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CRYPTOSPORIDIUM PARVUM AMONG COPROLITES FROM LA CUEVA DE LOS MUERTOS CHIQUITOS (600–800 CE), RIO ZAPE VALLEY, DURANGO, MEXICO

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ABSTRACT: In the present study, 90 coprolites from La Cueva de los Muertos Chiquitos (CMC) were subjected to enzyme-linked immunosorbent assay (ELISA) tests for 3 diarrhea-inducing protozoan parasites, *Entamoeba histolytica*, *Giardia duodenalis*, and *Cryptosporidium parvum*, to determine whether these parasites were present among the people who utilized this cave 1,200–1,400 yr ago. These people, the Loma San Gabriel, developed as a culture out of the Archaic Los Caracoles population and lived throughout much of present-day Durango and Zacatecas in Mexico. The Loma San Gabriel persisted through a mixed subsistence strategy of hunting-gathering and agricultural production. The results of ELISA testing were negative for both *E. histolytica* and *G. duodenalis* across all coprolites. A total of 66/90 (~73% prevalence) coprolites tested positive or likely positive for *C. parvum*. The high prevalence of *C. parvum* among CMC coprolites contributes to our growing knowledge of the pathoecology among the Loma San Gabriel who utilized CMC. Herein, we report the successful recovery of *C. parvum* coproantigens from prehistoric coprolites. The recovery of these coproantigens demonstrates the existence of *C. parvum* in Mesoamerica before European contact in the 1400s.

Using enzyme-linked immunosorbent assays (ELISAs) as a means of examining archaeological materials for evidence of diarrhea-inducing protozoan parasites has become increasingly more routine among archaeoparasitological researchers (Allison et al., 1999; Gonçalves et al., 2002, 2003, 2004, 2005; Le Bailly and Bouchet, 2006; Le Bailly et al., 2006, 2008, 2014; Mitchell et al., 2008; Friás et al., 2013; Yeh et al., 2014, 2015). Much of the existing work has focused on the recovery of protozoan parasite antigens from latrine sediments (Gonçalves et al., 2004; Le Bailly et al., 2008, 2014, Mitchell et al., 2008; Yeh et al., 2014, 2015). In addition to sediments, Allison et al. (1999) used ELISAs to test coprolites from the intestinal tracts of mummies for *Cryptosporidium* sp. and *Giardia* sp. antigens. Another study tested coprolites for *Giardia duodenalis* antigens and found 4/84 to be positive (Gonçalves et al., 2002). Gonçalves et al. (2004) examined 22 coprolites from Brazil, Chile, and Sudan using an ELISA kit for detecting *Entamoeba histolytica* antigens and did not find any positive samples. The present study represents an effort to analyze a large number of coprolites (n = 90) from a single archaeological site for the presence of 3 common diarrhea-inducing species of protozoans.

It is reasonable that the occurrence of these parasites contributed to prehistoric childhood mortality rates in ways similar to their significance in modern human populations of developing countries. Presently, approximately 15–31% of child deaths are attributed to diarrheal diseases, rates that are often higher in less developed nations (Walker et al., 2012; Pop et al., 2014).

Immunodiagnositic methods offer the promise of increased sensitivity and specificity for the detection of diarrhea-inducing protozoan parasites preserved in fecal samples from prehistoric human populations where microscopic evidence of cysts may be lacking because of infrequent shedding or degradation. The present study utilized commercially available ELISA kits for the detection of *E. histolytica*, *G. duodenalis*, and *Cryptosporidium parvum* coproantigens in preserved fecal samples to better understand the pathoecology of parasitism among the Loma San Gabriel people residing in a cave known as La Cueva de los Muertos Chiquitos (CMC). Studies that explore the parasites of these coprolites yield insights into the pathoecology of the Loma San Gabriel who utilized CMC (Jiménez et al., 2012) and provide data for understanding how parasitism may have affected their daily lives.

The Rio Zape Valley, a region approximately 18 km southeast of Guanaceví in Durango, Mexico, is home to several caves and rock shelters utilized by the Loma San Gabriel. The region illustrates a transition zone between the northernmost edge of Mesoamerica and the greater American Southwest (Kelley, 1956, 1971; Brooks and Brooks, 1980). CMC itself was used year round as a temporary habitation by the Loma San Gabriel between 1,200 and 1,400 yr ago (Brooks et al., 1962; Foster, 1986). This cave housed an abundance of botanical artifacts, the skeletons of 7 children (ages several months to 5 yr at the time of death), and nearly 500 coprolites, all sealed beneath adobe floors (Brooks et al., 1962; Brooks and Brooks, 1978, 1980; Phillips, 1989). Previous analyses of coprolites reported excellent preservation of parasitic helminth eggs (Jiménez et al., 2012) and bacterial DNA (Tito et al., 2012). The people who utilized CMC subsisted using a mixed strategy of agricultural production and hunting-gathering, both of which fluctuated seasonally (Brooks et al., 1962; Foster, 1984; Hammerl et al., 2015).

Previous work at CMC focused on helminth infections (Jiménez et al., 2012). From this work we infer that community sanitation was poor by modern standards. Artifacts from CMC (e.g., bones from fish, rodents, and other mammals) demonstrate that there was potential for the transmission of zoonotic parasites. Our understanding of the Loma San Gabriel culture suggests multiple potential pathways for parasite transmission. Studies have shown that those utilizing CMC had living and nutritional behavior patterns that likely perpetuated the life cycles of parasites associated with poor sanitation. As these people modified their environments for food production and took up residence in fecal-contaminated shelters, they inadvertently promoted the transmission of diarrhea-inducing pathogens. The presence of diarrhea is reflected in the morphologies of some CMC coprolites that showed evidence of diarrheal events (Jiménez et al., 2012). Some of these events may have resulted from infections of diarrhea-inducing protozoan parasites (Fig. 1; Table I).

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FIGURE 1. Formed La Cueva de los Muertos Chiquitos (CMC) coprolites CMC-12 (**A**) and CMC-14 (**B**) compared with coprolites CMC-82 (**C**) and CMC-81 (**D**) displaying evidence of diarrheal events. Color version available online.

MATERIALS AND METHODS

Coprolite selection and processing

The CMC coprolites used for the present study were gathered from the materials housed within the Pathoecology Laboratory in the School of Natural Resources at the University of Nebraska-Lincoln in Lincoln, Nebraska (Table I). To prevent modern contamination of material, nitrile gloves were worn throughout processing. A total of 100 coprolites was given a laboratory identification number for analysis and photographed using a Sony Cybershot 18.2 megapixel camera. The sample was diversified by selection of coprolites from every stratum from every grid square. The morphology of each coprolite was inspected. Coprolites that were identifiable as fecal deposits but lacked cylindrical morphology, or otherwise appeared to be the product of loose stool formations before desiccation, were classified as diarrheal events (Fig. 1; Table I). After this inspection, the surfaces of the coprolites were cleaned via surface brushing. Cleaning coprolite surfaces is a necessary step as these coprolites had previously been stored together in communal bags and transported repeatedly, allowing for surface cross-contamination.

Of the cleaned coprolites, 90 were deemed suitable in size for ELISA testing. Subsamples of approximately 1 g were extracted from each of the 90 coprolites. The subsamples were rehydrated in a 0.5% trisodium phosphate solution for 24 hr before being disaggregated, and then screened through a 250-μm mesh. Macroscopic remains (those larger than 250 μm) were dried and stored for future analysis. Microscopic remains (those smaller than 250 μm) were collected into plastic 15-ml screw-cap conical tubes and centrifuged. Pellets from these tubes were used for ELISA testing.

Immunoassay procedures

Aliquots of processed coprolite material were assayed for *E. histolytica, G. duodenalis*, and *C. parvum* antigens using commercially available coproantigen detection kits. ELISA kits were purchased from 2 suppliers: TechLab® (Blacksburg, Virginia) and Epitope Diagnostics, Inc. (San

TABLE I. *Cryptosporidium parvum* enzyme-linked immunosorbent assay results for La Cueva de los Muertos Chiquitos (CMC) coprolites. An asterisk (*) indicates an observed coprolite morphology indicative of a diarrheal event.

Sample ID	OD† Value #1	OD Value #2	Avg. OD	Designation
Positive cutoff	0.2651	0.2574	0.26125	n/a
Negative cutoff	0.2169	0.2106	0.21375	n/a
CMC-1	0.505	0.283	0.394	Positive
CMC-2	0.653	0.311	0.482	Positive
CMC-3	0.106	0.081	0.0935	Negative
CMC-4	0.084	0.117	0.1005	Negative
CMC-5*	0.082	0.079	0.0805	Negative
CMC-6*	0.131	0.099	0.115	Negative
CMC-7	0.386	0.204	0.295	Likely positive
CMC-9*	0.195	0.094	0.1445	Negative
CMC-10	1.314	0.472	0.893	Positive
CMC-11	0.23	0.146	0.188	Likely negative
CMC-12	0.205	0.245	0.225	Equivocal
CMC-13	0.161	0.542	0.3515	Likely positive
CMC-14	0.154	0.374	0.264	Likely positive
CMC-15*	0.168	0.561	0.3645	Likely positive
CMC-16	0.188	0.303	0.2455	Equivocal
CMC-17	0.237	0.331	0.284	Likely positive
CMC-18	0.396	0.423	0.4095	Positive
CMC-19	0.696	1.212	0.954	Positive
CMC-20	0.694	0.46	0.577	Positive
CMC-21*	0.622	0.64	0.631	Positive
CMC-22	0.489	0.525	0.507	Positive
CMC-23	0.263	0.405	0.334	Positive
CMC-24	0.539	0.797	0.668	Positive
CMC-25	0.352	0.652	0.502	Positive
CMC-26	0.495	0.952	0.7235	Positive
CMC-27*	0.165	0.717	0.441	Likely positive
CMC-28	0.84	0.227	0.5335	Likely positive
CMC-29	0.372	0.216	0.294	Likely positive
CMC-30*	0.594	0.25	0.422	Likely positive
CMC-31	0.786	0.488	0.637	Positive
CMC-32*	0.745	0.662	0.7035	Positive
CMC-33*	0.413	0.409	0.411	Positive
CMC-34	0.487	0.525	0.506	Positive
CMC-35*	0.605	1.006	0.8055	Positive
CMC-36	0.196	0.378	0.287	Likely positive
CMC-37	0.141	0.237	0.189	Likely negative
CMC-38	0.552	0.793	0.6725	Positive
CMC-39*	0.259	0.389	0.324	Likely positive
CMC-40*	0.356	0.621	0.4885	Positive
CMC-41	0.293	0.36	0.3265	Positive
CMC-42	0.488	0.692	0.59	Positive
CMC-43	0.243	0.362	0.3025	Likely positive
CMC-45	0.194	0.733	0.4635	Likely positive
CMC-47	0.23	0.599	0.4145	Likely positive
CMC-48*	0.232	0.722	0.477	Likely positive
CMC-49	0.11	0.298	0.204	Likely negative
CMC-50	0.207	0.338	0.2725	Likely positive
CMC-51*	0.722	0.856	0.789	Positive
CMC-52	0.288	0.328	0.308	Positive
CMC-53	0.499	0.349	0.424	Positive
CMC-55*	0.099	0.267	0.183	Likely negative
CMC-56	0.101	0.42	0.2605	Equivocal
CMC-57	0.21	0.624	0.417	Likely positive
CMC-58	0.092	0.204	0.148	Negative
CMC-59	0.084	0.118	0.101	Negative
CMC-60	0.128	0.149	0.1385	Negative
CMC-61	0.176	0.165	0.1705	Negative
CMC-62*	0.14	0.153	0.1465	Negative
CMC-63*	0.362	0.429	0.3955	Positive
CMC-64	0.382	0.568	0.475	Positive
CMC-65*	0.461	0.581	0.521	Positive
CMC-66	0.363	0.644	0.5035	Positive
CMC-67	0.333	0.581	0.457	Positive
CMC-68	0.575	1.221	0.898	Positive
CMC-69	0.488	1.093	0.7905	Positive
CMC-70*	0.687	1.388	1.0375	Positive
CMC-71*	0.435	0.293	0.364	Positive
CMC-72*	0.194	0.169	0.1815	Negative
CMC-73	0.505	0.538	0.5215	Positive
CMC-74	0.394	0.717	0.5555	Positive
CMC-76	0.268	0.559	0.4135	Positive
CMC-77	0.172	0.308	0.24	Equivocal
CMC-78	0.264	0.559	0.4115	Positive
CMC-79	0.265	0.522	0.3935	Positive
CMC-80	0.318	0.671	0.4945	Positive
CMC-81*	0.291	0.414	0.3525	Positive
CMC-82*	0.319	0.804	0.5615	Positive
CMC-84	0.082	0.177	0.1295	Negative
CMC-85*	0.189	0.49	0.3395	Likely positive
CMC-86	0.094	0.204	0.149	Negative
CMC-87	0.261	0.621	0.441	Likely positive
CMC-90	0.278	0.209	0.2435	Equivocal
CMC-92	0.307	1.079	0.693	Positive
CMC-93	0.165	0.309	0.237	Equivocal
CMC-95	0.306	0.801	0.5535	Positive
CMC-96	0.207	0.477	0.342	Likely positive
CMC-97	0.375	0.556	0.4655	Positive
CMC-98	0.434	0.741	0.5875	Positive
CMC-99	0.23	0.394	0.312	Likely positive
CMC-100	0.235	0.285	0.26	Equivocal

† OD, optical density; n/a, not applicable.

Diego, California). The *E. histolytica II* kit from TechLab uses immobilized polyclonal antibodies that bind to *E. histolytica*/*disbar* adhesin and a monoclonal antibody–peroxidase conjugate specific for *E. histolytica* adhesin. This kit has a sensitivity of approximately 0.2–0.5 ng/well of adhesin from *E. histolytica* and does not cross-react with *Ascaris lumbricoides, Blastocystis hominis, Clostridium difficile, Cryptosporidium* sp., *Endolimax* sp., *Entamoeba coli, G. duodenalis,* rotavirus, *Salmonella typhimurium, Shigella sonnei,* or *Trichuris trichura* (http://www.techlab.com).

The fecal *Giardia* antigen ELISA kit from Epitope Diagnostics features microplates coated with highly purified, polyclonal anti-*Giardia* antibodies used to bind *Giardia* antigens. This kit has a sensitivity of approximately 5 ng/ml of *Giardia* antigen and does not cross-react with *C. parvum*, rotavirus, or adenovirus (http://www.epitopediagnostics.com).

The fecal *C. parvum* antigen ELISA kit utilizes a highly purified polyclonal anti-*C. parvum* antibody to bind *C. parvum* antigen. This kit has a sensitivity of approximately 5 ng/ml of *C. parvum* antigen and does not cross-react with *Giardia,* rotavirus, or adenovirus (http://www.epitopediagnostics.com).

The 90 samples were tested for *E. histolytica, G. duodenalis,* and *C. parvum* antigens in duplicate along with positive and negative controls provided by the manufacturers. Aliquots of sediments from the pellets within each centrifuge tube were subjected to ELISA testing following the manufacturer's procedural instructions. A visual inspection was conducted and optical density (OD) values were collected with the aid of an Omega ELISA plate reader.

OD interpretation

The OD readings were used to determine whether a sample was positive or negative following the manufacturers' instructions. For the TechLab kits, the readings of the negative controls were subtracted from the readings of the positive controls and the sample OD values. The resulting OD values were considered positive if they were 0.050 or higher and negative if they were lower than 0.050. For the Epitope Diagnostics kits, positive and negative cutoff values were determined using the OD readings for the positive and negative control wells. As specified by manufacturer, the average OD reading for each plate's negative control wells was used to calculate both the negative and positive cutoff values (positive cutoff = 1.1 × [average negative OD value + 0.10]; negative cutoff = 0.9 × [average negative OD value + 0.10]). The OD reading for each sample was then assessed in light of these cutoff values to determine whether the sample should be considered positive (greater than the positive cutoff), negative (less than the negative cutoff), or equivocal (between the positive and negative cutoffs) (Table I). Average OD values for samples were used when a single sample yielded two different results (e.g., 1 well was positive and the second well was negative or 1 well was positive or negative and the second well was equivocal for the same sample).

The OD values for duplicate sample wells were averaged and compared with the average positive and negative cutoff values calculated for the 4 control wells. The average OD values for controls and samples were used to designate these ambiguous samples as being likely positive (average OD value was positive), likely negative (average OD value was negative), or equivocal (average OD value was equivocal) (Table I).

RESULTS

Visual inspection of the ELISA plates was conducted after the collection of OD values via the ELISA plate reader. Positive/negative assessments were recorded on the basis of color change and later compared with sample designations using OD values. Visual inspection of all *E. histolytica* and *G. duodenalis* kits resulted in negative designations for all samples. Visual inspection assessments of *C. parvum* kits aligned with OD value designations 87.7% of the time (158/180 wells were consistent across methods). During ELISA testing for *C. parvum*, a total of 7 sample wells was designated as equivocal using OD readings. An additional 8 sample wells were visually inconsistent with OD value designations.

Data collected via the ELISA plate reader yielded negative results for both *E. histolytica* and *G. duodenalis* across all test kits. The OD values collected from the *C. parvum* kits yielded 66 positive/likely positive sample designations, 17 negative/likely negative sample designations, and 7 equivocal sample designations (Table I). Of these, 45 positive samples were determined to be positive on the basis of total agreement between both plates (i.e., sample wells for both plates yielded positive OD values). Another 13 samples were in total agreement of a negative designation (i.e., sample wells for both plates yielded negative OD values). Using the average OD values across both wells for each individual sample, 13 samples with a positive or negative reading in 1 well and an equivocal reading in the other were confirmed as being either positive or negative. Average OD values were also used to distinguish samples with 1 negative well and 1 positive well (12/90 samples) as being either likely positive or likely negative. By taking the average, 11 samples were designated as likely positive samples and 1 sample was designated as likely negative. In total, 55 samples were designated as being positive, 11 were designated as likely positive, 16 were designated as negative with an additional sample that was determined to be likely negative, and 7 samples were designated as being equivocal.

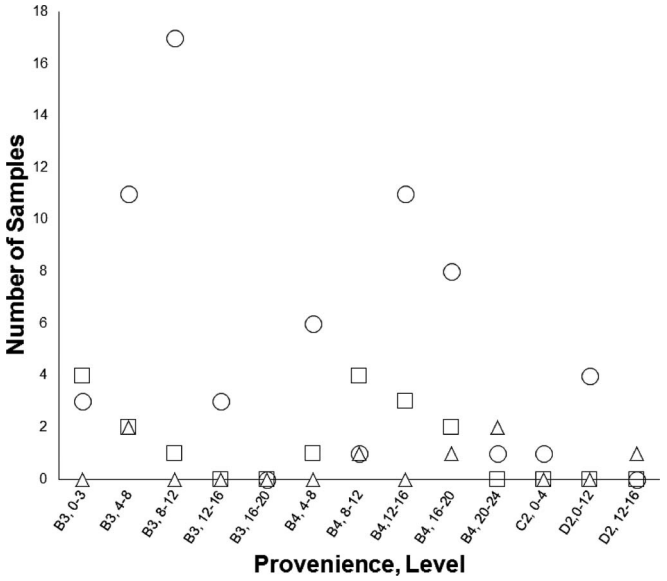

FIGURE 2. *Cryptosporidium parvum* among La Cueva de los Muertos Chiquitos coprolites by archaeological provenience and level. Open circles = positive; open squares = negative; open triangles = equivocal.

After collecting the raw data, samples were assessed according to archaeological collection data (Fig. 2). Samples from 4 proveniences (grid squares B3, B4, C2, and D2) were utilized. Coprolites from provenience B3 (n = 43) were collected from 4 different levels (0–3, 4–8, 8–12, and 12–16 inches below cave surface). Among these samples, 79% were designated as either positive or likely positive. The coprolites from provenience grid square B4 (n = 41) represented material from 5 different levels (4–8,8–12, 12–16,16–20, and 20–24 inches below cave surface) and approximately 66% of these coprolites were designated as either positive or likely positive. The only coprolite from C2 (level 0–4) tested positive, with agreement across both ELISA plates. A total of 5 coprolites was tested from provenience grid square D2 (levels 0–12 and 12–16 inches below cave surface) and 80% of these coprolites were designated as either positive or likely positive.

These data were used to compute the coefficient of determination (r^2) for samples in relation to their archaeological provenience. The r^2 values for positive, negative, and equivocal samples across proveniences and levels were 0.1717, 0.1956, and 0.0122, respectively. These low values indicate that sample positivity was not dependent on archaeological grid square or level below ground surface.

DISCUSSION

ELISA technology has been used to detect evidence of parasitic infection from latrine and burial sediments (Gonçalves et al., 2003, 2004; Le Bailly et al., 2014). However, few studies have successfully detected parasite antigens in coprolites associated with a human population at a single archaeological site (Gonçalves et al., 2002; Ortega and Bonavia, 2003; Yeh et al., 2015) and none has tested so many coprolites (n = 90) from a single archaeological site. Examination of this large sample provides a perspective for greater understanding of parasitism at the population level and the distribution of protozoan infections in the residents of CMC between 600 and 800 yr ago.

The results of this study contribute to our epidemiological understanding of protozoan parasitism within the population using CMC between 600 and 800 CE.

Previous examinations of coprolites have yielded relatively low *C. parvum* coproantigen prevalence. A study by Allison et al. (1999) reported *C. parvum* in 8 of 39 coprolites (20.5% prevalence) from the Andes using ELISA techniques. In a study of 22 coprolites from Peru, only 1 tested positive for *C. parvum*, demonstrating a 4.5% prevalence among the coprolites (Ortega and Bonavia, 2003). In the present study, 55/90 coprolites (61% prevalence) tested positive in both sample wells. Additionally, 11 coprolites were determined to be likely positive on the basis of average OD values. Including both positive and likely positive samples, an approximate prevalence of 73% was observed in the present study. The present study reports the highest prevalence of *C. parvum* ever observed among coprolites from a single archaeological site.

In modern contexts, prevalence values for cryptosporidiosis (the disease caused by infection with *Cryptosporidium* sp.) are often much lower. For instance, a study of modern cryptosporidiosis in India demonstrated only 9.8% prevalence among 591 diarrheal episodes (Ajjampur et al., 2007). In the present study, the high observed prevalence could be attributed to differences in the pathoecology of the Loma San Gabriel 1,300 yr ago as compared with modern populations in which *C. parvum* thrives, but it is equally possible that the present study includes some false-positive samples. False positives could arise from in-context fecal contamination of previously deposited feces as loose stools containing parasite oocysts were deposited. Similarly, coproantigens could have been transferred between fecal deposits within the same or adjacent proveniences over time. To minimize these potential complications, we sampled from as many different proveniences as possible, but it is important to note that some of our positive results could represent false positives.

The presence of *C. parvum* coproantigens among CMC coprolites is not surprising given the parasite's cosmopolitan nature and the ease with which it is spread in unsanitary environments. This parasite is typically spread via oocyst-contaminated water. The outer walls of the oocysts have been shown to be tolerant to chlorination, demonstrating the durability of this parasite. Today, cryptosporidiosis is the leading cause of waterborne illness in the United States, with about 748,000 cases reported annually (Centers for Disease Control and Prevention [CDC], 2015). The oocysts can also be spread via contaminated soil, food, or surfaces. They can also be transferred through contact with infected animals or people and can be autoinfective since the oocysts are sporulated upon excretion. Cryptosporidiosis is often asymptomatic in healthy people, but can cause watery diarrheal events, dehydration, fever, nausea, weight loss, vomiting, and abdominal cramps. Symptoms typically cease after 1–2 wk, but oocysts can continue to be shed in bowel movements for several weeks afterward. In people with weakened immune systems, the parasites can cause other gastrointestinal pathology and in some cases can spread to the respiratory tract (Leitch and He, 2012; CDC, 2015).

Coprolites from CMC used in the present study were selected on the basis of their size and provenience. Assay results and the distribution of coprolites exhibited low r^2 values and indicated that sample positivity was unrelated to horizontal and vertical provenience in the archaeological deposits. We selected coprolites representing as many different stratigraphic levels as possible for the analyses. In testing whether the provenience/level from which a coprolite originated was related to *C. parvum* antigen recovery, the coefficient for determination (r^2) values were low for all samples that were classified as being positive, negative, or equivocal. This shows that there was no significant correlation between collection locality and the presence of *C. parvum*.

Of the coprolites examined in the present study, 24 showed morphological evidence of a diarrheal event (Table I). Because many things can induce diarrhea in humans, such as bacteria, changes in diet, and several intestinal parasites, we decided to conduct both a standard archaeoparasitological analysis and a molecular analysis to test for 3 common diarrhea-inducing protozoan parasites. These samples were negative for both *E. histolytica* and *G. duodenalis*. The standard archaeoparasitological analyses are ongoing and will further elucidate the nature of parasitism among the Loma San Gabriel at CMC. Of the 24 coprolites with evidence of a diarrheal event, 12 of them tested positive for *C. parvum* with total agreement, 6 were likely positive, and 6 were designated as either negative or likely negative. It is likely that *C. parvum* contributed, at least in part, to the diarrheal events observed in the morphologies of 18 CMC coprolites. The 48 remaining coprolites designated as being either positive or likely positive that did not display such morphologies could have been deposited by individuals who were infected but not displaying diarrheal symptoms. In modern cases of cryptosporidiosis, patients generally do not start experiencing symptoms until several days postinfection. Some patients do not experience any symptoms at all. Other patients will retain infective oocysts, despite the cessation of diarrhea, for several weeks. Given these characteristics of modern cryptosporidiosis, it is not surprising that there is not a strong correlation between antigen presence and observed diarrheal morphologies. Additionally, the authors acknowledge that diarrhea can be caused by a great number of disease organisms and by dietary preferences.

The use of immunodiagnostic methods on archaeological materials is not a new concept. In the past, researchers have used both ELISA and immunofluorescent microscopy to identify protozoan parasites in such samples (Frías et al., 2013). Immunofluorescence microscopy is often used in clinical diagnostic settings because it is highly sensitive and specific (CDC, 2015). This technique has been applied to archaeological materials (Faulkner et al., 1989); however, it is subject to false negatives because protozoan cysts can be degraded by taphonomic processes (Gonçalves et al., 2005). Cysts may also not be present in samples from individuals who are infected because of infrequent shedding or being in the early stage of cryptosporidiosis before diarrheal symptoms and cyst shedding begin. These occurrences limit the usefulness of this technique when applied to archaeological materials.

ELISA tests have been shown to be more useful for archaeoparasitological research (Fouant et al., 1982; Allison et al., 1999; Gonçalves et al., 2002, 2003, 2004; Le Bailly and Bouchet, 2006; Le Bailly et al., 2006) than other immunodiagnostic techniques. The ELISA tests for parasite coproantigens are not dependent on the occurrence or microscopic visualization of cysts in fecal samples. This feature makes the use of ELISA excellent for the detection of protozoan parasite coproantigens in coprolites and other archaeological materials. However, because of the destructive nature of these analyses, researchers should

proceed with caution in conducting future ELISA tests of coprolites. We recommend that researchers never process more than 1 g of material from an individual coprolite when conducting ELISA tests.

However, ELISA methods have limitations. Parasite antigens in archaeological materials may be denatured and subject to degradation, with false-negative test results. Likewise, false positives may result from cross-reactivity of shared epitopes between parasite species and other biological residues in archaeological materials. The presence of bacteria and fungi within archaeological materials can compromise the validity of ELISA tests. For this reason, we processed our coprolites and kept the samples refrigerated overnight, then ran our tests over the course of the next 3 days. This prevented the growth of bacterial and fungal colonies and greatly reduced the likelihood that such organisms could have compromised our ELISA results. Despite these limitations, ELISA and other immunodiagnostic methods are effective tools for detection of parasitic infections in archaeological materials.

The pathoecology of cryptosporidiosis among the Loma San Gabriel who utilized CMC approximately 1,300 yr ago is complex. The subsistence patterns of these people consisted of some agricultural production combined with hunting-gathering strategies (Hammerl et al., 2015). This culture lived in a region that represents a transition zone between Mesoamerican and the greater American Southwest from an archaeological perspective (Kelley, 1956, 1971; Brooks and Brooks, 1980; Foster, 1984). They were known to have eaten several fruits and vegetables, both wild and cultivated, which could have easily become contaminated with *C. parvum* oocysts. The major water source near CMC could have easily been contaminated by humans and other animals living in region. Some of the coprolites from CMC contain the eggs of trematodes, which require a molluscan host as part of their life cycle. Eating freshwater gastropods or bivalves from the nearby river could have also been a source of *C. parvum* contamination, as this parasite has been found to be associated with wild molluscs (Miller et al., 2005; Neira et al., 2010). The rocky surfaces of the cave itself could also have become contaminated and acted as a source of infection. The parasites' abilities to be autoinfective could have also helped it to proliferate among the Loma San Gabriel at CMC.

Because the remains of children predominated the skeletal findings of CMC, the recovery of *C. parvum* antigens is especially interesting. Cryptosporidiosis most adversely affects immunocompromised adults and young children (CDC, 2015). It is unknown how many positive samples were associated with children buried in the cave, but the high prevalence may be informative of premature mortality and overall morbidity of the Loma San Gabriel who utilized CMC.

The present study represents the largest sample size reported to date for ELISA analysis of coprolites from a single archaeological site. The recovery of *C. parvum* coproantigens provides new information regarding the diversity of parasites infecting those who utilized CMC in prehistoric Mesoamerica. These data, along with previous reports of this parasite among coprolites from other New World sites (Allison et al., 1999; Ortega and Bonavia, 2003), are important for beginning to understand the paleoepidemiology of this parasite and its pathoecological role in the prehistoric Americas. Examinations of archaeological materials from Old World sites have not yielded positive results for *C. parvum* to date. This incongruence could be a product of differential antigen preservation among the collection sites or of the lower sample sizes available for ELISA examination.

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LITERATURE CITED

Ajjampur, S. S. R., B. P. Gladstone, D. Selvapandian, J. P. Muliyil, H. Ward, and G. Kang. 2007. Molecular and spatial epidemiology of cryptosporidiosis in children in a semiurban community in south India. Journal of Clinical Microbiology **45:** 915–920.

Allison, M. J., T. Bergman, and E. Gerszten. 1999. Further studies on fecal parasites in antiquity. American Journal of Clinical Pathology **112:** 605–609.

Brooks, R. H., L. Kaplan, H. C. Cutler, and T. W. Whitaker. 1962. Plant material from a cave on the Rio Zape, Durango, Mexico. American Antiquity **27:** 356–369.

Brooks, S. T., and R. H. Brooks. 1978. Paleoepidemiology as a possible interpretation of multiple child burials near Zape Chico, Durango, Mexico. *In* Across the Chichimec Sea: Papers in honor of J. Charles Kelley, J. C. Kelley, C. L. Riley, and B. C. Hedrick (eds.). Southern Illinois University Press, Carbondale, Illinois, p. 96–101.

Brooks, S. T., and R. H. Brooks. 1980. Cranial deformation: Possible evidence of Pochteca trading movements. Transactions of the Illinois State Academy of Sciences **72:** 1–12.

Centers for Disease Control and Prevention (CDC). 2015. Parasites—*Cryptosporidium* (also known as "Crypto"). Available at: http://www.cdc.gov/parasites/crypto/. Accessed 19 October 2015.

Faulkner, C. T., S. Patton, and S. S. Johnson. 1989. Prehistoric parasitism in Tennessee: Evidence from the analysis of desiccated fecal material collected from Big Bone Cave, Van Buren County, Tennessee. Journal of Parasitology **75:** 461–463.

Foster, M. S. 1984. Loma San Gabriel subsistence patterns: A preliminary discussion. Anthropology **8:** 13–30.

Foster, M. S. 1986. The Weicker site: A Loma San Gabriel hamlet in Durango, Mexico. Journal of Field Archaeology **13:** 7–19.

Fouant, M. M., M. Allison, E. Gerszten, and G. Focacci. 1982. Parasitos intestinales entre los indigenas precolombinos. Chungara **9:** 285–299.

Frías, L., D. Leles, and A. Araújo. 2013. Studies on protozoa in ancient remains—A review. Memórias do Instituto Oswaldo Cruz **108:** 1–12.

Gonçalves, M. L. C., A. Araújo, R. Duarte, J. P. da Silva, K. J. Reinhard, F. Bouchet, and L. F. Ferreira. 2002. Detection of *Giardia duodenalis* antigen in coprolites using a commercially available enzyme-linked immunosorbent assay. Transactions of the Royal Society of Tropical Medicine and Hygiene **96:** 640–643.

Gonçalves, M. L. C., A. Araújo, and L. F. Ferreira. 2003. Human intestinal parasites in the past: New findings and a review. Memorias do Instituto do Oswaldo Cruz **98**(Suppl. 1)**:** 103–118.

Gonçalves, M. L. C., V. L. da Silva, C. M. de Andrade, K. Reinhard, G. C. da Rocha, M. Le Bailly, F. Bouchet, L. F. Ferreira, and A. Araújo. 2004. Amoebiasis distribution in the past: First steps using an immunoassay technique. Transactions of the Royal Society of Tropical Medicine and Hygiene **98:** 88–91.

Gonçalves, M. L. C., C. Schnell, L. Sianto, F. Bouchet, M. Le Bailly, K. Reinhard, L. F. Ferreira, and A. Araújo. 2005. Protozoan infection in archaeological material. Journal of Biological Research **80:** 146–148.

Hammerl, E. E., M. A. Baier, and K. J. Reinhard. 2015. Agave chewing and dental wear: Evidence from quids. PLoS One **10:** e0133710. doi: 10.1371/journal.pone.0133710.

Jiménez, F. A., S. L. Gardner, A. Araújo, M. H. Fugassa, R. H. Brooks, E. Rácz, and K. J. Reinhard. 2012. Zoonotic and human parasites of inhabitants of Cueva de Los Muertos Chiquitos, Rio Zape Valley, Durango, Mexico. Journal of Parasitology **98:** 304–309.

KELLEY, J. C. 1956. Settlement patterns in North-Central Mexico. *In* Prehistoric settlement patterns in the New World, G. R. Willey (ed.). Viking Fund Publications in Anthropology, New York, New York, p. 128–129.

KELLEY, J. C. 1971. Archaeology of the northern frontier: Zacatecas and Durango. *In* Handbook of Middle American Indians, Vol. 11. Archaeology of Northern Mesoamerica, Part 2, G. F. Ekholm and I. Bernal (eds.). University of Texas Press, Austin, Texas, p. 768–801.

LE BAILLY, M., AND F. BOUCHET. 2006. Paléoparasitologie et immunologie: l'Exemple d'*Entamoeba histolytica*. Revue Archéométrie **30:** 129–135.

LE BAILLY, M., M. L. C. GONÇALVES, S. HARTER-LAILHEUGUE, F. PRODÉO, A. ARAÚJO, AND F. BOUCHET. 2008. New finding of *Giardia intestinalis* (Eukaryote, Metamonad) in Old World archaeological site using immunofluorescence and enzyme-linked immunosorbent assays. Memórias do Instituto Oswaldo Cruz **103:** 298–300.

LE BAILLY, M., M. L. C. GONÇALVES, C. LEFÈVRE, D. ROPER, J. W. PYE, A. ARAÚJO, AND F. BOUCHET. 2006. Parasitism in Kansas in the 1800s—A glimpse to the past through the analysis of grave sediments from Meadowlark cemetery. Memórias do Instituto Oswaldo Cruz **101:** 53–56.

LE BAILLY, M., TH. ROMON, AND S. KACKI. 2014. New evidence of *Entamoeba histolytica* infections in pre-Columbian and colonial cemeteries in the Caribbean. Journal of Parasitology **100:** 684–686.

LEITCH, G. J., AND Q. HE. 2012. Cryptosporidiosis—An overview. Journal of Biomedical Research **25:** 1–16.

MILLER, W. A., E. R. ATWILL, I. A. GARDNER, M. A. MILLER, H. M. FRITZ, R. P. HEDRICK, A. C. MELLI, N. M. BARNES, AND P. A. CONRAD. 2005. Clams (*Corbicula fluminea*) as bioindicators of fecal contamination with *Cryptosporidium* and *Giardia* spp. in freshwater ecosystems in California. International Journal for Parasitology **35:** 673–684.

MITCHELL, P. D., E. STERN, AND Y. TEPPER. 2008. Dysentery in the crusader kingdom of Jerusalem: An ELISA analysis of two medieval latrines in the city of Acre (Israel). Journal of Archaeological Science **35:** 1849–1853.

NEIRA, O. P., S. N. MUÑOZ, V. B. STANLEY, C. M. GOSH, AND L. M. J. ROSALES. 2010. *Cryptosporidium parvum* in wild gastropods as bioindicators of fecal contamination in terrestrial ecosystems. Revista. Chilena Infectología **27:** 211–218.

ORTEGA, Y. R., AND D. BONAVIA. 2003. *Cryptosporidium*, *Giardia* and *Cyclospora* in ancient Peruvians. Journal of Parasitology **89:** 635–636.

PHILLIPS JR., D. A. 1989. Prehistory of Chihuahua and Sonora, Mexico. Journal of World Prehistory **3:** 373–401.

POP, M., A. W. WALKER, J. PAULSON, B. LINDSAY, M. ANTONIO, M. A. HOSSAIN, J. OUNDO, B. TAMBOURA, V. MAI, I. ASTROVSKAYA, ET AL. 2014. Diarrhea in young children from low-income countries leads to large-scale alterations in intestinal microbiota composition. Genome Biology **15:** R76.

TITO, R. Y., D. KNIGHTS, J. METCALF, A. J. OBREGON-TITO, L. CLEELAND, F. NAJAR, B. ROE, K. REINHARD, K. SOBOLIK, S. BELKNAP, ET AL. 2012. Insights from characterizing extinct human gut microbiomes. PLoS ONE **7:** e51146.

WALKER, C. L. F., J. PERIN, M. J. ARYEE, C. BOSCHI-PINTO, AND R. E. BLACK. 2012. Diarrhea incidence in low- and middle-income countries in 1990 and 2010: A systematic review. BMC Public Health **12:** 220.

YEH, H. Y., A. PLUSKOWSKI, U. KALĒJS, AND P. D. MITCHELL. 2014. Intestinal parasites in a mid-14th century latrine from Riga, Latvia: Fish tapeworm and the consumption of uncooked fish in the medieval eastern Baltic region. Journal of Archaeological Science **49:** 83–89.

YEH, H. Y., K. PRAG, C. CLAMER, J. B. HUMBERT, AND P. D. MITCHELL. 2015. Human intestinal parasites from a Mamluk Period cesspool in the Christian quarter of Jerusalem: Potential indicators of long distance travel in the 15th century AD. International Journal of Paleopathology **9:** 69–75.