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# confiabilidad de los zimodemos de *Entamoeba histolytica* para el diagnóstico de laboratorio clínico

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# the reliability of *Entamoeba histolytica* zymodemes in clinical laboratory diagnosis

Resumen

Las infecciones por *Entamoeba histolytica* se consideran sintomáticas o asintomáticas. La enfermedad sintomática se puede manifestar de muchas maneras, en ocasiones con lesiones viscerales como secuelas. Las infecciones asintomáticas suelen conservarse ocultas hasta que se revelan de manera fortuita. Sólo las infecciones sintomáticas originan anticuerpos sanguíneos demostrables en títulos crecientes. Puede añadirse la caracterización enzimática del propio parásito infectante como tercer aspecto para medir su estado. Aunque no hay duda de que la enfermedad sintomática requiere tratamiento, cabe sugerir que no lo requieren la mayor parte de los sujetos que albergan a *E. histolytica* no patógena, puesto que su presencia en el intestino ocurre en calidad de comensal y, en muchas ocasiones, es sólo transitoria. No parece que la presencia de ciertas bacterias, e incluso de algunos virus, ejerza influencia de algún tipo sobre las amibas, aunque los otros microorganismos mencionados tengan importancia clínica notable, para volver patológicas a las últimas.

Abstract

Infections with *Entamoeba histolytica* are described clinically as symptomatic or asymptomatic. Symptomatic disse may present in many cases with. on occasions, visceral lesions as the sequelae. Asymptomatic infections are usually occult until reveled by chance. Only symptomatic infections give rise to demonstrable antibodies in increasing titres in blood. Enzyme characterization (zymodeme) of the infecting parasite itself can now be added as a third parameter to measure its status. Whilst there is no doubt that symptomatic disease requires treatment we suggest that most subjects harbouring non-pathogenic *E. histolytica* do not require treatment since its presence in the intestine is as a commensal and on many occasions is only transitory. No influence appears to be exerted on the amoebae by concomitant bacteria, or some viruses, even of notable clinical importance, to convert them to an invasive strain.

It has been shown in many surveys undertaken in various parts of the world, that *Entamoeba histolytica* isolated from man can be associated with either symptomatic disease or asymptomatic carriers. The association is based on the clinical presentation together with the mobiliy patterns of four isoenzyme systems, as shown in Figure 1, always used together and never in any other fashion, exclusively on thin layer starch gel electrophoresis (Sargeaunt *et al.* 1982a, 1984).

Isolation of amoebae from biological material has been by inoculation into a monoxenic biphasic medium, usually that of Robinson (1968) and estimation of isoenzyme characteristics of those amoebae has been undertaken in the shortest possible time. This has in most circumstances been accomplished within approximately 6 sub passages, amounting to 12 days after isolation. Time span in culture has inevitably been associated with country of origin and time in travel. Thus, for example, stocks from Australia have been passaged 9 times whilst those isolated from the United Kingdom, where all electrophoresis has been undertaken, have been passaged usually only twice. Frequently, however, primary cultures have been used for isoenzyme characterization (zymodemes).

Diagnosis of a strain of *E. histolytica* has therefore followed the usual well trodden pathway of strict clinical laboratory practice. That is, isolation and identification of the causative organism of disease in the shortest possible time, thus preserving integrity of that organism.

Using the system briefly outlined we have examined over a period of 10 years, some six thousand separate isolates of *E. histolytica*. Practically all the enzyme characterization work has been undertaken without previous knowledge of clinical presentation and consequently results have been completely unifluenced by any such factors.

A blood sample was taken from many of the subjects providing specimens of faeces and all of those from whom pus was aspirated from the liver. The blood was used for a battery of specific amoebic serology, such as, Indirect Fluorescent Antibody Test, Indirect Haemagglutination Antibody Test, and Gel Diffusion Test.

Then matching of the three parameters, that is:

a. Clinical presentation

b. Isoenzyme characters

c. Serology

has shown the following types of presentation whicl can be categorised as:

1. Clinically assessed symptomatic amoebiasis is associated with a selection of isoenzyme patterns (Sargeaunt *et al.* 1982a, 1984, etc. etc.) all of which show the absence of an $\alpha$ band and the presence of a $\beta$ band in phosphoglucomutase (PGM) and fast running bands (with the exception of zymodeme XIII) in hexokinase (HK), Figure 1, together with "positive" serology.

2. Clinically assessed asymptomatic cyst passing is associated with a selection of isoenzyme patterns (Sargeaunt *et al.* 1982a, 1984, etc. etc.) all of which show the presence of an $\alpha$ band in (PGM) and show slow running bands in hexokinase (HK), Figure 1, together with "negative" serology.

3. Clinically assessed asymptomatic cyst passing is associated with isoenzyme patterns, and serology as in category 1.

4. Clinically assessed asymptomatic cyst passing is associated with isoenzyme patterns as in Category 2 and serology as in category 1.

Using the symbols + (positive) and - (negative) for serology and using the symbols + for absence of an $\alpha$ band in PGM and - for presence of an $\alpha$ band in PGM, a summary of the above categories can be shown as follows:

| Clinical presentation | Symptomatic | Asymptomatic | Asymptomatic | Asymptomatic |
|---|---|---|---|---|
| zymodeme | + | - | + | + |
| serology | + | - | + | - |
| category | 1 | 2 | 3 | 4 |

Categories 1 and 2 are self explanatory, their description is legend and they form the vast majority of subjects examined.

Category 3 contains a small minority of cases found in surveys in *E. histolytica* endemic areas (Sargeaunt *et al.* 1982, 1984) and represent subjects in a "prepatent" period of infection leading to amoebiasis.

Category 4 represents three cases found in India, and it is known that at least one of these developed clinical amoebiasis, associated with haematophagus trophozoites in a rectal biopsy within 2 weeks of zymodeme diagnosis.

From the assessment described, it can be seen that the three parameters, a, b and c are inextricably linked, and consequently any one of those parameters can be used as an index of disease, specific diagnosis, and indicator for drug therapy. It follows therefore that the convenient label of "pathogenic" or "non-pathogenic" can be used as a descriptive term for the outcome of isoenzyme characterization, and as with "positive" or "negative" serology is an immediately comprehensible clinical term.

As the higher primates with man as the pinnacle are the definitive hosts of *E. histolytica,* a suitable model for demonstration of factors influencing the life history of the parasite have never been completely satisfactory. For example, myriads of papers have been published regarding the pathogenicity or virulence of particular strains of the organism and just two illustrate exactly how difficult it is to understand this one factor.

Both the papers were presented at Amoebic Seminars in Mexico City, one by Diamond *et al.* in 1974 and the second by Mattern *et al.* in 1982. These reports show widely varying virulence, as assessed in hamster liver lesions, of various strains of *E. histolytica,* but however, the strains used were in axenic culture.

Since our work on zymodeme characterization has been very firmly linked to clinical effect, and hence the definitive host, we have always endeavoured to retain what we believe is the integrity of the parasite by using it only at short passage from primary isolation. Primary isolation has usually been made in what many think of as a polyxenic culture system. But that culture system only becomes polyxenic with the inoculation of the bacteria with the faeces, prior to that the medium contains only one species of bacterium. Therefore we look at the isoenzyme characteristics of the parasite with its concomitant bacteria intact, and since the parasite is in short passage we believe this culture system represents the conditions in the intestine of the host. We have of course compared enzyme results using the culture system both with and without the preaddition of bacteria. We have also compared various other culture systems such as isolation of *E. histolytica* from liver pus in both monoxenic culture and polyxenic culture. Again we have taken a strain of amoebae growing only with a species of crithidia and reintroduced it to a monoxenic culture and a polyxenic culture system. None of these variables which we have been testing over a long period of time has ever shown a change in the enzyme pattern form non-pathogenic to pathogenic or vice versa.

Despite the ability of the parasite to maintain its isoenzyme stability under the various conditions described, and also because of the recent publication of Mirelman, Bracha and Sargeaunt (1982) where they describe virulence enhancement of isoenzyme stable parasites, we continued to challenge strains of *E. histolytica* by varing their concomitant bacterial flora.

## Materials and methods

Various strains of isoenzyme characterized *E. histolytica* were retrieved from liquid nitrogen storage and inoculated into both Robinson's (1968) and Jones' (1946) media. The bacterial flora from the stocks were separated from the amoeba by inoculation into 0.5 per cent peptone water. All the bacteria from all the strains used were identified by inoculation of solid media, and single colonies of these were identified by special specific media, using standard laboratory procedures. Isoenzyme "non-pathogenic" strains were inoculated with bacteria from isoenzyme "pathogenic" strains.

Identity of bacterial strains present in culture:
*Escherichia coli, Pseudomona aeruginosa, Serratia marcescens, Klebsiella aerogenes, Proteus morgani, Klebsiella oxytoca, Streptococcus faecalis, Pseudomonas maltophilia, Klebsiella aerogenes. Enterobacter cloacae, Citrobactor freundii, Proteus mirabilis, Clostridium perfringens, Bacteroides fragilis.*

IDENTITY OF STRAINS USED IMS4/D123AG

Sargeaunt & Williams = SAW
CLONE = CL

Pathogenic = P

Non-pathogenic = NP

| | *Zymodeme* | |
|---|---|---|
| *SAW 142 Cl* | *III* | *NP* |
| *408 Cl* | *II* | *P* |
| *760 Cl* | *IX* | *NP* |
| *981 Cl (Cl)* | *III* | *NP* |
| *1638* | *IIα-* | P |
| 1706 | XIX | P |
| 1710 | XIX | P |
| 1714 | XIX | P |
| 1719 | I | NP |
| 1721 | XIX | P |
| 1728 | XIX | P |
| 1734 Cl | III | NP |
| 1751 | III | NP |
| 1649 | II | P |
| 1652 | III | NP |

Mixtures of amoebae and bacterial "cocktail"

| These non-pathogenic zymodemes received the bacteria from | | | These pathogenic zymodemes | | |
|---|---|---|---|---|---|
| SAW | 142 Cl | III | Saw | 1728 | XIX |
| | 981 Cl (Cl) | III | | 1721 | XIX |
| | 1734 Cl | III | | 1714 | XIX |
| | 760 Cl | IX | | 1710 | XIX |
| | 1751 | III | | 1706 | XIX |
| | 1719 | I | | 1638 | IIα- |
| | | | | 408 Cl | II |
| | | | | 1649 | II |

Each "non-pathogenic" zymodeme received separately the bacteria from each "pathogenic" zymodeme separately. SAW 1734 Cl, zymodeme III, had its bacteria taken to extinction with antibiotics, with the exception of *Pseudomonas aeruginosa,* and then reintroduced to species of bacteria in the above list. SAW 1652 zymodeme III received the bacteria from SAW 408 Cl, zymodeme II only.

Whilst most of the work described above was undertaken in Jones' (1946) medium, many of the experiments were repeated and controlled by using Robinson's (1968) medium. Most of the cultures were maintained over a period of fourteen days, i.e., seven sub passages, and were constantly monitored by enzyme profiles.

As a culmination of continuous efforts to ensure that no change occurred in the host, of zymodeme characters of their *E. histolytica* infection, we took the opportunity to examine a true clinical situation. Eighty two subjects, sixty two of whom were infected with various clinically notable microorganisms or viruses, but all

being clinically free of signs and symptoms of amoebiasis could be split into two groups as follows:

*Group 1 contained* subjects with
13 Human T lymphocyte virus III (HIV)
3 rectal *Herpes simplex*
1 *Neisseria gonorrhoeae*
2 *Chlamydia trachomatis*
1 *Shigella sonnei*
11 *Mycoplasma hominis*
12 *Ureaplasma urealyticum*
17 *Spirochaetosis*
1 *Yersinia fredericksenii* (USING *et al.* 1980)

One subject proven to have "non-pathogenic" *E. histolytica,* was diagnosed 6 weeks later as a case of acquired immune deficiency disease, and shown to be positive for *Pneumocystis carinii. Group 2 contained* the remaining 20 subjects who were free of the organisms shown in Group 1 and consequently act as controls.

All the subjects in both groups had various mixtures of bacteria in their faeces, as shown in the identity list above.

## Results

All the cultures of amoebae used contained more than a single species of bacterium and many species appeared randomly throughout. Consequently although a culture may already have contained a particular species it would be inoculated with the same species of bacterium from another culture. By using monoxenic and polyxenic culture systems, and by changing the concomitant bacterial flora, no zymodeme at any time by the conditions described could be induced to change its characteristics.

### *E. histolytica in subjects with concomitant infections*

Examination of these two groups over time in many cases up to one year, and all longer than 5 weeks showed all cases, with a few exceptions, had the continuing presence of a "non-pathogenic" *E. histolytica* infection. The exceptions were some subjects who had lost their infection spontaneously whilst others were proven to lose their infection and then become reinfected. All the 84 subjects had rectal biopsies examined both by culture and histology. Their blood was also tested for amoebic antibodies. On no single occasion was invasion of the rectal mucosa or "positive" serology found (Goldmeier *et al.* 1986; Allison-Jones *et al.* 1986), whilst all positive cultures showed *E. histolytica* expressing a "non-pathogenic" zymodeme.

Parts of the research described were recently independently confirmed by Jackson *et al.* (1985) and Gathiram and Jackson (1985). These workers used a total of 1381 subjects and also assessed results using the three parameters, a. Clinical presentation, b. Isoenzyme characters and c. Serology. Their results exactly parallel those described in this paper.

### *Drug treatment*

The present rationale for drug treatment of subjects with *E. histolytica* infections is that in endemic areas no treatment is required for asymptomatic carriers.

Nanda *et al.* (1984) "believe that treatment of cyst passers is not justified. The possibility that these individuals are a source of infection to others is of little importance in an endemic country such as India, where a large proportion of the population harbour the parasite at any given time and are a source of infection to others. Therefore treatment of cyst passers should be limited to food handlers or those working in premature nurseries or caring for immune-compromised individuals. These recommendations would result in an enormous saving in the cost of drugs, since the prevalence rate of *E. histolytica* in India is about 15%. In terms of numbers this amounts to 105 million, and if the population of the world is taken into account, the figure is truly staggering".

Translation from the prevailing situation in endemic areas to *E. histolytica* infections in Europe and North America, for example, is difficult since the prevalence of this parasite in those areas is exceedingly low. However, male homosexuals provide a source for comparison since these subjects are known to harbour *E. histolytica* and the carriage rate is approximately 20% (Allason-Jones *et al.* 1986). Interpretation of the type of infection with *E. histolytica* in male homosexuals is that they are non-pathogenic, this conclusion being reached using the three parameters previously discussed in this text. More than one London hospital, each with a large clientele of male homosexuals attending sexually transmitted disease clinics, leave *E. histolytica* infections untreated. In this context, Weber (1985) reports from St. Mary's Hospital, London, that, "we have ceased treating our

homosexual cyst passers with metronidazole and reserve diloxanide furoate only for those with gut symptoms".

"The finding of trophozoites in the faeces is not absolute proof that amoebiasis is the cause of the patient's illness, and the finding of cysts is a strong indication for an alternative diagnosis, since cysts are rarely found in the stools of patients with amoebic dysentery" (Wilcocks & Manson-Bahr, 1972).

Choice of drug for treatment of carriers of non-pathogenic *E. histolytica* varies from country to country. Generally because the nitroimidazoles are accepted as tissue amoebicides and since non-pathogenic *E. histolytica* is by definition only a lumen dwelling organism, then drugs such as diloxanide furoate are available for treatment if required.

Subjects harbouring either pathogenic or non-pathogenic *E. histolytica,* diagnosed by clinical, serological or zymodeme characterization, may effect a self erradication of their infection (Goldmeier *et al.* 1986; Allason-Jones *et al.* 1986; Sargeaunt *et al.* 1982b). Similarly either type of infection may only be detected by serology (Gutierrez *et al.* 1976) or zymodeme characterization (Sargeaunt *et al.* 1982a,b,c).

Whatever arguments are raised regarding the possibility of treatment or non-treatment, the cardinal principle remains that decision is the responsibility of the consulting physician alone, irrespective of the fact that he may be influenced by laboratory results.

Treatment is unquestionably required for infections with pathogenic *E. histolytica,* however are diagnosed, and metronidazole is probably the drug of choice, certainly in the case of visceral lesions.

## *Conclusion*

In vitro challenge by various bacteria of "non-pathogenic" zymodemes extended over a period commensurate with standard practice for isolation does not alter the non-pathogenic status. In vivo studies in patients with "non-pathogenic" zymodemes of *E. histolytica* together with clinically notable bacterial or viral infections show a stability of the zymodemes over periods up to one year.

To fulfil the need for assistance in clinical practice, methods should be more precise and more accurate. We suggest therefore that zymodeme characterization of *E. histolytica* by development of isoenzyme mobilities of the four enzymes shown in figure 1 is a reliable, clinical, laboratory diagnostic tool. It matches clinical presentation and serological studies, all of which are indications for drug treatment, which may not be required for infections with non-pathogenic *E. histolytica.*

Referencias
References


1. ALLASON-JONES, E.; MINDEL, A.; SARGEAUNT, P.G. & WILLIAMS; P: Entamoeba histolytica *as a commensal intestinal parasite in homosexual men.* N. Eng. Med. 1986; *315:*353.
2. DIAMOND, L.S; PHILLIPS, B.P. & BARTGIS, I.L: *A comparison of the virulence of nine strains of axenically cultivated* E. histolytica *in hamster liver.* Arch. Inves. Med. (Mex.) 1974; *5,* (Suppl 2), 425-426.
3. GATHIRAM, V. & JACKSON; T.F.H.G. (1985): *Frequency distribution of* Entamoeba histolytica *zymodemes in a rural South African population.* Lancet. 1985; 719.
4. GOLDMEIER, D.; SARGEAUNT, P.G.; PRICE, A.B.; MUNDAY, P.E.; BILLINGTON, O.; DIXON, I.; BORRIELLO, P.; CARDER, J.M.; SHAW, A.; HILTON, J. & JEFFRIES, D.J. (1986): Is Entamoeba histolytica *in homosexual men a pathogen* Lancet. 1986; 641.
5. GUTIERREZ, G.; LUDLOW, A.; ESPINOSA, G.; HERRERA, S.; MUÑOS, O.; RATTONI, N.; SEPULVEDA, B. (1976): *National serologic survery: II Search for antibodies against* Entamoeba histolytica *in Mexico. Memorias de la Conferencia Internacional sobre Amibiasis.* B. Sepúlveda and L. Diamond Instituto Mexicano del Seguro Social, México, 609-618.
6. JACKSON, T.F.H.G.; GATHIRAM, V.; SIMJEE, A.E.; (1985): *Seroepidemiological study of antibody responses to the zymodemes of* Entamoeba histolytica. Lancet. 716.
7. JONES, W.R. (1946): *The experimental infection of rats with* Entamoeba histolytica; *with a method for*

*evaluating the anti-amoebic properites of new compounds.* Annals of Tropical Medicine and Parasitology, *40,* 130-140.

8. MATTERN; C.F.T.; KEISTER, D.B. & NATOVITZ, P.C: *Virulence of* Entamoeba histolytica *upon continuous axenic cultivation.* Invest. Méd. (Méx.), 13 (Suppl 3), 185-190.

9. MIRELMAN, D.; BRACH, R. & SARGEAUNT, P.G.: Entamoeba histolytica: *Virulence enhancement of isoenzyme-stable parasites. Exp. Parasitology,* 1984; *57,* 172-177.

10. NANDA, R.; BAVEJA, U. & ANAND, B.S. (1984). Entamoeba histolytica cyst passers: clinical features and outcome in untreated subjects. *The Lancet,* II, 301.

11. ROBINSON, G.L: *The laboratory diagnosis of human parasitic amoebae.* Trans. Roy. Soc. Trop. Med. Hyg. 1968; *62,* 285-294.

12. SARGEAUNT, P.G.; JACKSON, T.F.H.G. & SIMJEE, A.E. (1982a): *Biochemical homogeneity of* Entamoeba histolytica *isolates, especially those from liver abscess. Lancet,* 1982 I, 1386.

13. SARGEAUNT, P.G.; WILLIAMS, J.E.; JACKSON, T.F.H.G. SIMJEE, A.E. (1982b). *A zymodeme study of* Entamoeba histolytica *in a group of South African schoolchildren.* Trans. Roy. Soc. Trop. Med. Hyg., *76,*3, 401-402.

14. SARGEAUNT, P.G.; PORTER, I.A. & SINTON, G.P.: *Importation of pathogenic* Entamoeba histolytica. (1982a). *Brit. Med., 284,* 507.

15. SARGEAUNT, P.G.; BAVEJA, U.K.; NANDA, R. & ANAND, B.S. (1984): *Influence of geographical factors in the distributions of pathogenic zymodemes of* Entamoeba histolytica: *identification of zymodeme XIV in India. Transactions of the Royal Society of Tropical Medicine and Hygiene, 78,* 96-101.

16. USING, J.; BRENNEN, D.J.; BERCOVIER, H.; FARRING, G.R., STEIGERWALT, A.G.; BRAULT J. & MOLLART, H.H. (1980). Yersinia frederiksenii *A new species of Enterobacteriaceae composed of rhamrose-positive strains (formerly called atypical* Yersinia enterocolitica *or* Yersinia enterocolitica-like). Current Microbiology, *4,* 213-217.

17. WEBER, J. (1985). *Sexually acquired parasitic infections in homosexual men.* Parasitology Today, 4, 93-95.

18. WILCOCKS, C. & MANSON-BAHR, P.E.C. (1972): *Manson's Tropical Diseases 17 th Edition. Bailliere Tindall London.*

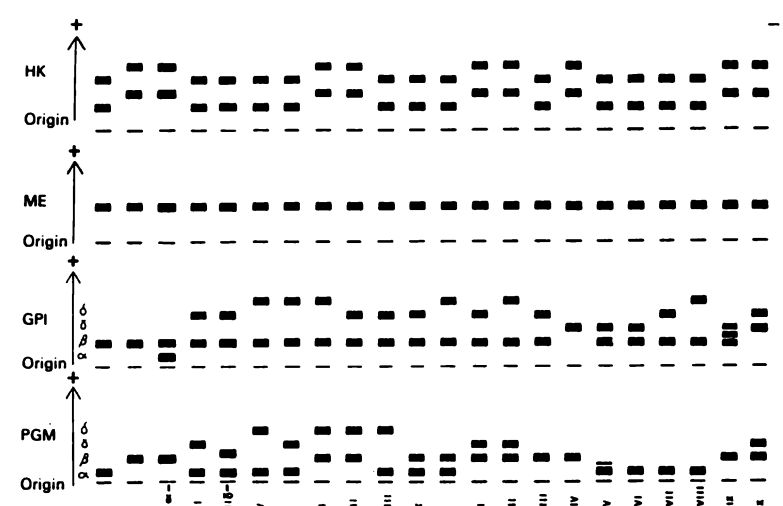


Figura 1. Zymodemes of *Entamoeba histolytica* identified using EC 5319 glucose phosphate isomerase (GPI); EC 11140 L-malate; NADP + oxidoreductase (oxaloacetate decarboxylating) (ME); EC 2751 phosphoglucomutase (PGM); and EC 2711 hexokinase (HK).

A zymodeme is a population of amoebae differing from similar populations in the electrophoretic mobility of certain enzymes. The markers for pathogenicity are the absence of the $\alpha$ band together with the presence of the $\beta$ band in PGM. Advanced bands in HK confirm the PGM results. The only exception in zymodeme XIII which lacks advanced HK bands.