenic E. histolytica is capable of expressing different isoenzyme patterns concurs with the hypothesis of Sargeaunt et al (1984) that the spectrum of zymodemes of E. histolytica that have been recorded are phenotypes of the parasite and that the amoebae in which single bands are recorded in PGM and GPI are the most important key zymodemes. The zymodemes with single bands in PGM and GPI viz I, II & XIV could therefore be considered as the original strains of E. histolytica while the other zymodemes may be phenotypes derived from them as a result of some environmental factor. This hypothesis may explain the observations in Chapter 2 that two different pathogenic zymodemes could be isolated simultaneously from two different sites (liver and colon) from the same individual (Table 2.5) and the apparent change in zymodeme that occurred following treatment of another patient (Table 2.6). It may also explain the apparent shift in the most prevalent zymodemes from zymodeme I (Chapter 3) to zymodeme III during the drought of 1983 (see Chapter 4).

TABLE 6.2 : Compares the frequency distribution of pathogenic zymodemes of E. histolytica in the three categories amoebic liver abscess (ALA) amoebic dysentery (AD) and asymptomatic cyst passers. These are the cumulative data from Chapters 2, 3 and 4.

	ZYMODEME			
	II	XI	VII	XX
ALA	82,1%	14,3%		3,6%
AD	80%	20%		
Asymptomatic carriers	42,8%	47,6%	4,8%	4,8%

When the frequency distribution of pathogenic zymodemes in the three categories, ALA, AD and asymptomatic cysts passers was compared an interesting trend emerged (Table 6.2). Apparently there is an almost equal distribution of pathogenic zymodemes II and XI in asymptomatic carriers; however, zymodeme II seems to be involved more frequently in deeper tissue invasion. Whether or not this propensity for deeper tissue invasion and clinical disease is zymodeme-related should be investigated further in future epidemiological investigations. Alternatively, it is possible that this observation relates to the previously mentioned phenomenon of pathogenic zymodemes apparently changing from one characteristic pattern to another. The change in environment from the polyxenic conditions in the gut to the axenic conditions in deeper tissue

invasion might be an example of a trigger that can induce the 'switching' of isoenzymes.

CHAPTER 7

A CASE STUDY OF RECURRENT AMOEBIC LIVER ABSCESS : EPIDEMIOLOGIC FEATURES

7.1 INTRODUCTION

Recurrence of amoebic liver abscess is rare (Wilmot, 1962). In a recent drug trial conducted in Durban (Simjee et al 1985 and Chapter 2) only three out of 48 patients with amoebic liver abscess (ALA) experienced recurrent ALA; in one patient recurrence occurred on 3 occasions. Epidemiological investigation of the family of the patient with multiple recurrence of ALA was performed; the results are presented here as they highlight the value of following up the contacts of patients with invasive amoebiasis.

7.2 Patient description:

The patient was a 46 year old Black male (patient number 19 in Tables 2.6 and 2.7) employed by the South African Navy in Durban; his home was in the Eshowe district approximately 100 km from Durban; he lived in a men's hostel in Durban and visited his family occasionally (once in every two to three months). He was admitted to King Edward VIII Hospital for the first time in February 1981 with ALA. The diagnosis of ALA was made on clinical history and physical examination and confirmed by ultrasound examination of the liver which showed an echo-free lesion in the right lobe. In addition, bacteriologically sterile pus was aspirated from the liver abscess and the patient responded to treatment with

metronidazole (800 mg three times per day for 5 consecutive days). E. histolytica was isolated from the liver pus in Robinson's culture medium (Robinson, 1968); isoenzyme electrophoresis proved the isolate to be zymodeme II (Table 2.6). At this stage, absolute T and B-Cell numbers as well as T-cell function were estimated (Simjee et al, 1985); the latter was assessed by the ability of T-lymphocytes to undergo mitogenic transformation in the presence of 20 ug amoebic antigen as well as phytohaemagglutinin (Simjee et al, 1985); both qualitative and quantitative evaluation of T and B-cell function were normal (Simjee et al 1985). The patient improved clinically and was discharged after 10 days. He was followed up as outlined in Chapter 2, and remained well; the defect in the liver was monitored by ultrasound examination and progressively decreased in size and showed signs of healing.

In April 1981 the patient again complained of right upper quadrant abdominal pain and pyrexia. Ultrasound examination now showed an increase in abscess size which had, in addition, developed uneven edges. The liver aspirate was again bacteriologically serile and E. histolytica which proved to be zymodeme II was isolated (Table 2.7). The patient was treated with metronidazole (800 mg tds x 5 days) and discharged, apparently well, after 10 days. He was again followed up at monthly intervals; the liver abscess had completely healed by August 1981.

On a third occasion in September 1981 the patient presented (Table 2.7) with complaints of chest pain and dyspnoea on exertion. He was febrile with an elevated jugular venous pressure, the pulse rate

was 120/min with pulsus paradoxus. A pericardial friction rub was clearly audible. He had a 3cm hepatomegaly which was tender in the epigastrum. ECG showed sinus rhythm; a rate of 120/min and ST elevation in the standard as well as the anterior chest leads. The chest radiograph revealed a globular heart. Ultrasound examination showed an anechoic defect in the left liver lobe close to the left hemi-diaphragm; there was also a defect in the left hemidiaphragm and a 2 cm pericardial effusion was present. A left lobe ALA with rupture into the pericardium was diagnosed. Drug treatment with metronidazole (800 mg tds) was commenced and the pericardial effusion as well as the liver abscess were drained surgically. The patient made an uneventful recovery and was discharged after 2 weeks. Subsequent ultrasound examinations revealed complete healing of the abscess.

The patient was readmitted in June 1984 with symptoms of right upper quadrant abdominal pain and pyrexia. Diagnosis of a right lobe ALA was made; this was confirmed by ultrasound examination. E. histolytica (zymodeme II) was isolated from the liver abscess (Table 2.7). Treatment with metronidazole successfully cured the ALA. Qualitative and quantitative evaluation of T and B-cell function were again normal.

Serological testing amoebic gel diffusion (AGDT) was done at the time of each admission and two months following discharge from hospital by means of the amoebic gel diffusion test (AGDT); on each occasion precipitins were seen at 20 hours (ie strong seropositivity - Chapter 5). Microscopy of stools and cultures for

E. histolytica were done on the day of discharge and a month later on every occasion the patient was admitted to hospital. E. histolytica-zymodeme II was isolated from a post-treatment stool following the initial admission (Table 2.6). Subsequent post-treatment stool cultures failed to isolate E. histolytica. The patient did not have any concomitant disease and had never been treated with steroids.

7.3 EPIDEMIOLOGICAL INVESTIGATIONS

Having established that person-to-person contact may be a factor in disease transmission (Chapters 4 and 6) a complete coprological and serological evaluation of the family of this patient was conducted to detect the presence of pathogenic E. histolytica.

The family consisted of 20 people (other than the patient himself), ranging in age from 2 to 40 years (Table 7.1). The kraal consisted of 3 mud huts; there was no latrine and water, drawn from a nearby stream, was not boiled or chlorinated prior to drinking.

A brief history with regard to gastrointestinal symptoms was obtained from each subject; in addition a freshly passed stool specimen as well as 5mℓ of blood were taken. A portion of each stool specimen was cultured in Robinson's medium (Robinson, 1968) and whenever E. histolytica was isolated the zymodeme was determined. A portion of the stool specimen was also examined microscopically for parasites. Serological testing for E. histolytica antibodies was done using the AGDT (Chapter 5).

7.4 RESULTS

Two family members were free of intestinal parasites on microscopical examination; in the remainder the level of parasitic infestation was rather high. The following parasites were isolated: E. histolytica (4), E. hartmanni (5); E. coli (10); Iodamoeba butchlii (4); Giardia lamblia (2), Chilomastix meslinii (2); Ascaris lumbricoides (11) and Trichuris trichiura (16).

Table 7.1 provides a summary of the results obtained. A notable finding is that E. histolytica was successfully cultured from 7 (35%) subjects and pathogenic zymodeme II was present in one (5%) of these. Seven of the 20 subjects were seropositive with two of them being strongly positive.

7.5 DISCUSSION

The antibody responses that are present in invasive amoebiasis do not appear to be effective in controlling or protecting against invasive disease (Salata & Ravdin 1986). Cellular sensitisation to amoebic antigen has been demonstrated (Harris & Bray, 1976; Ganguly et al 1979, 1981; Savanat et al 1973; Aust Kettis & Sundqvist, 1982; Simjee et al 1985); however this appears to be a transient phenomenon since no evidence of cellular sensitisation could be demonstrated two months after treatment of ALA (Simjee et al,

TABLE 7.1 Serological and parasitological evaluation of family members of a patient with recurrent amoebic liver abscess

Subject No	Relationship to patient	Age	Sex	*E. histolytica* zymodeme	AGDT
1	Wife	30	F	III	Negative
2	Wife	40	F	III	Negative
3	Brother	40	M	N.G.	40 hr*
4	Daughter	20	F	III	40 hr*
5	Son	18	M	N.G.	Negative
6	Daughter	18	F	N.G.	40 hr*
7	Daughter	18	F	III	Negative
8	Son	16	M	N.G.	Negative
9	Daughter	11	F	N.G.	20 hr*
10	Daughter	18	F	VIII	Negative
11	Brother	41	M	N.G.	40 hr*
12	Daughter	12	F	N.G.	Negative
13	Daughter-in-law	30	F	N.G.	40 hr*
14	Daughter	17	F	N.G.	Negative
15	Daughter	16	F	N.G.	Negative
16	Sister-in-law	40	F	N.G.	Negative
17	Grand-son	4	M	XI	20 hr*
18	Wife	35	F	XVII	Negative
19	Daughter	5	F	N.G.	Negative
20	Daughter	2	F	N.G.	Negative

NOTES : N.G. = No growth
M = Male
F = Female
40hr = Weakly positive AGDT
20hr = Strongly Positive AGDT
* = Positive and Strongly Positive

1985). Whether or not the development of cell mediated immunity protects against recurrence of ALA is not clear.

The high prevalence of seropositivity (35%) implies that an appreciable proportion of the family members had been infected with pathogenic E. histolytica at some stage (Jackson et al, 1985; Chapter 5); furthermore one subject proved to be a carrier of a pathogenic zymodeme. The results again highlight person-to-person transmission of the parasite. It is proposed that recurrence of ALA in this patient probably occurred as a result of repeated exposure to pathogenic zymodemes at his home because complete cure was apparently achieved on each hospital admission.

It is interesting that only a single member of this family developed ALA and also that multiple recurrences were noted in the patient; the results imply that he had a predisposition to developing invasive amoebiasis. An immunological deficiency could not be demonstrated by the tests of humoral and cellular immune functions performed; however these tests are fairly crude and may miss subtle deficiencies.

In an epidemiologic study of two cases of ALA in children Harrison et al (1979) found that in the family of one of them, three of four members had serologic evidence of infection and one of 17 members of the family of the other child had positive serology while eight were cyst passers; they concluded from this study that investigation of the family or close contacts may be as important a duty as care of the patient (Harrison et al, 1979). Spencer et al (1977) conducted an epidemiological investigation of the extended

family of two cousins who developed ALA; they found that 45% of 162 family members had a positive indirect haemagglutination test for amoebiasis and 12,6% of 111 were passing cysts of E. histolytica; only 0,3% of a random sample of the remainder of the community were seropositive. They (Spencer et al, 1977) conclude that investigation of the family and close contacts of patients with amoebiasis may disclose many additional infected individuals who often can be treated as outpatients with minimal cost and inconvenience.

CHAPTER 8

MONAXENIC AND AXENIC CULTIVATION OF PATHOGENIC AND NON-PATHOGENIC STRAINS OF ENTAMOEBA HISTOLYTICA

8.1 INTRODUCTION

According to Bos (1975) axenic cultivation of E. histolytica has been successfully attempted by only a few workers. Furthermore, where this has been possible only those strains of E. histolytica that were originally isolated from symptomatic cases of amoebiasis have been adapted to axenic cultivation (Bos, 1975). Bos (1975) was able to monaxenise and subsequently axenise a further 8 strains of E. histolytica; 5 of these were from patients with invasive amoebiasis and the remaining 3 were from asymptomatic carriers. Using hamster liver infections to test for virulence Bos and Hage (1975) demonstrated that the 3 carrier strains were as virulent as the 8 patient strains. Furthermore, since these carrier strains originated from patients living in the same pavilion of a mental hospital he (Bos 1975) concluded that these carrier strains might be one and the same having been introduced to the hospital ward by a nurse who 'showed signs of amoebic dysentery'. Bos (1975) was unable to monaxenise other carrier strains of E. histolytica that showed either "low or no pathogenic potential".

Sargeaunt et al (1980a) determined the zymodemes of a large number of axenic strains of E. histolytica which were derived from various geographical localities and concluded that all of them belonged to

pathogenic zymodemes. These results suggest that the pathogenic strains of E. histolytica are more easily adapted to monaxenic and axenic growth than the non-pathogenic strains.

More recently, Mirelman and co-workers (Mirelmen, 1987, Mirelman et al 1986a,b) have been able to adapt two non-pathogenic zymodemes to axenic growth in an artificial environment supplemented by powerful antibiotics and lethally irradiated bacteria. This group of workers were also able to demonstrate that during the process of axenisation the non-pathogenic zymodemes apparently underwent a complete metamorphosis in that their isoenzyme banding converted to characteristic pathogenic zymodeme patterns and furthermore the amoebae apparently showed an increase in virulence.

The main objective of the study described in this chapter was to attempt monaxenisation and axenisation of known pathogenic and non-pathogenic zymodemes and to study the effects of these changes in culture on the isoenzyme patterns of E. histolytica.

8.2 MATERIALS AND METHODS

8.2.1 Strains used.

Eight strains of E. histolytica were used; the source and date of isolation of these are depicted in Table 8.1. All strains were initially isolated in Robinson's medium (Robinson, 1968) and then transferred to TYS-GM medium (Diamond, 1982b).

TABLE 8.1 Data on E. histolytica strains used for monaxenic and axenic cultivation

STRAIN	ISOLATED ON	ZYMODEME	SOURCE
2218	March 1984	III	Asymptomatic carrier - Malagasy
C1602	September 1984	II	Patient - amoebic liver abscess
Cape 221	September 1984	XI	Asymptomatic carrier - Phillipi
Cape 222	September 1984	XI	Asymptomatic carrier - Phillipi
842	June 1985	II	Asymptomatic carrier - Valley Trust
1036	June 1985	III	Asymptomatic carrier - Valley Trust
1029	June 1985	III	Asymptomatic carrier - Valley Trust
1056	June 1985	VII	Asymptomatic carrier - Valley Trust

Notes:

1) Malagasy is the study area described in Chapters 3 and 4 (see Table 4.1).

2) Phillipi was described in Chapter 5

3) Valley Trust is a rural area approximately 50km west of Durban.

4) Strain C1602 was isolated from the liver aspirate of a patient with amoebic liver abscess.

8.2.2 Elimination of bacteria and monaxenisation

This was carried out by first suspending the sediments of culture tubes in TYS-GM medium together with a solution of 20mg/mℓ neomycin sulphate. After incubation for approximately 16 hours the sediment was transferred to TYI-S33 culture medium (Diamond, 1978b); 0,25mℓ of a Trypanosomi cruzi stock culture maintained in Toby's medium (Tobie et al, 1950) was added to each tube together with 150ug of streptomycin and 300 Units penicillin. Every 48 hours the cultures were inspected under an inverted microscope (Olympus, Japan) with a 10x objective; the tubes were not opened but the glass wall was inspected for the number of actively moving amoebae adhering to it. A semiquantitative evaluation of growth was used (+ to ++++); + representing approximately 10 amoebae per field and ++++ when amoebae completely covered the observed area (Bos, 1975). Four tubes of every strain were permanently kept as stock cultures. Every 48 hours, amoebae from the two tubes showing the best growths were sub-cultured into a further four tubes; sub-culturing was performed by chilling the parent tubes for 5 minutes in an iced water bath, centrifuging it for 5 min at 2000xg before carefully decanting the supernatent fluid and harvesting the sediment by drawing it into a Pasteur pipette; the sediment was divided into two daughter tubes with fresh TYI-S33 medium, trypanosomes and antibiotics as described above. Antibiotics were no longer required after the organisms had been sub-cultured 10-15 times.

In order to determine whether bacterial contamination was present material from the cultures was examined bacteriologically as

described above (chapter 2.2.3) whenever sub-culturing was performed.

8.2.3 Axenisation of E. histolytica

Axenisation was attempted by transferring all the sediment from a parent tube into a single tube with fresh TYI-S33 medium and by halving the number of trypanosomes added to this and subsequent cultures until the trypanosomes were no longer required to maintain growth. This was found to be possible only when growths had attained ++++. Cultures were examined every 24 hours and sub-culturing was performed every 48 hours. Additional trypanosomes were added whenever it was found that the intensity of growth had diminished.

Various methods were employed in an attempt to increase the growth of strain 2218 (zymodeme III) to facilitate adaptation to axenic growth; these are outlined below:

i The pH of the TYI-S33 culture medium in which they were grown was varied over the pH-range 5-8 in increments of 0,2 to determine whether a change in the redox potential might influence growth. Cultures grew best at a pH 7 and never attained more than ++ growth intensity.

ii Cultures were grown in TYI-S33 medium with a supplement of cysteine HCl on an ionagar slope; both of these factors had previously been shown to increase the growth of axenic

cultures (Bos, 1975). Neither of these manipulations increased the growth of strain 2218.

iii Freeze-dried, heat inactivated Escherichia coli were added to the cultures in an attempt to provide nutritional support but this failed to improve their growth.

iv A known axenic pathogenic zymodeme II of E. histolytica (strain 1602) kept at the RIDTE was homogenised; the homogenate was then filtered through a 0,22um Millipore filter and the filtrate added to a number of monaxenic cultures of strain 2218. Here an attempt was made to transfer viruses which had been shown to occur concomitantly with strain 1602 (Olivier et al, 1984) into the non-pathogenic zymodeme. This procedure only resulted in rapid lysis of the trophozoites in the culture of strain 2218; an increase in growth was never observed.

The zymodeme of Strain 2281 was repeated after 2 months and 1 year of continuous monaxenic cultivation.

8.3 RESULTS

The following notable observations were made:

a) All strains were monaxenised on the first attempt.

b) The pathogenic strains C1602 (zymodeme II) and 1056 (zymodeme VII), were both successfully axenised, the former after 3 months and the latter after 10 weeks in monaxenic culture.

c) The pathogenic strains Cape 221, Cape 222 and 842, could not be axenised, however, they all showed better growth (+++ to ++++) in monaxenic culture than the non-pathogenic strains, ie 2218, 1036 and 1029 (usually ++).

d) Strain 2218 (zymodeme III) the first to be monaxenised was kept in monaxenic culture for a total of 18 months. Numerous attempts to axenise this culture proved unsuccessful.

e) Strain 2218 still proved to be zymodeme III when isoenzyme electrophoresis was repeated at both two months and one year after continuous monaxenic cultivation.

f) Strain 1036 (zymodeme III) was maintained in continuous monaxenic culture for 5 months. Both strains 2218 and 1036 eventually died out as a result of bacterial infection introduced into the culture with bacterially contaminated trypanosome supplement.

g) Monaxenic strains 842 (zymodeme II), Cape 221 (zymodeme XI), and Cape 222 (zymodeme XI), survived for 8 months, 4 months and 4 months respectively in monaxenic culture. Axenic strain C1602 was maintained in culture for 8 months. All of these cultures succumbed to bacterial contamination.

h) Axenic strains 1056 and monaxenic strain 1029 were maintained in culture for 4 weeks and 8 months respectively; both of them eventually died out due to a malfunctioning incubator.

8.4 DISCUSSION

Only pathogenic zymodemes (strains C1602 and 1056) were successfully axenised and sustained in axenic culture. Furthermore, of those strains that could not be axenised, the monaxenic cultures of the pathogenic strains (Cape 221, Cape 222 and 842) showed better growths than the non-pathogenic strains (2218, 1036 and 1029). The results are in agreement with those of Bos (1975) and indicate that the pathogenic zymodemes are easier to axenise than the non-pathogenic zymodemes. These observations concur with the hypothesis of Bos (1975) that the 'non invasive carrier strains' (non-pathogenic zymodemes) have been selected over a long period by adaptation to the intestinal environment; these strains grow very poorly in the absence of concomitant micro-organisms which probably provide nutritional support for them. The pathogenic zymodemes seem to adapt more readily to bacteria-free media. This property possibly confers on them the capability of tissue invasion and growth in a bacteriologically sterile environment such as a liver abscess (see Chapter 2).

It is interesting that strain 2218 maintained in continuous monaxenic culture for 18 months continued to express its original non-pathogenic isoenzyme patterns (zymodeme III) when tested a year after the culture was initiated. Furthermore, addition of

heat-inactivated bacteria failed to increase the growth of this strain and did not enhance the potential of this strain to be axenised.

Since the work discussed above was completed, similar studies by Mirelman and co-workers (Mirelman et al, 1986a,b) were reported in which non-pathogenic zymodemes were apparently adapted to axenic growth within 3 weeks by the addition of lethally irradiated bacteria to the cultures to provide nutritional support. They claim to have successfully adapted two non-pathogenic zymodemes to axenic growth; in the process of axenisation these non-pathogenic zymodemes apparently 'converted' to pathogenic zymodemes; this apparent 'conversion' was accompanied by an increase in the virulence of the previously non-pathogenic zymodemes. Similar experiments aimed at converting a third non-pathogenic zymodeme have repeatedly proved unsuccessful (Mirelman, 1987a). Furthermore, Mirelman's group also claimed that they could convert pathogenic zymodemes to non-pathogenic zymodemes by reassociating the former with bacterial flora derived from a xenic culture of a non-pathogenic zymodeme (Mirelman, 1987a).

Sargeaunt et al (1988) conducted a series of experiments in which bacteria from xenic cultures of known pathogenic zymodemes were added to cultures of non-pathogenic zymodemes. These latter cultures were then maintained for a period of two weeks (subcultures being performed every 48 hours) and the zymodemes were then determined. They were unable to demonstrate any change in the zymodemes of the non-pathogenic strains by simply manipulating the

nature of the concomitant bacteria. Similarly, Neal (1956) was unable to demonstrate an increase in the virulence (ability to produce caecal ulceration in mice) of a (xenic) non-pathogenic strain of E. histolytica to which bacterial flora from a pathogenic strain had been added. More recently Jackson and co-workers (pers comm) at the Research Institute for Diseases in a Tropical Environment in Durban have attempted to repeat the experiments of Mirelman's group; preliminary results suggest that axenisation of non-pathogenic zymodemes by the addition of lethally irradiated bacteria is not possible and that changes in pathogenicity based on isoenzyme electrophoretic patterns does not occur on prolonged monaxenic cultivation of E. histolytica.

In the longitudinal population studies described in this thesis (Chapters 4 and 6) changes in zymodemes have been observed but these have always been from one non-pathogenic to another non-pathogenic zymodeme (eg I to III) or from one pathogenic to another pathogenic zymodeme (eg II to XI). A change in zymodeme from non-pathogenic to pathogenic or vice versa has never been recorded in these in vivo studies.

The results of Mirelman et al (1986a,b) are interesting in that they imply that the pathogenicity as well as zymodemes of E. histolytica are not stable properties of the amoeba. However, they admit that this phenomenon cannot be reproducibly demonstrated (Mirelman 1987a); furthermore, their observations have not been reproduced by other workers and have not been observed in the natural host of E. histolytica ie man. Whether or not this test

tube phenomenon, induced by the artificial culture environment incorporating mixtures of powerful antibiotics can be reproduced in the natural environment of the amoeba (ie the gut of man) will be borne out by further research and should be urgently pursued. Nevertheless, the observations of Mirelman's group have received support from some quarters as evidenced by a recent editorial in the New England Journal of Medicine (Krogstad, 1986). The main contention surrounding this issue relates to whether or not asymptomatic E. histolytica cyst passers should be treated; the "Mirelman" camp propose that the treatment of all cyst passers is mandatory since all these carriers have the potential of developing invasive disease. It is significant that the Mirelman group (Garfinkel et al, 1989) have subsequently shown genetic differences between pathogenic and non-pathogenic zymodemes using DNA probes and have for the first time come to the startling conclusion: "...the molecular mechanisms involved in the conversions and changes in zymodemes are not yet known, and although they were also observed with a cloned culture, at present we cannot totally exclude the possibility that they were due to the selection of a previously existing but undetectable sub-population".

In contrast to the limited observations of Mirelman et al (1987a) it is noteworthy that the zymodeme determination of E. histolytica performed on approximately 6000 isolates, from humans worldwide, have proved that the division of E. histolytica into pathogenic and non-pathogenic zymodemes is a stable property of the parasite

(Sargeaunt 1987). This concept concurs with the observations recorded in this thesis (Chapters 4 and 6).

Recent editorials in 'The Lancet' (1979, 1985, 1986), the 'Nederlandse Tijdschrift vir Geneeskunde' (Kager 1987) and the 'South African Medical Journal' (Jackson et al, 1984, Jackson 1987) also support the hypothesis that E. histolytica consists of 2 morphologically indistinguishable species; one that is always pathogenic (E. dysenteriae of Brumpt, 1924) and the other that is non-pathogenic (E. dispar of Brumpt, 1924). It is reasoned therefore that only carriers of pathogenic zymodemes need be treated since treatment is expensive and the limited funding available in amoebiasis-endemic areas necessitates that chemotherapy be targeted specifically at the carriers of pathogenic zymodemes of E. histolytica.

CHAPTER 9

THE ESTABLISHMENT OF CYST PRODUCTION BY PATHOGENIC ZYMODEMES OF ENTAMOEBA HISTOLYTICA.

9.1 INTRODUCTION

In the introduction to this thesis the epidemiology of amoebiasis was presented in light of the 'Promethian', 'Commensalist' and the 'two species' hypotheses. As pointed out earlier the validity of the Promethian hypothesis is insupportable due to the lack of available experimental and clinical evidence. The commensalist hypothesis has gained renewed credibility recently resulting from the claim that non-pathogenic zymodemes can be converted to pathogenic zymodemes during the process of axenisation in a highly artificial environment (Mirelman, 1987a,b). However these observations relate to two strains of E. histolytica and the work has not been reproduced by other researchers; in fact Mirelman (1987) also admits that the phenomenon is not reliably reproducible. Furthermore, two crucial questions remain unanswered - does this phenomenon occur in vivo in humans and what critical advantage, if any, would such a conversion confer on this organism? On the other hand, the 'two species' hypothesis of Brumpt (1928) which maintains that E. histolytica consists of two morphological identical species ie E. dysenteriae which is pathogenic and E. dispar which is a commensal is supported by ample experimental data (Table 1.1) as well as epidemiological observations.

Epidemiological data presented in this thesis supports the hypothesis of Brumpt. The supportive observations are as follows:

1 Both the pathogenic and non-pathogenic strains have been shown to occur at a measurable prevalence in asymptomatic individuals in an endemic area.

2 The fact that the non-pathogenic zymodemes occur nine times more frequently than pathogenic zymodemes supports the epidemiological fact that the prevalence of cyst passers out-numbers the prevalence of invasive amoebiasis.

3 It has been shown that the pathogenic and non-pathogenic strains also vary physiologically in that the former always elicits an antibody response regardless of symptomology while the latter does not.

4 The division of E. histolytica into pathogenic and non-pathonegic zymodemes is a stable property of the parasite and this is supported by the fact that in vivo changes from pathogenic to non-pathogenic zymodemes and vice versa was not observed.

5 The production of cysts by the pathogenic strains has been implied but not conclusively proven in Chapter 6.

Logically, the existence of two morphologically indistinguishable strains which vary in their pathogenicity would depend on the

demonstration that the pathogenic variety can independently complete its life-cycle ie that they have the capability of producing the cystic stage thereby ensuring transmission of the parasite from one host to another. Proponents of the commensalist hypothesis refute this phenomenon (Elson-Dew, 1978). A study was thus initiated to confirm that pathogenic zymodemes of E. histolytica are capable of cyst production.

9.2 MATERIALS AND METHODS

9.2.1 Patients

Three subjects who were passing cysts of E. histolytica were chosen for the study - one had an amoebic liver abscess (ALA) the other amoebic dysentery (AD) and the last one was asymptomatic.

9.2.1.1 Amoebic Liver Abscess

The patient was an adult male age 35 years. The diagnosis of ALA was made on clinical symptoms and signs, the demonstration of a transonic lesion in the liver by ultrasound examination, the aspiration of bacteriologically sterile pus from the abscess cavity and good clinical response to treatment with metronidazole (Flagyl, Maybaker) together with a strongly positive serological response using the amoebic gel diffusion test (AGDT) (Chapter 5). Stool examination revealed formed stools in which cysts of E. histolytica were present.

9.2.1.2 Amoebic Dysentery

A female child aged 7 years was admitted to the paediatric ward of King Edward VIII Hospital with kwashiorkor. The diagnosis of AD was made on a 2 week history of dysentery and the demonstration of haematophagus E. histolytica, pus, erythrocytes and quadrinucleate cysts of E. histolytica in the stools. This patient also responded to treatment with metronidazole (50mg/kg/day in 3 divided doses) for 10 days. The experimental stool specimen was collected 3 days after commencement of treatment with metronidazole, at which stage no trophozoites were detected but the patient was still passing numerous cysts.

9.2.1.3 Asymptomatic Cyst Passer

A male subject aged 9 years, identified during a parasitological survey of 50 schoolchildren in an area +/- 6 km from the study area described in Chapter 3. He was found to be a carrier of a pathogenic zymodeme of E. histolytica. Although he was asymptomatic the AGDT performed on his serum was strongly positive (20 hour). Stool examination revealed formed stools with cysts of E. histolytica; there was no pus or blood.

9.2.2 Separation of cysts

Approximately 10g of stool was suspended in distilled water. The suspension was first passed through a 75 micron filter to remove the larger faecal particles. The filtrate was thereafter further

filtered consecutively through a series of filters: 50, 30, 20, 15, 10 and 7 microns; the residue obtained after filtration through the 7 micron membrane was harvested; this was suspended in distilled water at room temperature for 48 hours. In the case of the patient with ALA and the asymptomatic cyst passer the cysts in the residue were further separated on a discontinuous Percol (Pharmacia Fine Chemicals, Uppsala, Sweden) gradient prepared in dilutions of 10%, 20%, 30% ...100% (v/v) in phosphate buffered saline. The cysts were harvested from the interface between 50% and 60% Percol. Cysts from the patient with AD was suspended in a sucrose solution with a specific gravity of 1,180 and centrifuged for one minute; the cysts accumulated in a layer on the surface and were collected with a wire loop. All the harvested cysts where then washed a further three times in distilled water by resuspension and centrifugation. The end product was examined microscopically by two experienced laboratory technologists to exclude the presence of E. histolytica trophozoites.

9.2.3 Culture and electrophoresis

Cultures of fresh stool specimens from each subject were initiated in Robinson's (Robinson, 1968) medium. The separated cysts were also inoculated into the same medium. Isoenzyme electrophoresis was performed on cultured amoebae to determine their zymodemes.

TABLE 9.1 Zymodemes of E. histolytica cultures from cyst preparations and stools

	Zymodeme from cyst culture	Zymodeme from stool culture
ALA	II	II
AD	II	II
Asymptomatic cyst passer	XIV	XIV

9.3 RESULTS

All the cultures attempted from fresh stools and from separated cyst suspensions grew in Robinson's medium. The results are summarised in Table 9.1. It will be noticed that amoebae harvested from cyst and stool cultures were of the same pathogenic zymodeme. Unexpectedly zymodeme XIV was isolated from one of the subjects. Prior to this isolation, zymodeme XIV had been only found in India; the implications of this isolate was discussed in Chapter 3.

9.4 DISCUSSION

This study has established that pathogenic zymodemes of E. histolytica are capable of producing cysts and can therefore independently complete their life-cycles. Surprisingly, the elegant in vivo experiments of Walker and Sellards (1913) conclusively

demonstrated that dysentery could be produced in humans by feeding them cysts of E. histolytica (Fig. 1.2). In their study, 3 out of 12 human volunteers fed cysts from a convalescent carrier developed dysentery and of 4 volunteers fed cysts from an asymptomatic cyst passer one developed invasive amoebiasis. Quincke and Roos (1893) reported that dysentery may be produced in cats not only by feeding them with trophozoites but also with E. histolytica cysts derived from dysenteric patients. Scrutinisation of Neal's (1965) work reveals that he demonstrated that a pathogenic strain of E. histolytica could produce cysts in vitro and that there was no loss of virulence during the process of encystation. Geekie et al (1958) also demonstrated that amoebae derived from ALA patients (and therefore pathogenic) were capable of producing cysts. However, the work of these researchers became clouded in the quest to explain the discrepancy between the grossly low prevalence of invasive amoebiasis in comparison to the high prevalence of asymptomatic cyst passers in endemic amoebiasis areas. Earlier workers simplistically explained this disproportion by adopting either the 'Promethean' or 'Commensalist' hypothesis; scientific proof to substantiate these theories, however, was sorely lacking. The more logical approach of Brumpt (1928), based on epidemiological observations, which divided E. histolytica into two morphologically identical species viz E. dysenteriae and E. dispar was ignored mainly because there was no acceptable method of distinguishing them.

Thus, the status of the cyst passer in terms of the pathogenesis of amoebiasis can now be revised as follows:

1 The division of E. histolytica into pathogenic and non-pathogenic zymodemes is a stable property of the amoeba and conversion from one form to the other has never been satisfactorily demonstrated by either in vivo (Chapters 4 and 6) or in vitro (Sargeaunt 1987, Chapter 8) studies.

2 Pathogenic zymodemes are very likely to independently complete their life-cycles ie they can produce cysts (Chapter 9).

3 A carrier state of the pathogenic zymodemes of E. histolytica can occur (Chapter 6).

4 Pathogenic zymodemes can be transferred by the faecal-oral route resulting in family clustering of infections (Chapter 6).

5 Carriers of non-pathogenic zymodemes do not develop invasive amoebiasis and they are sero-negative (Jackson et al, 1985, Chapter 5) unless they have had previous exposure to pathogenic zymodemes.

6 Invasive amoebiasis is always caused by pathogenic zymodemes (Sargeaunt et al, 1978, 1980b, 1982a,b,c, 1984, Jackson et al, 1982, Chapter 2).

7 A strongly positive serological response is indicative of tissue invasion and occurs in patients with AD, ALA as well as

asymptomatic cyst passers harbouring pathogenic zymodemes of E. histolytica (Jackson et al, 1985, Chapter 5).

8 Spontaneous clearance of infections with both pathogenic and non-pathogenic zymodemes occurs (Nanda et al, 1984, Gathiram and Jackson, 1987, Chapters 4 and 6).

9 A small proportion of carriers of pathogenic zymodemes actually develop overt invasive disease viz AD and ALA and the factors inducing this are not understood (Chapter 6).

Treatment of asymptomatic carriers of pathogenic zymodemes of E. histolytica is as necessary as treatment of patients with invasive amoebiasis and in both cases all contacts of affected individuals must also be treated if they are found to harbour pathogenic zymodemes of E. histolytica in order to ensure control of disease transmission. Where facilities for zymodeme analysis are not available, serological testing is invaluable, since asymptomatic carriers of pathogenic zymodemes have high antibody titres which have been shown to be comparable to those recorded in patients with ALA and AD. On the other hand, carriers of non-pathogenic zymodemes tend either to be seronegative or have low antibody titres (Jackson et al, 1985, Chapter 5). This approach has particular merit when limited funding prohibits unrestricted treatment of all cyst passers and would be targeted specifically at the carriers of pathogenic zymodemes of E. histolytica. In the absence of supportive tests treatment relies on clinical suspicion and becomes the responsibility of the consulting physician.

CHAPTER 10

RESUMé, CONCLUSIONS AND RECOMMENDATIONS

The existence of pathogenic and non-pathogenic strains of E. histolytica is now becoming more widely accepted (Pérez-Tomayo, 1986, Editorials 1979, 1986, 1987; Kager 1987). However, there are no known microscopic or ultrastructural differences between the pathogenic and non-pathogenic strains of the amoebae (Pérez-Tomayo, 1986). Thus, earlier investigators were faced with the dilemma of explaining how E. histolytica could live as a commensal in most people harbouring the parasite and yet also cause debilitating disease and even death in others. Therefore, it was argued that either all E. histolytica strains are pathogenic and that changes in natural host immunity leads to tissue invasion (ie Promethean theory) or that the commensal amoeba suddenly becomes pathogenic and causes disease in the host (ie commensalist theory). These theories became widely accepted even in the absence of firm scientific evidence to support them.

More recently several pathophysiological differences between the pathogenic and non-pathogenic strains have been identified (Table 1.1) which support the hypothesis of Brumpt (1928) that E. histolytica comprises two strains or even species that are morphologically indistinguishable viz E. dysenteriae (pathogenic) and E. dispar (non-pathogenic). The discovery that pathogenic and non-pathogenic amoebae could reliably and reproducibly be distinguished by characteristic isoenzymes of hexokinase (HK) and phosphoglucomutase (PGM) (Sargeaunt & Williams 1979; Farri et al, 1980) heralded in a new era in research into the epidemiology of amoebiasis. All zymodemes (strains identified by

characteristic isoenzyme factors) belonging to pathogenic E. histolytica strains share the β band in PGM and fast moving bands of HK. However, here again research perspectives have once again become divided. While on the one hand there is good evidence to prove that zymodemes are stable genotypic properties of the organism, some researchers believe that these zymodemes are merely markers of pathogenicity and are an unstable phenotypic property of E. histolytica.

Considering the latter of the above-two standpoints of view, it is interesting to note that Mirelman et al (1986a,b) have recently recorded that changes in zymodeme from pathogenic to non-pathogenic and vice versa can be induced in vitro under controlled laboratory conditions. Apparently axenisation of non-pathogenic E. histolytica induces in these amoebae a metamorphosis which causes a change to pathogenic zymodemes with a concomitant increase in virulence. It must be emphasised that these changes have only been observed in the test tube under highly artificial culture conditions supplemented by the use of powerful antibiotics and irradiated bacteria. Whether or not such conditions could occur in the natural environment of E. histolytica ie the gut of man, is questionable. If the observations of Mirelman et al (1986a,b) are borne out in the in vivo situation then the implications regarding chemotherapy of cyst-passers is vital and would imply that all cyst passers need to be treated since all have the potential to develop invasive disease.

Since the publication of the experiments of Mirelman et al (1986a,b) two further notable observations have been made which would support the existence of two distinct strains or even species of E. histolytica (pathogenic and non-pathogenic). One is a monoclonal antibody study in the

UK (Strachan et al, 1988) which proved that at least two distinct antigens exist on the surface of pathogenic zymodemes of E. histolytica which cannot be demonstrated on non-pathogenic zymodemes. The second study also from the UK (Allason-Jones et al, 1988) emphasises the inability of non-pathogenic zymodemes to invade its natural host even in severely immunocompromised patients with AIDS. More recently DNA differences between pathogenic and non-pathogenic zymodemes have also been reported (Garfinkel et al, 1989; Tannich et al, 1989). During the course of the work described in this thesis a number of pathophysiological differences between pathogenic and non-pathogenic zymodemes were observed which would also support the existence of two distinct strains of E. histolytica.

In the amoebiasis endemic area of Durban, a 10% prevalence of E. histolytica infections was noted. The majority (90%) of infected individuals were found to harbour non-pathogenic zymodemes and were asymptomatic. Infection with pathogenic zymodemes occurred in 1% of the population and resulted in an asymptomatic carrier state in the majority of cases. A small proportion (10%) of individuals infected with pathogenic zymodemes developed invasive amoebiasis. This observation provides an explanation of why the majority of individuals infected with E. histolytica fail to get sick.

Infections with non-pathogenic zymodemes I and III occurred most frequently - a pattern observed previously in Durban as well as in Mexico and India. In asymptomatic subjects infected with pathogenic E. histolytica zymodemes II and XI occurred with equal frequency whereas in symptomatic cases - amoebic liver abscess (ALA) and amoebic dysentery (AD) - only pathogenic zymodemes were isolated with zymodeme II occurring more

frequently (80%). Whether or not the tissue invasive potential (predisposition to disease) is zymodeme-related needs to be investigated.

Two new zymodemes viz XIX (non-pathogenic) and XX (pathogenic) have been characterised. Both of these occur at a very low frequency. Zymodeme XIV - the only pathogenic zymodeme found in India - was observed in a single instance only and is thought to have been the result of a point mutation. The absence of zymodeme XIV in Durban is noteworthy considering the migration of a large population of Indians to Durban between 1860 and 1960 and would indicate that invasive amoebiasis was not introduced into Durban from India.

Changes in zymodeme from non-pathogenic to pathogenic and vice-versa were not observed in a longitudinal study; all new infections with pathogenic zymodemes occurred *de novo*. Therefore, the observations of Mirelman *et al* (1986a,b) have not been recorded *in vivo*. Repeated zymodeme determinations at frequent intervals is urgently indicated to substantiate this observation. However, apparent changes from one pathogenic to another pathogenic zymodeme (eg II to XI) or from one non-pathogenic to another non-pathogenic (eg I to III) zymodeme have been noted *in vivo*. The cause of this phenomenon is unknown, and it is hypothesised that changes in the environment of the amoeba (ie the gut of man) may be responsible. This leads to the conclusion that zymodemes are phenotypic expressions of genotypically distinct strains of *E. histolytica* viz pathogenic and non-pathogenic. This concurs with the observation of Sargeaunt *et al* (1984) that those zymodemes expressing single bands in PGM and glucophosphate isomerase (GPI) (viz I, II and XIV) are the most important

key zymodemes and the spectrum of zymodemes observed are phenotypes of these.

Infection with both pathogenic and non-pathogenic zymodemes occurred by the faecal-oral route. This is indicated by the clustering of infections in family units, the higher rates of infection observed where unhygienic standards (viz the absence of latrines) prevaled and the higher prevalence rates of other intestinal parasites in the E. histolytica infected subjects.

Females were found to have higher prevalence levels of non-pathogenic zymodemes than males while the prevalence of the pathogenic zymodemes were equivalent in the two sex groups. This contrasts sharply with the male preponderance of both ALA and AD. Furthermore, the majority (90%) of individuals infected with pathogenic zymodemes apparently spontaneously lose their infections without becoming symptomatic. Therefore, the propensity to develop deeper tissue invasion is apparently both host- and zymodeme related; the factors predisposing to deep tissue invasion other than the sex of the individual need to be identified.

The serological responses of asymptomatic subjects harbouring pathogenic zymodemes was identical to patients with ALA and AD with all being seropositive with a high proportion (94-100%) of them being strongly seropositive. Carriers of non-pathogenic zymodemes on the other hand had a similar serological response to uninfected subjects (21% v/s 13,5%) and to a random population sample (21% v/s 19% seropositive). The frequency of strong seropositivity in subjects infected with non-pathogenic zymodemes (3%) is equivalent to those who are not infected at all and these are in

turn similar to the general population (4%). Therefore it is hypothesised that the absence of an antibody response in individuals harbouring infections with non-pathogenic zymodemes is due to its non-invasive behaviour. The high prevalence of antibody responses in asymptomatic subjects infected with pathogenic zymodemes implies contact of the amoeba with host tissues and thus at least subclinical tissue invasion in these subjects. Seroepidemiological surveys therefore cannot be used to assess the prevalence of E. histolytica in a community since it only identifies infections with pathogenic zymodemes. A comprehensive picture of the situation can only be ascertained by combining serological methods with zymodeme determination of the parasites since the latter also involves culturing them - a technique demonstrated to be up to 4 times more efficient than conventional microscopy for detecting E. histolytica.

Antibody profiles were prepared for the amoebiasis endemic area of Durban; a bimodal distribution of seropositive responses was recorded with the higher titres reflecting current or recent infections with pathogenic zymodemes and the lower titres indicating past infection. During an epidemic of invasive amoebiasis investigated at Philippi in the Cape Peninsula a higher proportion of E. histolytica isolations were found to be pathogenic zymodemes; here the antibody profile was again essentially bimodal but it was distinctly skewed towards the higher titres. In Gazankulu, an area free of invasive amoebiasis, only non-pathogenic zymodemes were isolated and all individuals tested were sero-negative. Therefore such antibody profiles are valuable for assessing the level of endemicity of E. histolytica - particularly the pathogenic zymodemes. Furthermore the observed variability in serological responses in

asymptomatic carriers of E. histolytica, in different communities can now be directly ascribed to exposure to pathogenic zymodemes in these people.

Rigorous efforts to axenise a non-pathogenic zymodeme were repeatedly unsuccessful. Pathogenic zymodemes on the other hand could be adapted to axenic growth and this was achieved with two strains. Monaxenic cultivation of both pathogenic and non-pathogenic zymodemes was readily attained. However, growth of pathogenic zymodemes in monaxenic culture proved to be uniformly better than that of non-pathogenic zymodemes highlighting another important difference between these strains. The easy adaptability of pathogenic zymodemes to axenic growth (bacteria-free) may confer on them the capability of tissue invasion and growth in a bacteriologically sterile environment such as a liver abscess.

Asymptomatic subjects harbouring pathogenic zymodemes were found to pass cysts of E. histolytica. E. histolytica cysts were harvested from the stools of a patient each with ALA, AD and an asymptomatic cyst passer and cultured in vitro. These cysts were stored in distilled water for 48 hours after purification and concentration using density gradients and filtration exclusion methods. All the resultant cultures derived from these cyst preparations proved to be pathogenic zymodemes thus confirming the impression from in vivo observations that pathogenic zymodemes can produce cysts and therefore can independently complete their life-cycles. Consequently it can be stated that a conversion from non-pathogenic to pathogenic zymodeme is not a prerequisite for a carrier of E. histolytica to develop disease. The cysts of pathogenic zymodemes could be transferred by the faecal oral route resulting in the observed family clustering of infections.

Treatment of ALA and AD fortunately does not present a problem. All patients treated responded well to either metronidazole or tinidazole with rapid clinical improvement. In the case of ALA all abscesses were aspirated. However recurrence of ALA was observed in 3 patients and of AD in one patient and in three of these, recurrences were associated with incomplete clearance of the cystic stage from the gut. The evaluation of the nitroimidazoles in effecting complete cure is therefore indicated and is currently being pursued.

The treatment of cyst passers remains controversial in view of the observations of Mirelman et al (1986a,b). However, from the evidence accrued so far it is recommended that at least all asymptomatic cyst passers harbouring pathogenic zymodemes should be treated for two reasons. Firstly invasive disease can develop at any time and presently it is impossible to predict which subjects are at risk of developing invasive amoebiasis. Secondly, these carriers are a reservoir of pathogenic E. histolytica and thereby pose a health risk to themselves and their community. This especially applies to the amoebiasis-endemic areas where limited funding necessitates that treatment be targeted specifically at the carriers of pathogenic zymodemes of E. histolytica. Where facilities for culturing and isoenzyme electrophoresis are not available the medical practitioner could be guided by the serological results since carriers of pathogenic zymodemes have been shown to have strongly positive serological responses. Since infections tend to occur in family clusters it is recommended that contacts of patients with invasive amoebiasis also be investigated as this may disclose many additional infected individuals who often can be treated as outpatients with minimal cost and inconvenience. The majority of asymptomatic cyst passers however harbour non-pathogenic zymodemes and the

choice of treating them is left to the discretion of the consulting physician. The endemicity of the area in question as well as the socio-economic status of the patient would obviously influence the course of action followed.

It must be stressed that neither the nitronimidazoles (metronidazole and tinidazole) nor the luminal amoebicides (diloxanide furoate and diiodohydroxyquin) have been evaluated for their efficacy in termination of cyst passing in asymptomatic carriers of pathogenic zymodemes of E. histolytica. This situation needs to be rectified as a matter of urgency.

In conclusion it must be emphasised that E. histolytica is spread by the faecal-oral route. Consequently higher prevalence rates are seen in overcrowded situations especially when these are accompanied by poor sanitary conditions. In the long term only improvement of these living conditions will result in a decrease in the prevalence of the parasite.

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