Recognition of *Entamoeba histolytica* Gal-Lectin Heavy Subunit Epitopes by Serum IgA and IgG Antibodies from Diverse Populations

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Abstract: A fragment of the *Entamoeba histolytica* Gal-lectin heavy subunit (LC3) contains four epitopes recognized by IgA and IgG from a South Africa population immune to reinfection. We studied diverse endemic populations, including Tanzanian subjects (n=231) and USA refugees from Liberia (n=200), Somalia (n=200), or an ethnic Hmong population (n=200) by ELISA; among anti-LC3 seropositive subjects 55% to 100% possessed both serum IgG or IgA antibodies to at least one of the four LC3 epitopes ($p<0.001$). Recognition was most intense for epitopes 2 and 9 ($p<0.01$) compared to epitopes 11 or 12; the anti-IgA prevalence was highest for epitope 11 ($p<0.032$) in Liberian subjects, epitope 2 ($p<0.05$) in Hmong and Tanzanian subjects, epitopes 2, 9 and 11 ($p<0.034$) in Somalian subjects and epitope 9 ($p=0.013$) in the immune South African cohort. Therefore, these four putatively protective LC3 epitopes have potential for application in an amebiasis subunit vaccine.

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Key Words: Entamoeba histolytica, Gal-Lectin, Epitopes, Peptide Vaccine, Diverse Sera.

1. Introduction

Entamoeba histolytica is one of the leading parasitic causes of morbidity and mortality worldwide due to the occurrence of amebic colitis, liver abscess and other extra intestinal syndromes.[1] Prospective studies in Dacca, Bangladesh, and Durban, South Africa, have focused on the association of intestinal anti-Gal-lectin IgA antibodies with immunity to new infection, especially following cure of invasive amebic liver abscess (ALA), which results in a high amnestic intestinal anti-lectin IgA antibody response. [2-4] Our laboratory has used overlapping recombinant proteins derived from a highly antigenic portion of the heavy subunit (designated LC3, amino acids 758 to 1150), which also contains the galactose binding site.[5] Fine mapping with synthetic peptides demonstrated that IgA and IgG antibodies from subjects in Durban, South Africa that recently cured of ALA, recognized four discrete LC3 epitopes (aa 891-903, 918-936, 1114-1134 and 1128-1150).[6] These putative protective IgA epitopes were utilized to synthesize a synthetic peptide vaccine (MAPS) by linkage to a polylysine backbone, which when administered intranasally to baboons with cholera toxin as an adjuvant, elicited high titer serum and intestinal anti-peptide IgA antibody responses.[7] The MAPS vaccine elicited serum and intestinal IgA antibodies also recognized native Gal-lectin protein, bound to the surface of *E. histolytica* trophozoites and had neutralizing activity *in vitro*, as defined by inhibition of Gal-specific adherence of *E. histolytica* trophozoites to target cells.[7] Last, the vaccine was found to be highly efficacious in preventing experimental asymptomatic *E. histolytica* infection as well as invasive amebic colitis in baboons.[8] We sought to determine whether these discrete IgA peptide epitopes, candidates for study as a MAPS amebiasis subunit vaccine, are recognized by IgA and IgG antibodies from subjects with diverse geographic and therefore, presumed, genetic backgrounds. This finding would have significance in regard to this synthetic peptide vaccine had broad applicability and could be potentially used in a globally distributed manner.

2. Methods

To obtain serum antibodies for this study, we utilized a number of sources. First, previously unpublished data were used from a cross-sectional prevalence study in 2004 in Northern Tanzania; following informed consent, blood and fecal samples were obtained from 231 asymptomatic subjects aged 2 to 90 years. This study was approved by the IRB of the University of Minnesota, Selian Lutheran Hospital, and Nkoranga Lutheran Hospital, as well as the Tanzanian National authorities.

Second, sera from adult refugees and immigrants resettling to the U.S. were obtained from the Migrant Serum Bank at the Centers for Disease Control and Prevention, Division of Global Migration and Quarantine (http://www.cdc.gov/immigrantrefugeehealth/migrant-serum-bank.html). The serum was collected from persons 15 years or older during their pre-departure medical screening between 2003 and 2004 and used for routine blood testing for certain inadmissible conditions. Six hundred serum samples, 200 from each group, were randomly selected from each of the three U.S.-bound populations; Hmong (Hmong ethnicity) departing from Wat Tham Krabok near Bangkok, Thailand; Bantu Somali departing from Kakuma, Kenya; and Liberians departing from Abidajan, Cote d'lvoire. The protocol was reviewed and the study granted exemption by both the University of Minnesota and the CDC.

Last, subjects were randomly selected from a previous prospective controlled study of the incidence of *E. histolytica* over 36 months in a Zulu population that had either been treated for amebic liver abscess (ALA) or were asymptomatic controls and reside in the periurban areas of Durban, South Africa; this group included 14 seronegative asymptomatic uninfected controls, up to 26 asymptomatically infected seropositive controls, and 20 seropositive subjects recently cured of ALA. *E. histolytica* infection was determined by culture and zymodeme determination[3]. This study was approved by the IRBs at the University of Minnesota, University of Natal in Durban, and the Medical Research Council of South Africa.

Preparation of the polylysine-linked synthetic peptides antigen

Based on previous findings, four peptides that include amino acids 891 – 903, 918 – 936, 1114 – 1134, and 1128 – 1150 of the LC3 recombinant protein were selected.[6] Peptides were synthesized using a Perkin Elmer Pioneer Peptide synthesizer by solid-phase FMOC (fluorenylmethoxycarbonyl) chemistry. Peptides were cleaved from the resin and de-protected using Reagent R and then lyophilized. Lyophilized crude peptides were purified by preparative reverse-phase HPLC (Beckman 126) on a C4 column by VYDAC. Solvent A = 0.1% TFA in water, solvent B= 0.1%TFA in ACN (Acetonitrile) on a gradient of 0-60% B in 30 minutes. Purity and quality control of the peptides were done by an analytical HPLC, HP1090 on a C18 (VYDAC) column by using the same gradient and by mass spectrometry as on a HP MALDI_TOF.[9]

Peptide polylysine complexes were manufactured by Alpha Diagnostic international company, and shipped in a lyophilized form. Each polylysine molecule has six peptides linked to it as a random mixture of all four peptides. The polylysine-linked peptide vaccine preparation was suspended in H_2O and submitted to quantitative assay.

ELISA for detection of anti-LC3 and anti-peptide antibodies

ELISA was performed as described previously.[5] Recombinant 52 KDa LC3 protein was purified as described.[5] ELISA plates (96-well microtiter flat-bottomed polystyrene) were coated with LC3 protein, or synthetic peptides and non-reactive sites were blocked with 1% BSA. Serum samples were studied at 1:100 dilutions for IgG or IgA ELISAs, all in PBS-Tween (1% BSA) and incubated for 2 hours at room temperature or over night at 4C°. Alkaline phosphatase-conjugated goat anti-human IgG (Sigma, St. Louis, MO) or IgA antibodies (ICN Biomedicals (Costa Mesa, California) were diluted (at 1:5000 for IgG and 1:2500 for IgA) in PBS-Tween (1% BSA) for incubation in 100-*ul* wells for 2 hours at room temperature. Sera from samples taken in the U.S. from persons with no known risk factors for *E. histolytica* were used as controls and to calculate cut-off points. Control sera were run with each ELISA plate. As previously described, mean of + 3 standard deviation of ELISA Optical Density (OD) of the controls was used as the cut-off point for each assay (above this value was considered positive). All controls were negative for the tested antibody response. The plates were read and nonspecific background binding was corrected as described[10].

Statistics

Data collected during the study were coded, entered into an Excel spreadsheet and analyzed by using SPSS 10 (SPSS, Chicago, IL). In all instances, $P < 0.05$ was considered to be statistically significant. Results of ELISA optical density reading of controls were expressed as mean + 3 SD to define the cutoff points that was used to calculate the percent positive and negative sera. Unpaired student t-test was used to compare optical density means[11]. Two sample z tests with Yates continuity correction were used to compare proportions (Sigma Stat software).

3. Results

Sera from Hmong, Somali, and Liberian refugee populations were collected from persons 15 years of age or older while the Tanzanian subjects included children as young as two years. The South African ALA subjects were predominantly male, while the seronegative and seropositive controls had a higher percentage of females.[4] The percentage of subjects in each population that were ELISAs positive for anti-LC3 IgA or IgG antibodies is summarized in Table 2; by definition the South

African subjects were selected as being positive or negative by ELISA for serum anti-LC3 IgA and IgG antibodies. The four remaining populations did not differ significantly among themselves in regard to seropositivity for anti-LC3 IgA and IgG antibodies (Table2); however, each population had a prevalence of at least 10% for both serum anti-LC3 antibodies, indicating current or prior exposure to the parasite.

Table 1. Demographic information of the four new populations studied

	Somaliani n=200	*Liberian n=200*	*Hmong n=200*	*Tanzanian n=231*
Mean age in years	32.2	33.3	34.5	29.3
Percent male	49.0%	43.5%	53.5%	23.4%

Table 2. Prevalence of serum anti-LC3 IgA and IgG antibodies in each population studied [a]

Positive ELISA for:	*Somalian (n=200)*	*Liberian (n=200)*	*Hmong (n=200)*	*Tanzanian(n=231)*
Serum anti-LC3 IgA	13%	10%	10%	16%
Serum anti-LC3 IgG	15%	14%	10%	14%
Either anti-LC3 IgA or IgG were positive	16.0%	14.5%	11.5%	16.5%

[a.] There are no significant differences among the studied populations studied in regard to the prevalence of anti-LC3 IgG and IgA antibodies as determined by ELISA (p>0.05).

Table 3. Prevalence of anti-peptide IgA antibodies in seropositive study subjects.

Peptide Epitope as Antigen in ELISA	*Somalian (n=25)*	*Liberian (n=17)*	*Hmong (n=21)*	*Tanzanian (n=18)*	*South African, asymptomatic (n=25)*	*South African, ALA (n=20)*
Peptide 2 (aa 891-903)	52%[d]	82%[d]	71%[b]	50%[b,d]	92%[c,d]	85%[c,d]
Peptide 9 (aa 918-936)	52%[d]	82%[d]	29%[b]	33%[d]	96%[c,d]	85%[c,d]
Peptide 11 (aa 1114-1134)	25%[d]	88%[a,d]	43%	6%[b,d]	32%[c]	10%[c]
Peptide 12 (aa 1134-1150)	40%[d]	65%[d]	48%	22%[d]	64%[c,d]	75%[c,d]
Any one of the peptides recognized	68%	100%	81%	61%	100%	100%

[a.] The Liberian subjects had the highest percent recognition of LC3 epitope 11 compared to all other populations studied (p<0.032 for each comparison).
[b.] LC3 epitope 2 was recognized more frequently by serum IgA antibodies in the Hmong and Tanzanian populations (p<0.05).
[c.] Compared to epitopes 2,9 and 12, LC3 epitope 11 was less frequently recognized by sera from both South African populations (p<0.042).
[d.] The percent recognition of any one of the four LC3 peptides by IgA antibodies was higher in Liberian or both South African populations, compared to the Somalian or Tanzanian refugees (p<0.05), but not the Hmong population (p>0.05).

Table 4. Prevalence of anti-peptide IgG antibodies in seropositive study subjects.

Peptide Epitope as Antigen in ELISA	*Somalian (n=20)*	*Liberian (n=20)*	*Hmong (n=16)*	*Tanzanian (n=21)*	*South African, asymptomatic (n=26)*	*South African, ALA (n=20)*
Peptide 2 (a.a 891-903)	45%[a]	90%[d]	56%[d]	67%[d]	54%	85%[d]
Peptide 9 (aa 918-936)	100%[a,d]	75%	50%[d]	48%	54%	100%[c,d]
Peptide11 (aa 1114-1134)	100%[a,d]	80%[d]	44%	33%[d]	38%	65%[c,d]
Peptide 12 (aa 1134-1150)	15%[a,b]	75%[b]	19%[b,d]	43%	54%[d]	65%[c,d]
Any one of the peptides recognized	100%	95%	75%	76%	81%	100%

[a.] Serum IgG antibodies from the Somali population exhibited a higher frequency of recognition of LC3 epitopes 2, 9 and 11 compared to epitope 12 (p<0.034).
[b.] Serum IgG antibodies from the Liberian population exhibited a higher frequency of recognition of LC3 epitope 12 compared to the Somali (p<0.001) and Hmong (p<0.003) populations.
[c.] S. African ALA subjects had a higher prevalence of recognition of LC3 epitope 9 by serum IgG antibodies, compared to epitopes 11 and 12 (p=0.013).
[d.] Serum IgG antibodies from all populations studied recognized at least one of the four LC3 epitopes at a comparable level within the same population or on comparison with other population (p>0.05).

Figure 1A

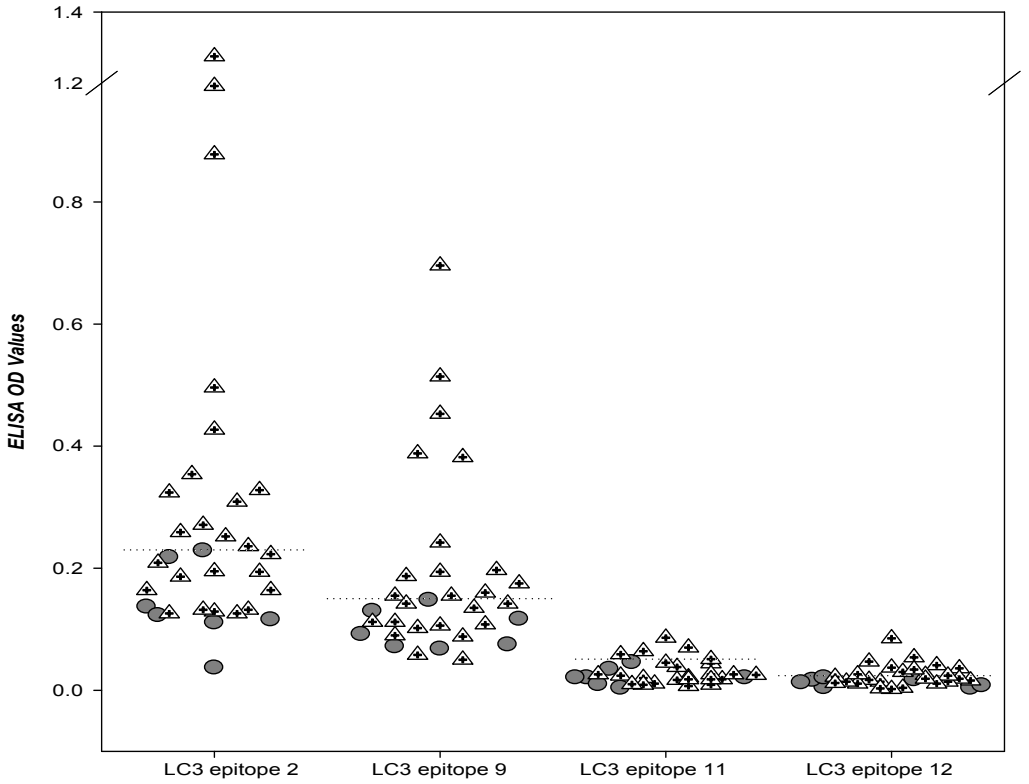

Figure 1B

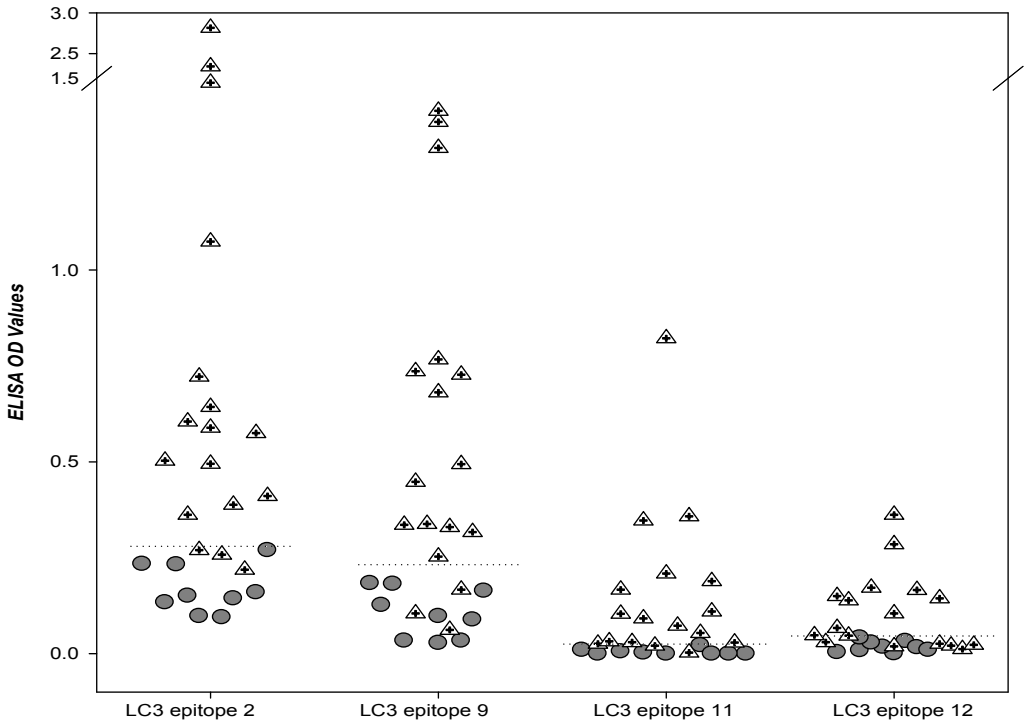

Figure 1C

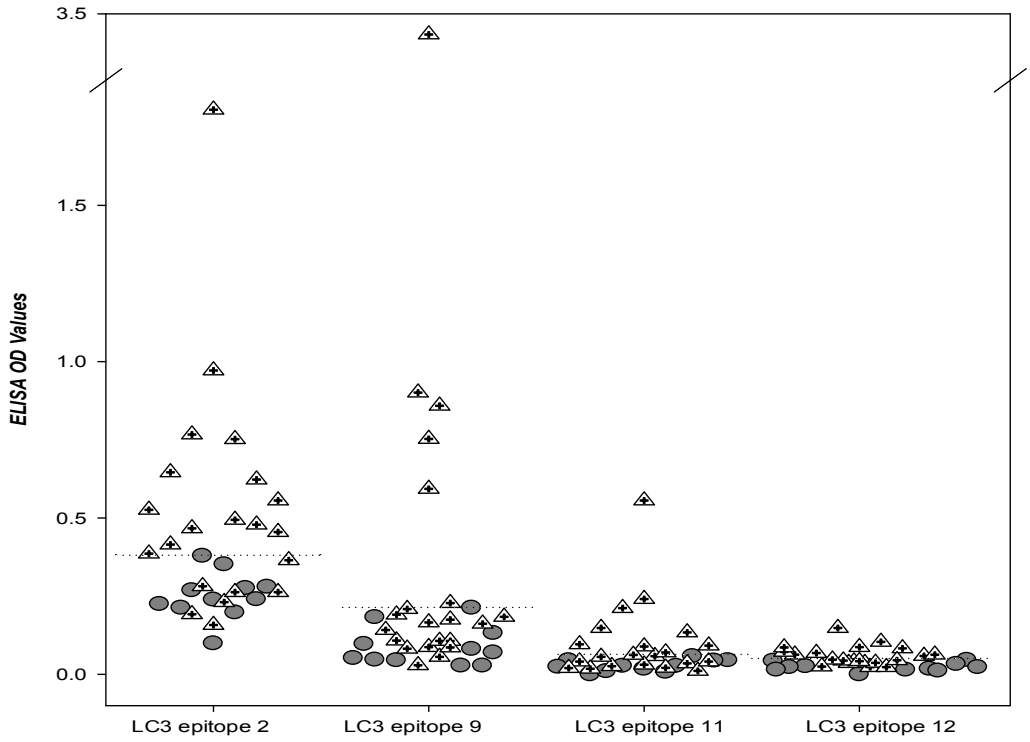

Figure 1D

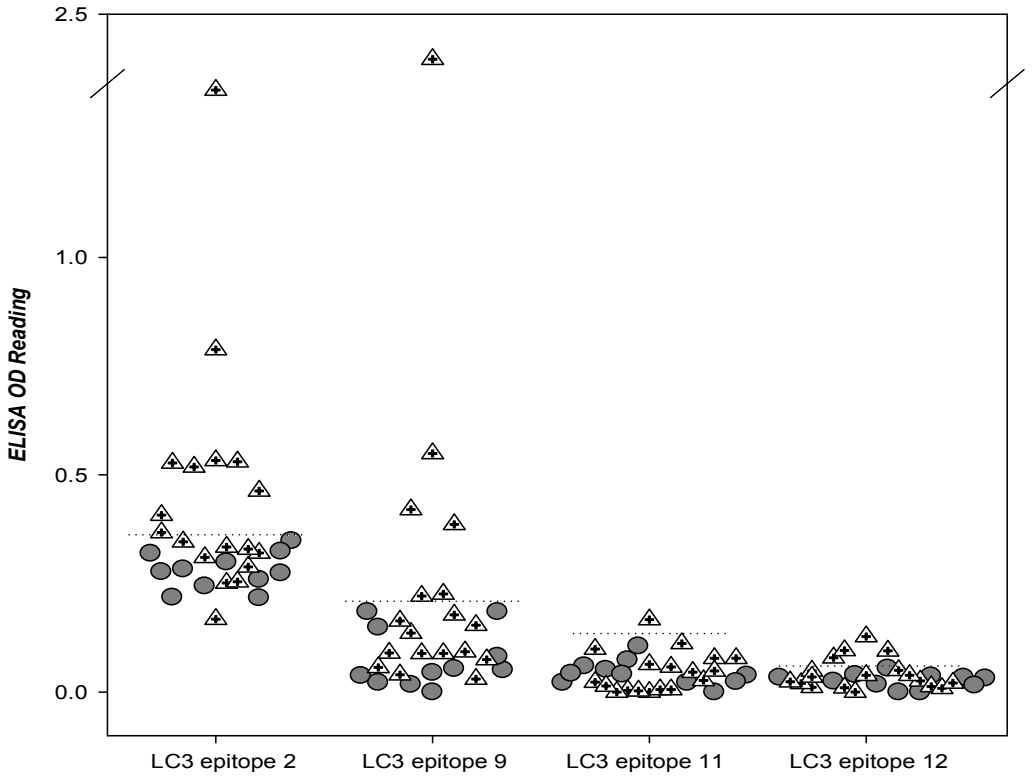

Figure 1E

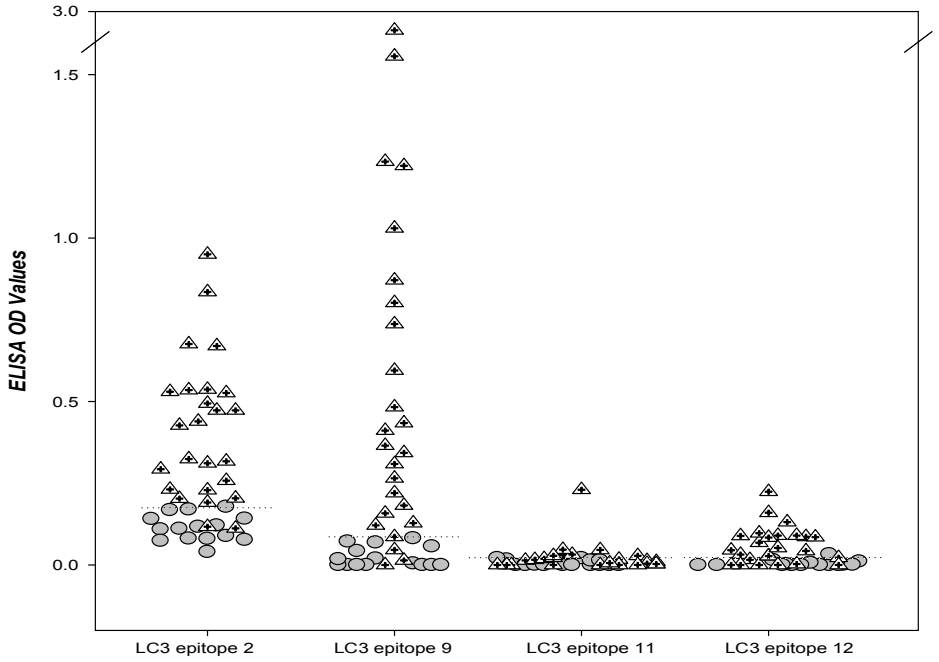

Figure 1F

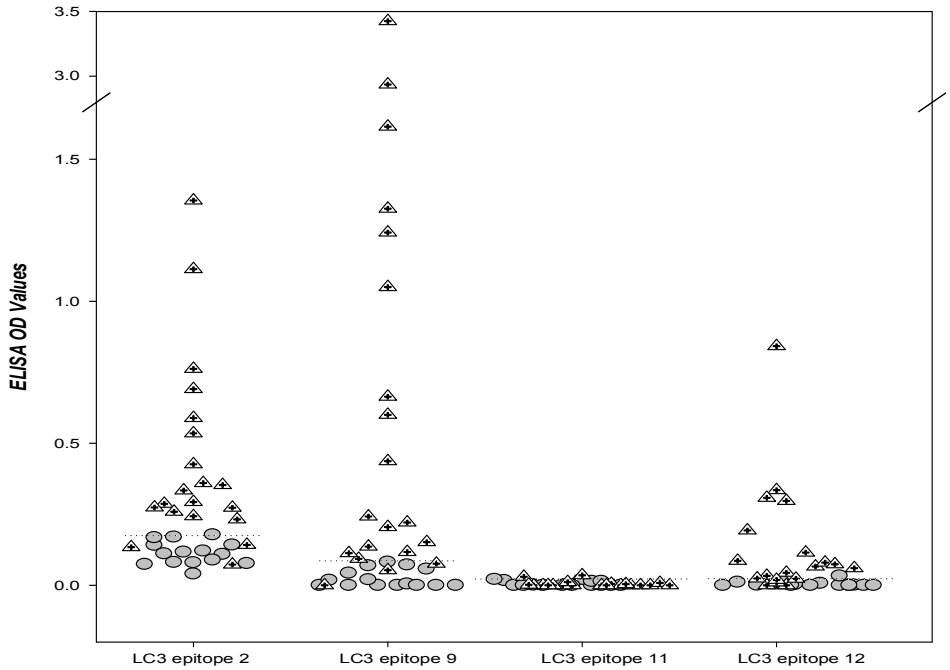

Figure 1. Recognition of LC3 epitopes 2,9,11 and 12 by serum anti-lC3 IgA antibodies from the Somalian (1A), Liberian (1B), Hmong (1C), Tanzanian (1D), asymptomatic, infected South Africans (1E), and South African subjects recently cured of amebic liver abscess (1F). Triangles (Δ) represent ELISA OD values for each seropositive individual studied and by each epitope (2,9, 11 and 12); circles (○) represent seronegative control subjects randomly selected from each population, for the South Africans these were selected from asymptomatic non-infected control subjects. Dotted lined represent the cutoff points for a positive assay for each epitope. Integrating the results of all populations studied, there was a greater intensity (ELISA OD value) for recognition of LC3 epitopes 2 and 9, compared to epitopes 11 and 12 (p<0.001).

Figure 2A

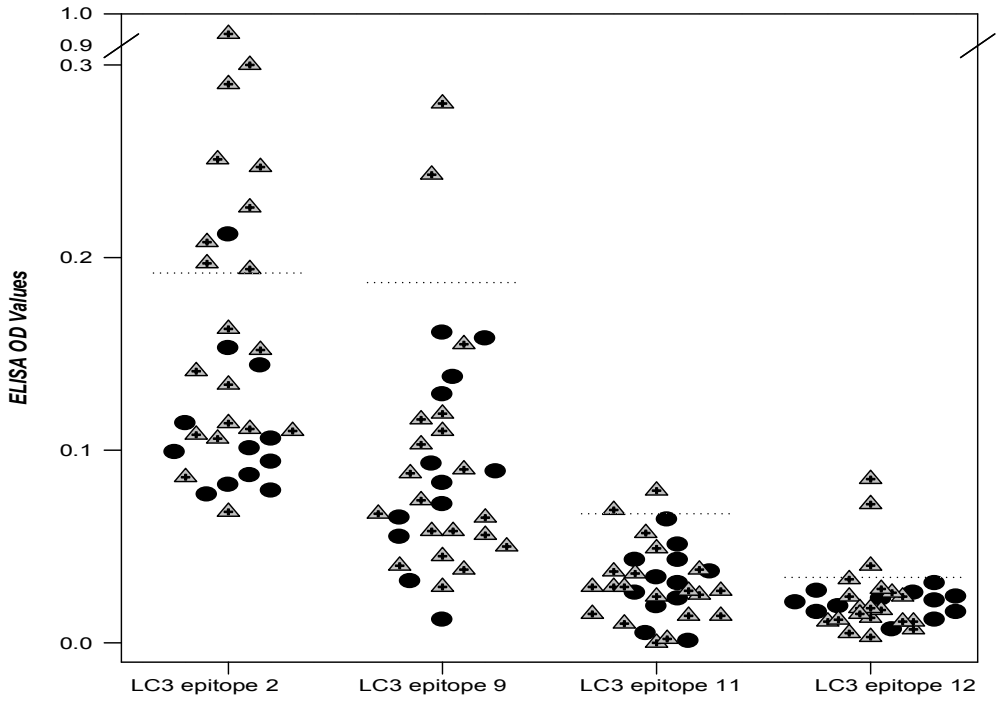

Figure 2B

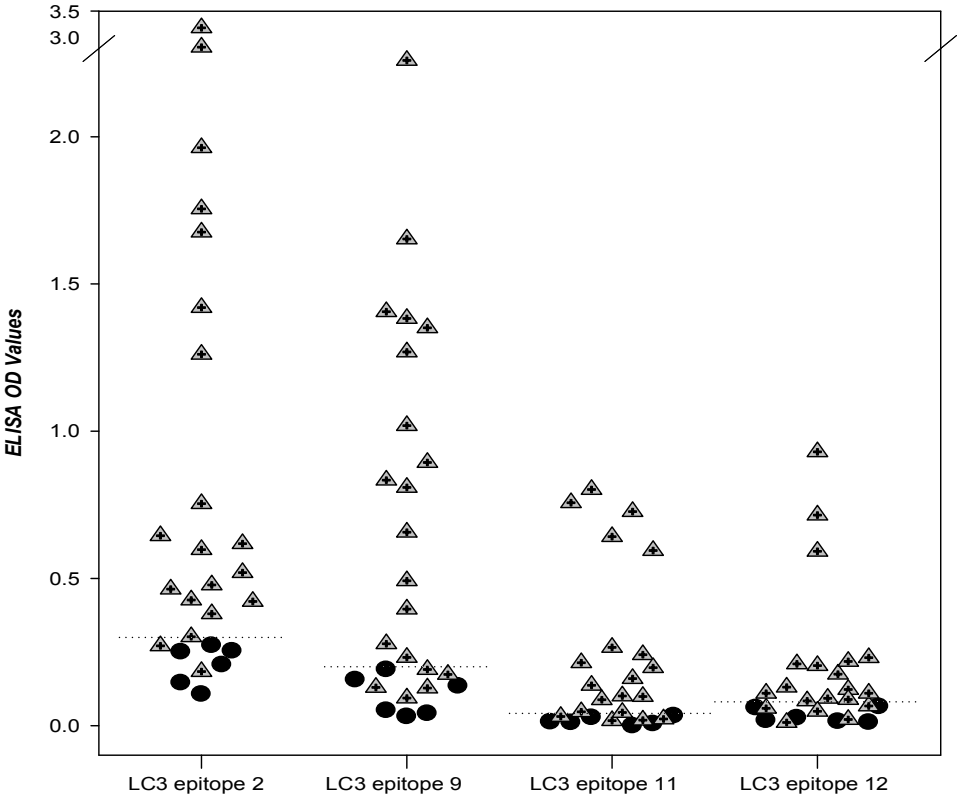

Figure 2C

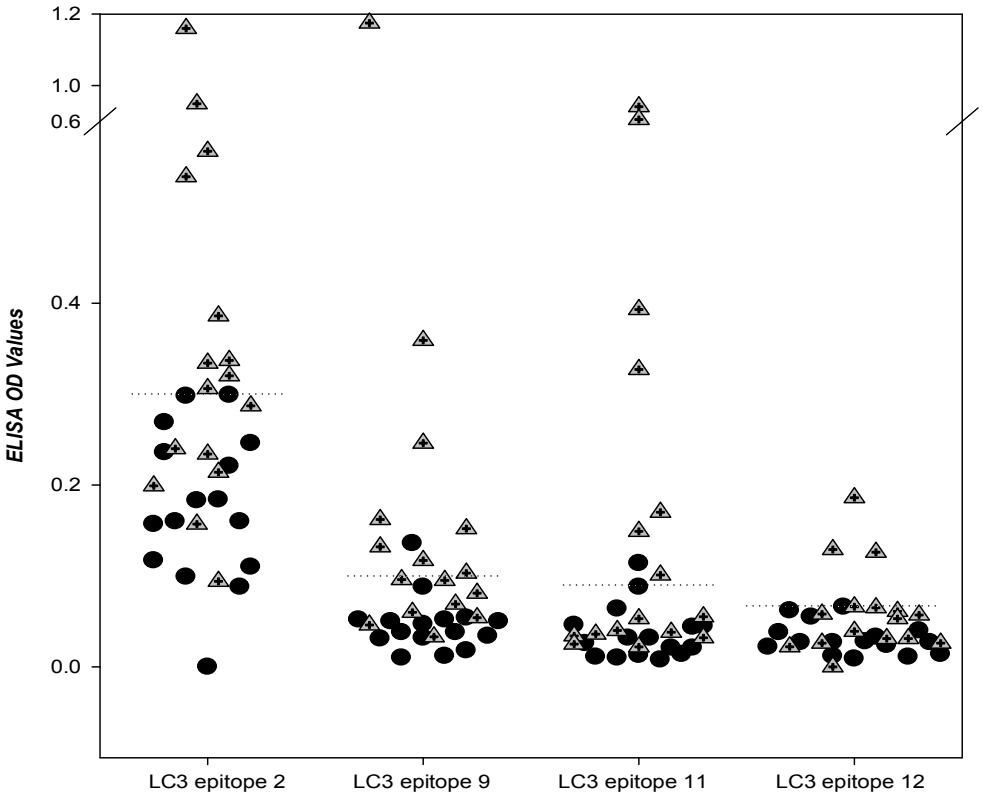

Figure 2D

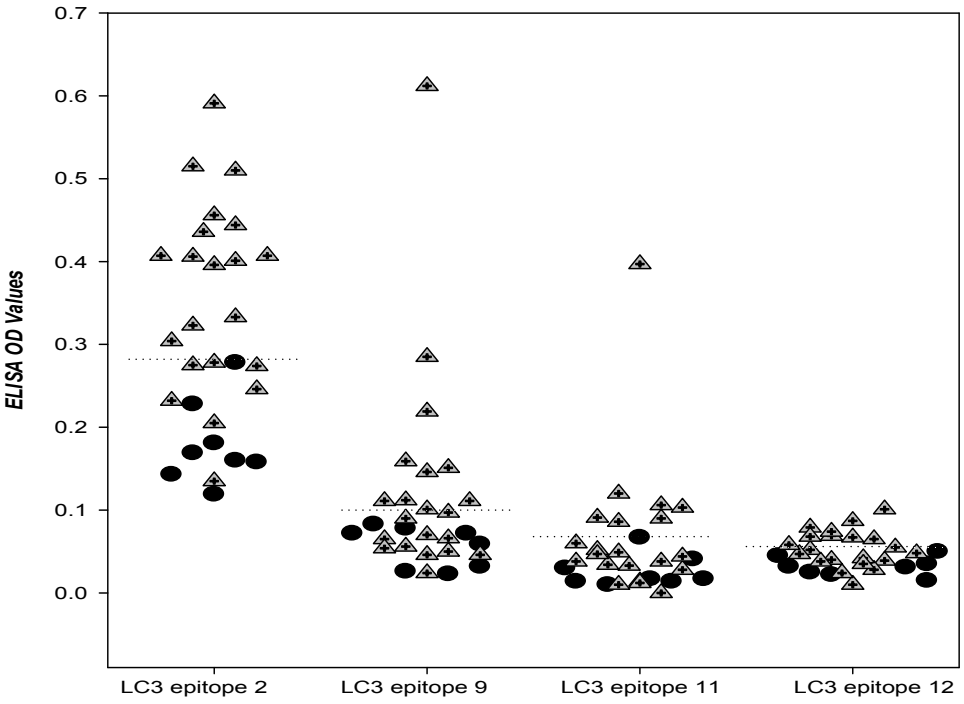

Figure 2E

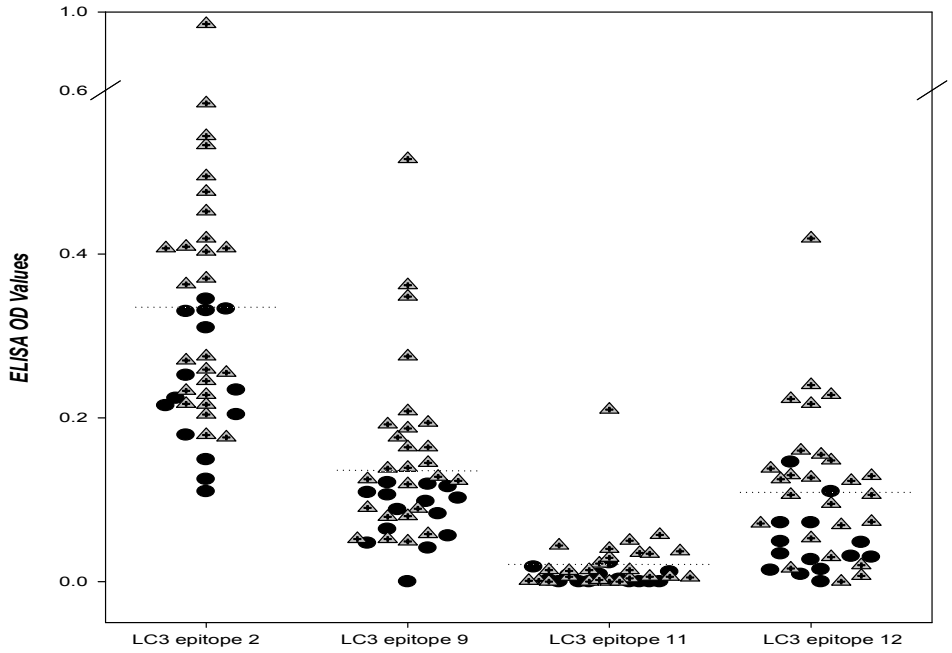

Figure 2F

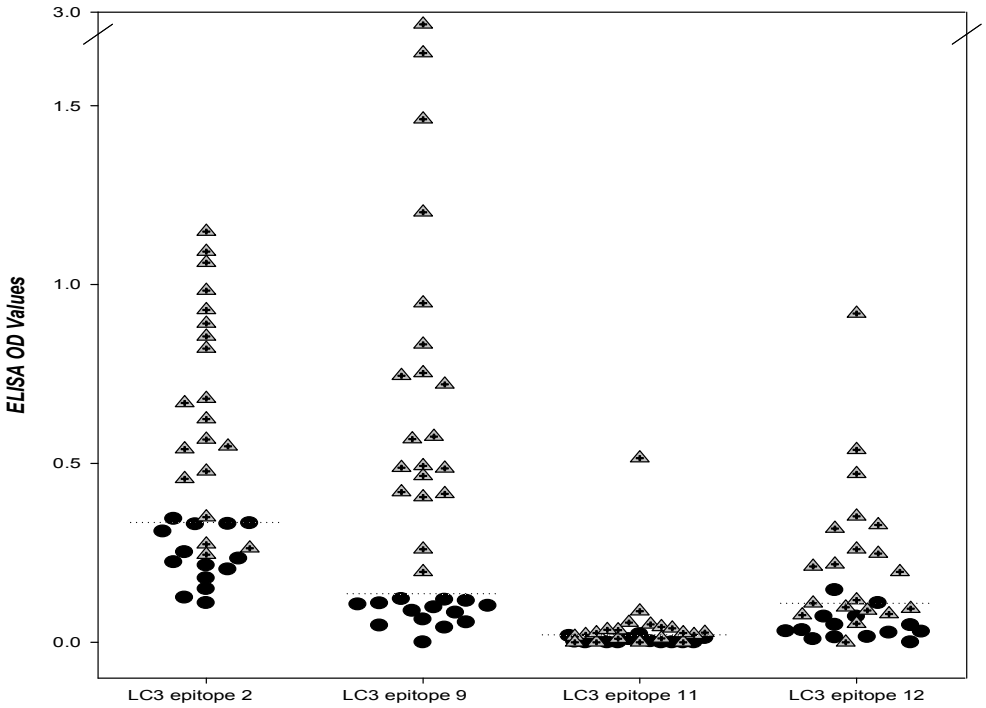

Figure 2. Recognition of LC3 epitopes 2,9,11 and 12 by serum anti-lC3 IgG antibodies from the Somalian (2A), Liberian (2B), Hmong (2C), Tanzanian (2D), asymptomatic infected South Africans (2E), and South African subjects recently cured of amebic liver abscess (2F). Triangles (Δ) represent ELISA OD values for each seropositive individual studied and by each epitope (2, 9, 11 and 12); circles (○) represent seronegative control subjects randomly selected from each population, for the S. Africans these were selected from asymptomatic non-infected control subjects. Dotted lined represent the cutoff points for a positive assay for each epitope. Integrating the results of all populations studied, there was a greater intensity (ELISA OD value) for recognition of LC3 epitopes 2 and 9, compared to epitopes 11 and 12 (p<0.001).

Among these subjects possessing either serum anti-LC3 IgA or IgG antibodies, we determined the percentage that also recognized the four LC3 peptide epitopes (2, 9, 11, 12) encompassing Gal-lectin heavy subunit amino acids 891 to 903, 918 to 936, 1114 to 1134 and 1134 to 1150 respectively, seronegative subjects in each population functioned as controls. The results are illustrated in Figures 1A-F, 2A-F and summarized in Tables 3 and 4. In all of the populations studied, subjects that were ELISA positive for anti-LC3 antibodies also had a high prevalence of IgG and IgA antibodies for one or more of the four synthetic peptides, with a range of 61% (Tanzanians) to 100% positive (Liberian and both reference South African cohorts), Table 3, $p<0.024$ compared to control serum. When all populations seropositive for anti-LC3 IgA antibodies were combined, there was a higher prevalence of serum IgA recognition for LC3 epitope 2 and LC3 epitope 9 (Table 3, $p<0.01$) and with a greater ELISA OD value (Figure 1A-E, $p<0.001$) than for ELISA studies using epitopes 11 and 12. Among the Somalian, Liberian, Hmong, and Tanzanian populations, the Liberian subjects exhibited the highest prevalence of IgA recognition for epitope 11 (Table 3, $p< 0.032$ for each comparison), whereas LC3 epitope 2 was recognized more frequently by serum IgA antibodies from the Hmong and Tanzanian populations (Table 3, $p<0.05$). Among the South African populations, LC3 epitope 11 was less frequently recognized by serum IgA antibodies, than epitopes 2, 9 or 12 (Table 3, $p<0.042$).The prevalence for recognition of any one of the four LC3 peptides by serum IgA antibodies was highest in the Liberian and both S. African populations, compared to either the Somalian or Tanzanian refugees ($p<0.05$), but not the Hmong population ($p>0.05$) (Table 3).

The results for serum anti-peptide IgG antibodies are summarized in Table 4 and Figure 2A-F. The range of prevalence of a positive ELISA for IgG antibodies to at least one of the four LC3 epitopes was 55 to 100%, ($p<0.01$) compared to seronegative specimens (Table 4). Anti-peptide IgG antibodies were of highest prevalence and intensity (ELISA OD value) for recognition of LC3 epitopes 2 and 9, compared to either epitopes 11 or 12 ($p<0.01$, Table 4 and $p<0.01$, Figure 2A-F). Within the Somalian population, there was a higher prevalence of IgG recognition of LC3 epitopes 2, 9 and 11, compared to epitope 12(Table 4, $p<0.034$). The Liberian population exhibited a higher prevalence of IgG antibody recognition of LC3 epitope 12 compared to both the Somalian ($p<0.001$) and Hmong ($p<0.003$) populations (Table 4). The South African ALA cohort exhibited a higher prevalence of IgG recognition of LC3 epitope 9, compared to epitopes 11 and 12 (Table 4, $p=0.013$). All six populations studied possessed serum IgG antibodies that recognized at least one of the four LC3 epitopes at a comparable frequency within the same population or with another population (Table 4, $p> 0.05$).

4. Discussion

E. histolytica is highly endemic throughout the world and leads to tremendous morbidity and mortality, yet currently there is no vaccine to prevent infection and disease. Only a small percentage of those infected develop invasive amebiasis; however, asymptomatic infection is very frequent and is associated with only a transient anti-amebic intestinal IgA antibody response and does not result in immunity to recurrent *E. histolytica* infection.[1,4,12] Therefore, populations residing in endemic areas are subject to recurring *E. histolytica* infections, with up to 70% being infected annually.[4,13-18] In addition, a small percentage of individuals may exhibit some level of innate immunity to *E. histolytica***;** however, in one study at least 80% of children were at risk for infection and disease.[19] Pharmacologic treatment of invasive amebic liver abscess results in development of high titer intestinal and serum anti-Gal-lectin IgA and IgG antibody responses and a brisk amnestic intestinal anti-Lectin IgA antibody response upon future challenge by the parasite.[12] This high titer response resulted in immunity to *E. histolytica* infection that was sustained for at least three years.[2-4]

Previously, we demonstrated that anti-lectin IgA and IgG antibodies from immune subjects (post-ALA treatment) in Durban, South African predominately recognized four epitopes on the LC3 (a.a 758 to 1150) portion of the Gal-lectin heavy subunit, which includes the lectin`s carbohydrate binding site.[6] We produced four synthetic peptides, which encode the putatively protective LC3 epitopes, which were effective as a polylysine linked intranasal MAPS vaccine, with cholera toxin as adjuvant, in eliciting adherence inhibitory intestinal IgA antibodies in baboons.[7] This intranasal subunit vaccine was also found to be highly efficacious in prevention of asymptomatic infection and colitis in an experimental baboon model.[8]

We asked the question: would anti-Gal lectin IgA and IgG antibodies resulting from *E. histolytica* infection of populations with a different geographic background than the South African Zulu population elicit serum IgA and IgG antibodies to the same four LC3 epitopes previously described [6,7,12] This response would increase the likelihood that a

epitope-based subunit vaccine, as studied in baboons[8] could be applicable to populations in many geographic regions (other than just Natal, South Africa).[4,7] We found that West and East Africa populations, as well as a Hmong population from Asia, possessed anti-LC3 antibodies that recognized one or more of the four peptide epitopes. Clearly, peptides 2 and 9 (aa 891-903 and 918-936) were dominant as determined by the prevalence of recognition and intensity (ELISA OD value) and were not statistically different in either measurement from that observed in simultaneous studies using an equal sample size from the control South African population.[4,6, 7] There were some statistically significant variation between individual populations for recognition of any one LC3 epitope, but all of the populations studied recognized at least one of the four LC3 epitopes at a comparable level.

In summary, this study demonstrated that antibody recognition of four specific peptide LC3 epitope is conserved across at least four widely geographically distributed populations, all of which are from highly endemic areas that would benefit from future availability of an efficacious amebiasis subunit vaccine. As the synthetic MAPS vaccine, consisting of peptides encoding these four LC3 epitopes, was effective in an experimental primate model of *E. histolytica* infection and colitis[8], this study provides further evidence supporting the wide utilization of these peptides in a vaccine adapted for and studied in humans.

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