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Prevalence of antibodies against *Entamoeba histolytica* in Mexico measured by ELISA

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SUMMARY

The prevalence of antibodies against *Entamoeba histolytica* was studied in the Mexican population using an immunoenzyme assay in solid phase (ELISA) and semiautomatic equipment. The antigen was a mixture of membrane proteins obtained by Triton X-100 extraction from an axenic culture of *Entamoeba histolytica* HM1-IMSS. The method was standardized by comparing serum samples from amoebic liver abscess patients with healthy volunteers. From the 60538 samples supplied by the National Seroepidemiology Survey, antibodies were found in 4·49% (4·32–4·65% at 95% confidence limit). More significant titres occurred in the central region of the country. The ratio female to male was 1·25:1. The population living in metropolitan areas had probably been infected at a younger age than those living in the country.

Important differences were found in the seroprevalence obtained by ELISA compared with a study which used indirect haemagglutination (IHA) in the same sample frame.

INTRODUCTION

Infection with *Entamoeba histolytica* is an endemic problem in many tropical and subtropical areas of the world where there are poor hygienic and sanitary conditions. According to the World Health Organization, 480 million people have the parasite. Although most of the infected patients are asymptomatic, some may present with clinical syndromes ranging from dysentery to abscesses of the liver, lungs, or brain. Between 40000 and 110000 people die each year of intestinal and extraintestinal complications of amoebiasis [1].

Amoebiasis is an important public health problem in Mexico. In 1989, 1516 cases of amoebic liver abscess (ALA) were reported (1·8/100000 inhabitants). However, the actual magnitude of amoebic disease is not known, because of insufficient epidemiological information.

Serological surveys may help to determine the endemicity of a disease since the

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antibody profile in a population is a record of the present and past experience with the pathogen [2]. A number of studies describing the prevalence of antibodies against amoebae in the general Mexican population have been published over the past 20 years [3–8], the most comprehensive of which was in 1974 and used counterimmunoelectrophoresis (CIE) techniques [7]. Prevalence ranged from 2·3 to 9·95% in different areas with an overall mean of 5·95%. Up to 29% of an urban population were found to have antibodies in a study where the indirect haemagglutination (IHA) method was used to detect them [9].

Here we describe the prevalence of serum antibodies to amoeba measured by an ELISA technique. this method was chosen because it is easily performed, can be automated and is highly sensitive and specific [10]. Those samples with antibody titres comparable to those found in ALA patients were considered to be positive. To our knowledge this is the only study where this criterion of classification has been used.

MATERIALS AND METHODS

Antigen

The antigen was prepared as described previously [11]. Briefly, an axenic culture of *Entamoeba histolytica* HM-1:IMSS was harvested by centrifugation and resuspended in Tris 0·05 M containing phenylmercuric acetate 0·1 mM (Sigma. Chemical Co., St Louis, MO, USA), phenylmethylsulfonylfluoride 0·1 M (Sigma) and $MgCl_2$ 1 mM. The trophozoites were lysed in an Omnimixer (Sorvall Inc., Norwalk, Conn.), the membranes were collected by ultracentrifugation 120000 g. 1 h (Beckman, Ins), and the pellet solubilized with 2% Triton X-100 in Tris 0·05 M pH 7·7 over 1 h at 20 °C. Finally, the suspension was ultracentrifuged for 1 h at 120000 g, the supernatant collected, and the protein concentration measured using the method described by Lowry and colleagues [12]. Aliquots of a single batch, in the presence of phenylmercuric acetate 0·1 mM and phenylmethyl sulfonylfluoride 0·1 M, were prepared and stored at −70 °C until used.

Serum samples

A total of 60538 samples of serum from the National Seroepidemiology Survey (NSS) that were representative of the Mexican population were supplied by the Instituto de Diagnóstico y Referencia Epidemiológicos, Secretaría de Salud, México [13]. The methodology for the design of the master sampling plan, the collection and storing of the sera and processing of the data have been reported elsewhere [14–17].

150 μl of each serum sample were transferred to 96-well silicon treated microtitre plates, which were then sealed and stored at −20 °C until use.

ELISA

A semiautomated method was used (BIOMEK-1000 apparatus, Beckman Inst.). 96 well microtitre plates (Nunc, Co.) were coated with 100 μl of a 20 μg/ml solution of the antigen in a carbonate buffer (pH 9·5) for 1 h at 37 °C and overnight at 4 °C. After blocking with 300 μl of PBS-2% nonfat dried milk for 1 h at 37 °C, the plates were incubated with the serum samples diluted 1:400 in PBS for 3 h at 37 °C. The plates were then incubated with a goat-antihuman-γglobulin-

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Table 1. *Antibodies against* E. histolytica *in Mexico determined by ELISA: distribution by geographic area*

	Region	Positive	Negative	Total	Rate
I	North west	293	8479	8772	3·34
II	North	534	8914	9448	5·65
III	North east	99	3502	3601	2·74
IV	South Pacific	101	4697	4798	2·10
V	Central west	497	11357	11854	4·19
VI	Central south	1028	11674	12702	8·09
VII	Gulf of Mexico	42	4555	4597	0·91
VIII	Yucatan Peninsula	130	4636	4766	2·72
	Total	2724	57814	60538	4·49

peroxidase conjugate diluted 1:1000 in PBS and the colour developed with OPD-H_2O_2 in citrate buffer pH 5·0. The reaction was arrested with 50 μl of 2·5 N sulphuric acid and the O.D. measured at 490 nm (Minireader, Dynatec or Biomek-1000, Beckman Ins). PBS-Tween 20 0·01% was used to wash to plates between incubations.

Standardization of the ELISA

The standardization of the assay has been described previously [11]. Eighty serum samples of patients with a clinical and radiographic diagnosis of ALA and 80 samples from healthy volunteers were analysed by a manual method. Antigen concentrations from 5 to 40 μg/ml were tested. With the serum diluted 1:400 and the antigen at 20 μg/ml a cut-off point was designated at 1 O.D. unit at 490 nm, where the greatest sensitivity and specificity were found (90·7 and 95%, respectively). One hundred samples were then assayed using the manual and the semiautomated methods. Similar O.D.s were found with both methods. In order to ensure the reproducibility of the test, the samples were assayed again if the variability coefficient was 10% or higher.

RESULTS

Of the 60538 samples analysed significant antibody titres against *Entamoeba histolytica* were found in 2724 cases, which represented 4·49% of the population (4·32–4·65%, 95% confidence limit). The prevalence of antibodies by geographical area is listed in Table 1. In 6 of the 32 states of Mexico, the prevalence was higher than 6%, and in 10 was less than 2·5%. The state of Queretaro had the highest rate (14·94%) and Quintana Roo the lowest with 0·46%. The prevalence of antibodies was higher in the central region of the republic, and lower in the southeast (Fig. 1). Antibody titres in 60258 of the samples could be analysed in association with sex, age, socioeconomic status and place of residence. The prevalence of antibodies in these samples was not different from the total sera analysed (4·46 and 4·49%, respectively). The distribution by age is shown in Fig. 2. About 3·5% of children under 10 years of age had positive titres and this figure increased to 5·5% in people of 25 years and older. The distribution of antibodies by sex is presented in Table 2; a higher prevalence was found in females, 4·87% than males, 3·88% ($\chi^2 = 4{\cdot}12$, $P = 0{\cdot}04$), the female-to-male ratio was 1·25:1.

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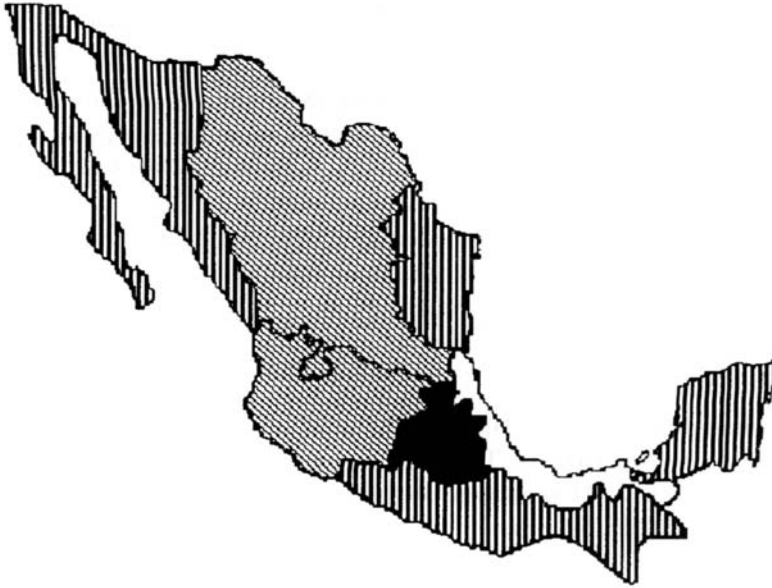

Fig. 1. Prevalence of antibodies against *E. histolytica* by geographic area. Rate: □, < 2%; ▥, 2–4%; ▧, 4–6%; ■, > 6%.

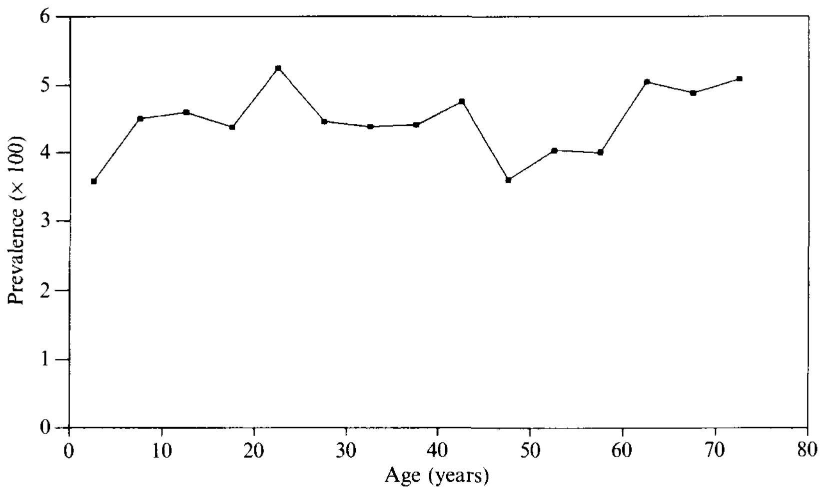

Fig. 2. Age distribution of antibodies against *E. histolytica* determined by ELISA.

Table 2. *Antibodies against* E. histolytica *determined by ELISA: distribution by sex*

Sex	Positives	Negatives	Total	%
Male	965	23922	24887	3·88
Female	1723	33648	33371	4·87
Total	2688	57570	60258	4·46

$\chi^2 = 4{\cdot}12$, $p = 0{\cdot}04$.

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Table 3. *Antibodies against* E. histolytica *according to socio-economic status*

Socio-economic status	Positives	Negatives	Total	%
Low	1064	21596	22660	4·69
Medium	704	15243	15947	4·41
High	920	20731	21651	4·20
Total	2688	57570	60258	4·46

$\chi^2 = 5{\cdot}28$, $P = 0{\cdot}07$.

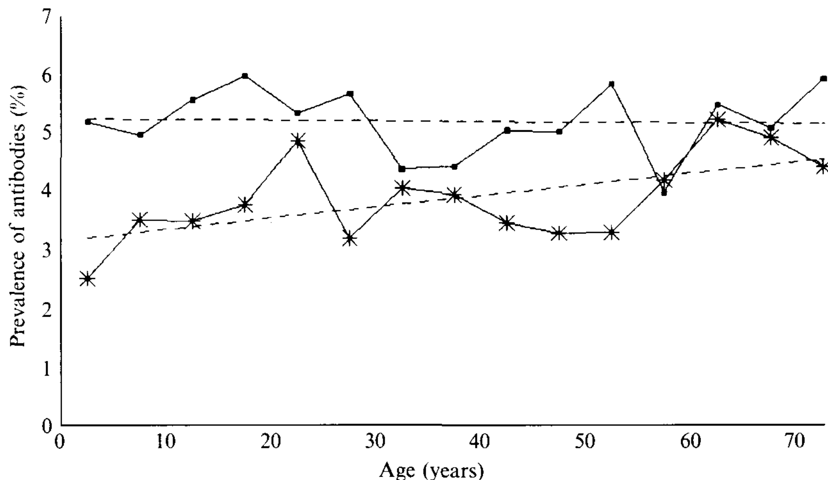

Fig. 3. Antibodies against *E. histolytica* determined by ELISA: distribution by place of residence and age. ■. Metropolitan area: ∗. rural area.

Table 4. *Antibodies against* E. histolytica *according to the 'crowding index'*

Crowding index	Positives	Negatives	Total	%
Low	649	15199	15848	4·10
Medium	744	15587	16331	4·56
High	1295	26784	28079	4·61
Total	2688	57570	60258	4·46

$\chi^2 = 6{\cdot}82$, $P = 0{\cdot}03$.

There is also a slight difference when comparing the prevalence rate by socio-economic status (Table 3), 4·69 % in the lowest group against 4·20 % in the highest ($\chi^2 = 5{\cdot}28$. $P = 0{\cdot}07$). Higher prevalence of antibodies against amoeba was found in metropolitan areas than in urban and rural areas: 5·18, 4·63 and 3·71 %, respectively ($\chi^2 = 44{\cdot}21$, $P < 0{\cdot}001$). Comparing the place of residence and the age distribution (Fig. 3), the prevalence of antibodies was higher in children from the metropolitan areas of the country (Mexico City, Guadalajara and Monterrey) than in those living in the rural areas (towns with 2000 or fewer inhabitants) (analysis of variance, $P = 0{\cdot}093$). In terms of life style, the prevalence of antibodies in families without crowding was 4·10 against 4·61 % in those living in a crowded family environment ($\chi^2 = 6{\cdot}82$, $P = 0{\cdot}03$) (Table 4), which is defined by the NSS as those families where there are four or more people living in the same room [17].

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Table 5. *Incidence of amoebiasis and prevalence of antibodies by geographic area in Mexico*

	Region	Prevalence of antibodies rate ×100	Incidence of amoebiasis rate ×100000	Incidence of ALA rate ×100000	Mortality by amoebiasis rate ×100000
I	North west	3·34	1267·5	0·5	9·34
II	North	5·65	1961·9	39·2	11·56
III	North east	2·74	1331·2	7·0	8·73
IV	South Pacific	2·10	1835·8	31·6	58·34
V	Central west	4·19	1608·2	26·0	19·96
VI	Central south	8·09	769·7	13·8	14·18
VII	Gulf of Mexico	0·91	1529·1	0	15·76
VIII	Yucatan Peninsula	2·72	2756·2	30·5	18·68

Pearson Correlation Coefficient = N.S.

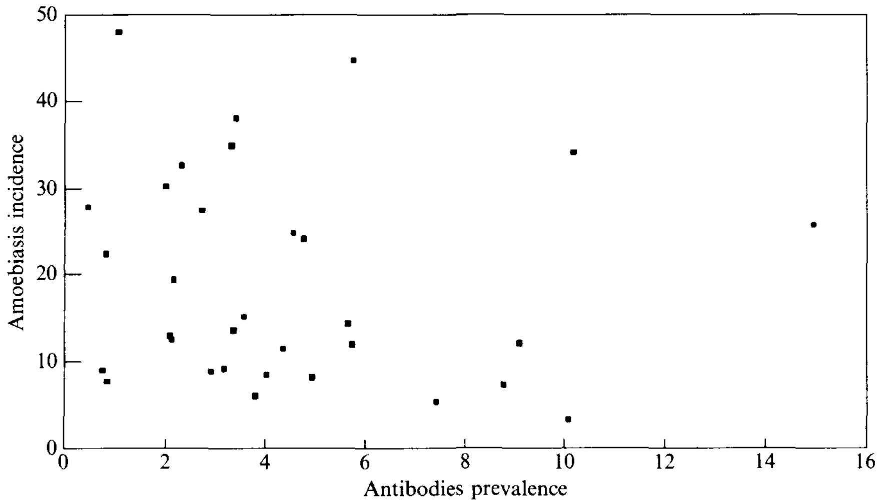

Fig. 4. Correlation between antibodies and incidence of amoebiasis. Rate ×1000 (1988).

Table 5 shows the prevalence of antibody, the incidence of amoebiasis, the incidence of ALA and mortality by geographical area. There was no significant correlation found between these values (Pearson's Correlation Coefficient). Fig. 4 shows the relation between antibody prevalence and incidence of amoebiasis in each state. Again no correlation was found.

DISCUSSION

The prevalence of the amoebic disease can be estimated by serological surveys. although the sampling procedures may make the results from different surveys difficult to compare. Most of the seroepidemiological studies in Mexico have been done in small samples which cannot be used to extrapolate observations to the general population. The sera in the current study are representative of the whole Mexican population, and will be used by the Mexican Ministry of Health to study the seroepidemiology of several diseases [15, 16]. Collection, distribution,

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processing and analysis of such large numbers of sera take some time and so the results reflect the seroprevalence of amoebiasis in the near past. Other problems that might make comparison of surveys difficult are associated with the antigen used. There are many different methods for the extraction of amoebic antigens using a variety of amoebic strains. The cultures may or may not be treated with protease inhibitors and disruption may be freeze-thawing, homogenization or by sonication of trophozoites followed by centrifugation. It is difficult to compare the immune response to such heterogeneous antigens [18] but in this survey a single batch of antigen was prepared and used throughout.

The statistical analysis showed poor correlation between prevalence of antibodies and age but if the place of residence was also considered (Fig. 3), people living in metropolitan were infected earlier in life than those from rural areas. These results suggest that the epidemiological behaviour of amoebiasis could be similar to other diseases where the main mechanism of transmission is the faecal–oral route. The epidemiological behaviour of poliomyelitis is well known. In countries with poor sanitation the infection occurs early in life in the urban areas and the disease is prevalent in children. Outbreaks are not frequent because most of the population is seropositive. When the sanitary conditions improve the infection rate decreases and outbreaks affect both young and old people. The observation of higher prevalence of antibodies in families living in a crowded environment is also consistent with this pattern.

No correlation was found between the presence of antibodies and the incidence of amoebiasis or ALA, or mortality by amoebiasis. However, the epidemiological information on the frequency of amoebic disease is probably inaccurate owing to a tendency to attribute any bloody diarrhoea to amoebiasis. Laboratory techniques may be difficult to carry out [9, 10] and are rarely used for confirmation of diagnosis.

The results presented in this study are not consistent with others made on the same sample from the NSS. A higher seroprevalence against amoebiasis (8·41%) was found when IHA was used [17]. Despite the differences in the prevalence of antibodies against *Entamoeba histolytica* found by ELISA or HIA in both surveys the highest prevalence was found in the South Central region which includes Mexico City whereas the lowest prevalence was found in the North region. Both studies found a higher prevalence of antibodies in females than in males. Differences in results from the two studies may be caused by differences in serological parameters. The cut-off point for the ELISA technique in this study distinguished between healthy people and ALA patients with 90·7% sensitivity and 95% specificity [11]. Since individuals with antibody titres comparable to ALA patients were considered as positives, the assay could be reflecting severe amoebic infection or chronic exposure to the parasite. A serum dilution 1:400 appears to be too high considering that other forms of extraintestinal amoebiasis, different from the ALA, induce lower antibody titres than the ALA. This criterion was selected because antibodies could play a role in the protective immunity against ALA. The study using the IHA was based on a previous publication [19] using modified techniques for sensitizing erythrocytes and with a different cut-off point of serum dilution [17, 19]. Such changes may alter the sensitivity and specificity of the test.

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There are other reports of discrepancies when comparing different methods. Some authors have found only 68% correlation between ELISA and IHA [20], others have reported lower titres against amoeba by ELISA compared with IHA [21]. In another study the rates obtained by IHA and CIE were different, and the authors concluded that CIE probably measures active invasive clinical amoebiasis while IHA may reflect both previous and present infections [2]. There is also poor correlation between the isolation of *Entamoeba histolytica* bearing pathogenic zymodemes in the stools of asymptomatic people, and the presence of antibodies measured by IHA [22].

There appears to be no single test of choice to evaluate the prevalence of amoebic antibodies, as each has advantages and disadvantages [23]. Agreement is required between workers on the analysis of data from immunoepidemiological studies if the results from different studies are to be compared.

Standardization of antigen extraction criteria and the use of the same antigen in all immunoassays would be helpful. The lipopeptidephosphogylcan (LPPG) may be among the antigens that might be used in epidemiological studies. It has been reported that patients with ALA have comparable antibody titres to LPFG isolated from *Entamoeba histolytica* HM-1:IMSS and its virulent clone C-A, but lower titres to the non-virulent L-6 clone [24].

Other methods which could be used to analyse the prevalence of amoebiasis include the Western blot with more purified antigens [25]. Methods searching for parasite elements such as the polymerase chain reaction could be more useful for prevalence surveys. These methods combined with Southern blot analysis and the isoenzyme pattern of the hexokinase may enable the identification of strains possessing pathogenic or non-pathogenic zymodemes. It is also possible to make direct measurements of virulence using the destruction of monolayer tissue culture cells, ability of phagocytosis and the ability to induce liver abscesses in the hamster [26]. These methods are expensive and time-consuming, making them impractical for epidemiological surveys. The commercial development of antigen detection kits and cDNA probes [27] for dot-blot hybridization may provide more rapid, accurate, and less costly diagnostic procedures for the future [28], but it is necessary to evaluate any advantages in epidemiological surveys.

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