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Secretory IgA as a marker of invasive amoebiasis

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Abstract

Amoebiasis is a common intestinal protozoan infection due to *Entamoeba histolytica* with an annual mortality between 50,000- 110,000 due to the various complications associated with the invasive disease. In India, the prevalence of the disease varies from 2-67%. Recently, asymptomatic carriage has been shown to be due to a non-invasive species *E.dispar* which is morphologically indistinguishable from the invasive *E.histolytica*. The only serological test available to detect the two parasites is a monoclonal based enzyme linked immune sorbent assay (ELISA) to detect specific lectin. Serological tests are available to detect antibodies but these do not really help in the diagnosis in endemic areas. Detection of salivary antibodies has been tried in some parasitic infections and may be good test as it also avoids any invasive procedure or collection of blood. In the present study, levels of specific IgA antibodies to *E.histolytica* have been measured in the saliva as well as serum with a view to analyze the role in diagnosis of invasive disease. The highest levels of specific secretory anti-amoebic IgA levels were observed in patients with intestinal amoebiasis (Group B) and ALA cases (Group A). There was no significant difference between the two groups but, there was a significant difference between the specific secretory IgA levels seen in these two groups when compared to the patients with other parasitic infections (Group C), or healthy controls (Group D). Specific anti-amoebic serum IgA levels showed a similar trend, except that ALA patients showed the highest levels which were even significantly different from the intestinal amoebiasis group. This indicates that presence of high titers of specific IgA levels in saliva may have a role in the seroepidemiological surveys to detect the persons who have had past invasive infection. In addition, there is the possibility that the detection of this specific antibody may have a role in the diagnosis of clinically suspected invasive disease.

Key words: Amoebiasis, secretory IgA, salivary antibodies, diagnosis

Introduction

Amoebiasis is a common intestinal protozoan infection due to *Entamoeba histolytica* with extra intestinal invasive manifestations. Worldwide annual mortality is estimated to be 50,000-110,000 due to the various complications associated with the invasive disease (1).

In India, the prevalence of *E.histolytica* on routine stool examination varies from 2-67% depending upon the population being screened (2). In Northern India, in hospital-based studies the prevalence is about 2%, while in the lower socioeconomic status populace in the field it is more than 10% (3) , probably due to the lack of sanitation and poor hygiene. Recently, asymptomatic carriage has been show to be due to a non-invasive species *E.dispar* (4). This is though to be responsible for the majority of the infections and the invasive *E.histolytica* is responsible for much fewer infections. However, morphologically these two forms are indistinguishable. In addition, the World Health Organization has recently stated that intestinal infection be diagnosed with an *E.histolytica* specific test (5). There are a few tests, based on use of Enzyme Linked Immunosorbent Assay (ELISA), which are available for the detection of *E.histolytica/ E.dispar* in fecal samples and cannot be routinely used in developing countries due to the high cost involved. Hence there is the need to develop a simple and non-invasive test, which would help in the diagnosis of invasive disease.Detection of antibodies in salivary fluid of infected patients would be one such test. This is because we do not need to collect the blood from patients and collection of saliva is easy and can also be accomplished with ease in children where collection of blood samples may be a problem. Saliva has been used recently for the diagnosis of neurocysticercosis to detect specific IgA antibodies with a high degree of specificity and sensitivity (6). In case of amoebiasis, there are a few studies, which have tried to use the detection of salivary IgA for diagnosis of invasive infection (7, 8, 9). These studies have given variable results with the diagnostic accuracy varying from 58-90%. In the present study, patients from an endemic area who had invasive disease or were carriers were analyzed for presence of secretory IgA in salivary samples with a view to evaluate its diagnostic potential.

Materials and methods

Patient groups

Forty-five patients with amoebiasis or other parasitic infections were included in the study. These patients were selected from those attending the Medical Out Patients Department (OPD) or admitted in the Nehru Hospital attached to the Postgraduate Institute of Medical Education and Research, Chandigarh, India. These were divided into the following groups:

Table 1. Specific anti-amoebic secretory IgA levels in saliva of different groups of patients and controls:

Group	Mean ± SD (salivary IgA; expressed as O.D. value)	Range (Optical density value on ELISA)
ALA (Group A)	0.39 ± 0.06	0.29 - 0.52
Intestinal amoebiasis (Group B)	0.48 ± 0.39	0.10 – 1.46
Other parasitic infections (Group C)	0.17 ± 0.06	0.10 – 0.33
Healthy controls	0.27 ± 0.87	0.12 – 0.47

Group A

Consisted of fifteen cases of proven amoebic liver abscess (ALA). These were diagnosed on the presence of minimum of two findings out of the following: (i) Sonographic diagnosis and the response to anti-amoebic therapy, (ii) positive amoebic serology by Indirect Haemagglutination Assay (IHA), (iii) Detection of *E.histolytica* in drained liver aspirate.

Group B

Consisted of 15 patients of intestinal amoebiasis diagnosed on the basis of finding of either cysts or trophozoites of *E.histolytica* in faecal samples on microscopic examination of three stool samples.

Group C:

Consisted of 15 patients with intestinal parasitic infections other than amoebiasis. These included patients suffering from giardiasis and helminthic infections. They were negative for *E.histolytica* on three consecutive samples and were also negative for amoebic serology.

***Group D*:**

Consisted of 15 normal healthy controls with no history of any intestinal infection in the past 6 months and were negative for *E.histolytica* on repeated stool examinations and also negative for amoebic serology.

Stool Examination

Three consecutive faecal samples from all the patients were collected in wide mouth plastic containers and examined for parasites by direct smear examination and after formalin ether concentration technique (10).

Examination of liver aspirate

Direct examination of liver aspirate was done under the microscope to detect trophozoites of *E.histolytica*.

Amebic serology for detection of anti-amoebic antibodies

Antigen was prepared from the standard strain NIH-200 maintained axenically in TYI-S-33 medium (11). Log phase cultures were harvested after chilling at 4^oC and the antigen prepared by sonication and the supernatant used as the antigen to coat the walls of microtitre plates. Amoebic serology was carried out according to the methods of (12) and (13) by Indirect haemagglutination assay (IHA).

Collection of saliva samples

About 4-5 ml of saliva was collected from patients as well as controls. The persons were instructed to dribble into a plastic cup. The saliva collected was then centrifuged at 2500xg for 30 minutes. The supernatant was collected and stored at -20^oC till use.

Estimation of salivary IgA antibodies to *E.histolytica*

This was carried out according to the method described by Acosta (7). Briefly, the amount of antigen to be used for coating of the microtitre plates was standardized by Chessboard titration according to the standard technique. Antigen prepared as described above by sonication was coated onto the microtitre plates (Dynatech, Alexandia, VA, USA) as described previously. After overnight incubation at 4^0C, the wells were washed thrice with PBS containing 0.05% Tween-20 (PBS-Tween) and blocked with 1% serum albumin in PBS-Tween. Next, the wells were incubated for 2 hr at 36^0C with undiluted clarified saliva (or serum) washed, and further incubated with alkaline phosphatase-conjugated goat antihuman IgA (A-3150 or A-3400, Sigma, St. Louis, MO), diluted 1 : 1,000. After washing excess conjugate, the well were incubated for 30 min. at 37^0C with freshly prepared p-nitrophenyl phosphate in diethanolamine buffer, pH 9.8. Substrate conversion was assessed at 405 nm using a multichannel spectrophotometer (Dynatech, Alexandia, VA, USA). Results were expressed as Optical Density Values (O.D.).

Results

Of the 15 ALA patients, 6 were females while 9 were males. A similar trend was observed in intestinal amoebiasis patients where 8 were males and 7 were females. In the group C patients, the majority were males (14/15 patients).

Parasites detected in Group C patients (other parasitic infections)

This group was one of the control groups and was included to exclude the possibility that other parasitic infections may be cross reacting with amoebic antigens and leading to high titers of anti-amoebic antibodies. The group largely consisted of males and the parasites detected in stool samples were *Giardia lamblia* (73.3%), *Hymenolepis nana* (20%) and *Enterobius vermicularis* (6.7%).

Results of amoebic serology

This was carried out on blood samples collected from all the groups. In case of ALA cases all the patients were positive with

Table 2. Levels of specific anti-amoebic serum IgA levels in different groups of patients and controls (results expressed as optical density (O.D.) values obtained with ELISA):

Group	Mean ± SD (serum IgA; expressed as O.D. value)	Range (Optical density value on ELISA)
ALA (Group A)	0.51± 0.28	0.16 - 0.97
Intestinal amoebiasis (Group B)	0.28 ± 0.16	0.11 - 1.68
Other parasitic infections (Group C)	0.33 ± 0.14	0.13 - 0.65
Healthy controls	0.32 ± 0.35	0.07 - 1.43

titers ranging from 1:512 to 1:2048 (the significant titer above which it is considered as positive is 1:256 in the laboratory). In the intestinal amoebiasis group (Group B), the titers were quite low and ranged between 1:8 to 1:32. A similar range of titers was observed in control group (Group D), where the titers were lower than 1:32 in all the persons tested.

Salivary IgA levels in different groups of patients and controls

The highest levels of specific secretory anti-amoebic IgA levels were observed in patients with intestinal amoebiasis (Group B) and ALA cases (Group A). There was no significant difference between the two groups (Table 1), but there was a significant difference between the specific secretory IgA levels seen in these two groups when compared to the patients with other parasitic infections (Group C), or controls (Group D). Specific anti-amoebic serum IgA levels showed a similar trend, except that ALA patients showed the highest levels which were even significantly different from the intestinal amoebiasis group (Group B; Table 2).

Discussion

The diagnosis of amoebiasis is mainly dependent on the examination of stool samples by microscopy. This technique is labor intensive and requires an experienced microscopist for accurate diagnosis. Furthermore, the sensitivity of a single stool examination is low as this usually detects only 50% of the infections (14). Serological tests, especially those using the detection of IgG antibodies are of value only as epidemiological tools in endemic areas as majority of the populace may have been exposed or infected in the past and the antibody titers persist for long periods. To detect acute infections, IgM antibodies are usually measured in various microbial infections but these are of marginal help in the diagnosis of acute amoebiasis (15). The only antigen to which the antibody response is short lived is the Gal-Gal Nac lectin. The drawback of this test is that it requires the use of purified lectin in the system, which may not be available at all places.

While the role of sIgA is not defined in the natural history of invasive amoebic disease, a number of studies have demonstrated the presence of anti-amoebic IgA antibodies in mucosal secretions such as stool, saliva and breast milk. Colostral anti-amoebic IgA has been detected in 31% of post-partal women in Kenya (16) and coproantibodies are present in 80% patients with invasive amoebiasis. At the moment, there are no studies available which have evaluated the role of salivary IgA in diagnosis of amoebiasis. There are a few studies in other parasites, which indicate that these may help in the diagnosis and coupled with the simple non-invasive technique for saliva collection it may prove to be a test, which could be of use in the field. In the present study, IgA antibody to *E.histolytica* was detected in saliva using ELISA test. It was observed that statistically significant differences between the levels of salivary IgA were noticed in patients with ALA or intestinal amoebiasis as compared to patients with other intestinal infections or controls (Table 1). A similar increase in IgA levels was observed in serum samples of these groups of patients. But, here the difference between the groups of ALA and controls was not statistically significant (Table 2), probably due to past infection by the parasite and persistence of some amount of antibody response in the endemic population. This indicates that increased serum IgA levels may not be of help in differentiating a recent invasive infection from past infection. On the other hand, salivary IgA levels showed a statistically significant difference between the ALA group, intestinal amoebiasis group and the other parasitic infection and control groups. This indicates that presence of high titers of specific IgA levels in the saliva may have a role in the seroepidemiological surveys to detect the persons who have had past invasive infection. In addition, there is the possibility that the detection of this specific antibody may have a role in the diagnosis of invasive disease. A recent study has shown that certain antigens of *E.histolytica* are recognized more frequently (>90%) by secretory IgA from patients with intestinal amoebiasis as compared to that from healthy subjects (<10%) (17). Though, the use of specific antigens is a better option, they are difficult to purify. Hence, in the present study we have used crude antigen to analyze the role of IgA antibodies as a marker of invasive amoebiasis. Some drawbacks of the present investigation are that the number of patients, which have been enrolled in the different groups, is small and also no follow up of the patients has been done to see for how long the antibodies persist. It would also be interesting to evaluate the presence and persistence of salivary IgA to the lectin antibody, which also helps in the diagnosis of invasive disease. Further studies in this regard are being planned and also evaluation of the test in the field situation is needed and is being planned.

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