PARASITIC EGGS IN ANCIENT COPROLITES FROM ARCHEOLOGICAL SITES IN PUERTO RICO

by

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ABSTRACT

Parasites have accompanied the human species since its origin and through their migrations around the world. Evidence of ancient parasitic diseases has been recovered from ancient human remains since 1910. The present work is the first paleoparasitological study in Puerto Rico and the Caribbean. Coprolites from the Saladoid and Huecoid pre-Columbian cultures recovered from archeological excavations in the municipalities of Guayanilla and Vieques were analyzed. A process of rehydration and spontaneous sedimentation was used with one gram of each of 34 samples. For each coprolite sample ten microscopic slide preparations of 50 μL of sediment and a drop of glycerin were scanned. All parasite eggs and larvae found were measured and photographed. A total of 15 different intestinal parasitic eggs were found. The most common species were: *Ascaris lumbricoides, Trichuris trichiura*, *Enterobius vermicularis* and cestodes. A statistically significant difference was found in richness of parasite species between Saladoid and Huecoid cultures in Vieques. The Jaccard and Whittaker β diversity index showed more similarity between the parasite communities present in the Saladoid culture from both archeological sites. Different stages of hookworm infection were detected in the Saladoid culture from Guayanilla. These findings add evidence for presence of this parasite in pre-Columbian America as suggested by previous investigations and also provide parasitological evidence for current research on human migrations to the New World.

RESUMEN

Los parásitos han acompañado la raza humana desde sus inicios y a través de sus migraciones alrededor del mundo. Evidencia sobre antiguas infecciones parasíticas han sido recuperadas desde 1910 en restos humanos antiguos. En Puerto Rico, este es el primer estudio paleoparasitológico realizado. Los coprolitos analizados corresponden a las culturas Saladoide y Huecoide, recuperados de yacimientos arqueológicos pre-colombinos en los municipios de Guayanilla y Vieques. Se llevó a cabo un proceso de rehidratación y sedimentación espontánea para un gramo de cada uno de los 34 coprolitos analizados. Un total de diez laminilla fueron preparadas por cada muestra con 50 μL de sedimento y una gota de glicerina para ser analizadas bajo el microscopio. Todos los huevos y larvas parasíticas fueron medidos y fotografiados. Se recuperaron un total de 15 huevos parasíticos distintos. Las especies más comunes fueron: *Ascaris lumbricoides*, *Trichuris trichiura*, *Enterobius vermicularis* y céstodos. Se halló diferencia estadística significativa entre la riqueza total de especies de la cultura Saladoide y Huecoide en Vieques. Tanto el índice de Jaccard como el de diversidad β de Whittaker mostraron mayor similaridad entre la comunidad de parásitos presente en la cultura Saladoide de ambos sitios arqueológicos. Se detectaron diferentes estadios de "hookworms" en la cultura Saladoide del yacimiento arqueológico de Guayanilla. Estos resultados proveen evidencia de la infección con éste parásito en la etapa pre-colombina como sugieren investigaciones previas. También proveen evidencia parasitológica para investigaciones actuales sobre las migraciones humanas al Nuevo Mundo.

DEDICATION

I dedicate this work to my family for supporting me during all these years since I started my career till this important moment of my academic life. Especially I dedicate this achievement to my husband the love of my life for being with me every step of the way.

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CHAPTER 1

GENERAL INTRODUCTION AND LITERATURE REVIEW

Scientists from many disciplines search for evidence to explain events of the past as a way to understand the present. For parasitologists, the study of host-parasite relationships has enabled them to discern the constant evolutionary arms race between these organisms. Paleoparasitologists study latrines, soil, coprolites and corpses to learn and understand how parasites affected ancient populations. Paleoparasitology offers tools and gives a unique opportunity to study ancient host-parasite relationships. It has been thought that parasites do not leave fossils but their remains have been found in paleontological sites well preserved in organic material. Paleoparasitologists find eggs and larvae of cestodes, trematodes and nematodes preserved by natural conditions. This type of study could provide evidence of human migration routes and important estimates of parasite prevalence and intensity of infections. Coprolites, desiccated or fossilized feces, can be found in archeological sites during excavations or inside mummified corpses and provide an ideal biological sample for parasitological studies. Paleoparasitology still continues its development and has made great contributions to the knowledge of ancient host-parasite relations, allowing us to understand how these dynamics worked in different populations throughout time.

The study of parasites from ancient materials has been developed for over a century and can be divided into three phases: pioneer, development and refinement of the field (Araújo et al. 1998). The pioneer period is marked by the first study of ancient human parasites published by Sir Marc Armand Ruffer in 1910, describing the eggs of *Schistosoma haematobium* in kidneys of Egyptian mummies. After this first report, additional studies reported parasites from ancient

remains (Helbaek 1958, Pizzi and Schenone 1954, Szidat 1944 and Taylor 1955 cited in Araújo et al. 1998). Another part of the pioneer period involved the development of various flotation techniques to recover parasites from soil and latrine sediments with the use of salts such as zinc sulphate (Pike 1967 cited in Araújo et al. 1998), zinc chloride with hydrochloric acid and sodium chloride (Bouchet et al. 2003, Reinhard et al. 1986). A zinc phosphate modified technique was applied to coprolites by Fry in 1977 (Araújo et al. 1998). Rehydration techniques were proposed during the same period with trisodium phosphate solutions (TSP) (Samuel 1965, Van Cleave and Ross 1947 cited in Araújo et al. 1998). TSP was originally used for rehydration of zoological specimens from museums, such as arthropods (Araújo et al. 1998), eventually the technique was modified for coprolite rehydration by Callen and Cameron in 1960 (Beltrame et al. 2010, Ferreira et al. 1984, Fugassa et al. 2006, Han et al. 2003, Santoro et al. 2003). Cockburn (1967) was the first to state the importance of coprolite analysis in order to describe evolution in a cultural context. At the end of the pioneer period Ferreira (1979) finally named the field Paleoparasitology.

From 1980 until approximately 1997 paleoparasitological studies developed new tools, including quantitative techniques using *Lycopodium* spore tablets to estimate number of eggs present, scanning electron microscopy (SEM), immunology (ELISA) and the most recent polymerase chain reaction (PCR) analyses (Pablo 1989 and 1990 cited in Araújo et al. 1998). From approximately 1998 to the present, paleoparasitological techniques have been refined and the knowledge has expanded throughout the continents (Minvielle et al. 2008, Beltrame et al. 2010, Araújo et al. 2011, Gijón et al. 2010, Lee et al. 2011). The rehydration process with TSP is now considered a standard method for processing coprolite samples. Araújo et al. (1981)

proposed the addition of acetic formalin to rehydrated samples in order to avoid bacterial and fungal proliferation.

Paleoparasitological studies have aided the understanding of diet and health of ancient populations from different cultures. Archeological sites studied for parasites, include Iquique in Chile (Ferreira et al. 1984), caves in Nevada (Lin et al. 1978), a rock shelter in Val Verde, Texas (Sobolik, 1991) among others. These studies have confirmed parasitic infections in humans before Columbus arrived to the New World (Araújo et al. 1985, Gonçalves et al. 2003, Horne 1985, Rick et al. 2002). Ancient parasite eggs of common species have been found in North, South America and other parts of the world but not in the Caribbean (Figure 1.1). Examples of paleoparasitological research are excavations in Minas Gerais, Brazil in which Gonçalves et al. (2003) reported the presence of *Ascaris sp.* and *Trichuris sp.* eggs. Other examples are excavations in San Pedro de Atacama in Chile, where Ferreira et al. 1989 reported *Trichuris sp.* and *Enterobius sp* eggs. Patrucco et al. (1983) reported the presence of *Ascaris sp.*, *Trichuris sp.* and *Enterobius sp.* during the excavations in Huarmey Valley, Peru (Gonçalves et al. 2003). Finally, Enterobius sp. eggs were reported from excavations in Durango, Mexico (Reinhard et al. 1989) and Pie de Palo, Argentina (Zimmerman and Morilla 1983 cited in Gonçalves et al. 2003). Other findings in the field show evidence of important migrations and parasite epidemics (Montenegro et al. 2006, Rick et al. 2002). Creation of evolutionary traces for parasites and knowledge of host-parasite relationships including losses of parasites and acquisition of new infections through time, are some of the contributions of this field to science (Araújo and Ferrerira, 2000, Araújo et al. 1998). Most importantly, knowledge of the spread of parasitic diseases around the world, discovery of significant outbreaks and other events may shed light on

the impact parasites have had on the development of human populations (Johnson et al. 2008, Reinhard and Bryant 2008, Araújo et al. 2008).

Many archeological studies have been conducted around the Caribbean but none has included coprolite analysis. Such a study of Puerto Rico's native people, specifically Saladoid and Huecoid cultures could provide information on their health, intensity of parasitic infection, parasite diversity and changes in abundance and diversity over time. In addition, some habits of these people can be inferred by the types of parasites present. Comparisons with other such studies can indicate the mode of living of these populations. More advanced studies using PCR to detect DNA and immunological studies to detect antibodies could add more specific and detailed data on parasite infection of these people.

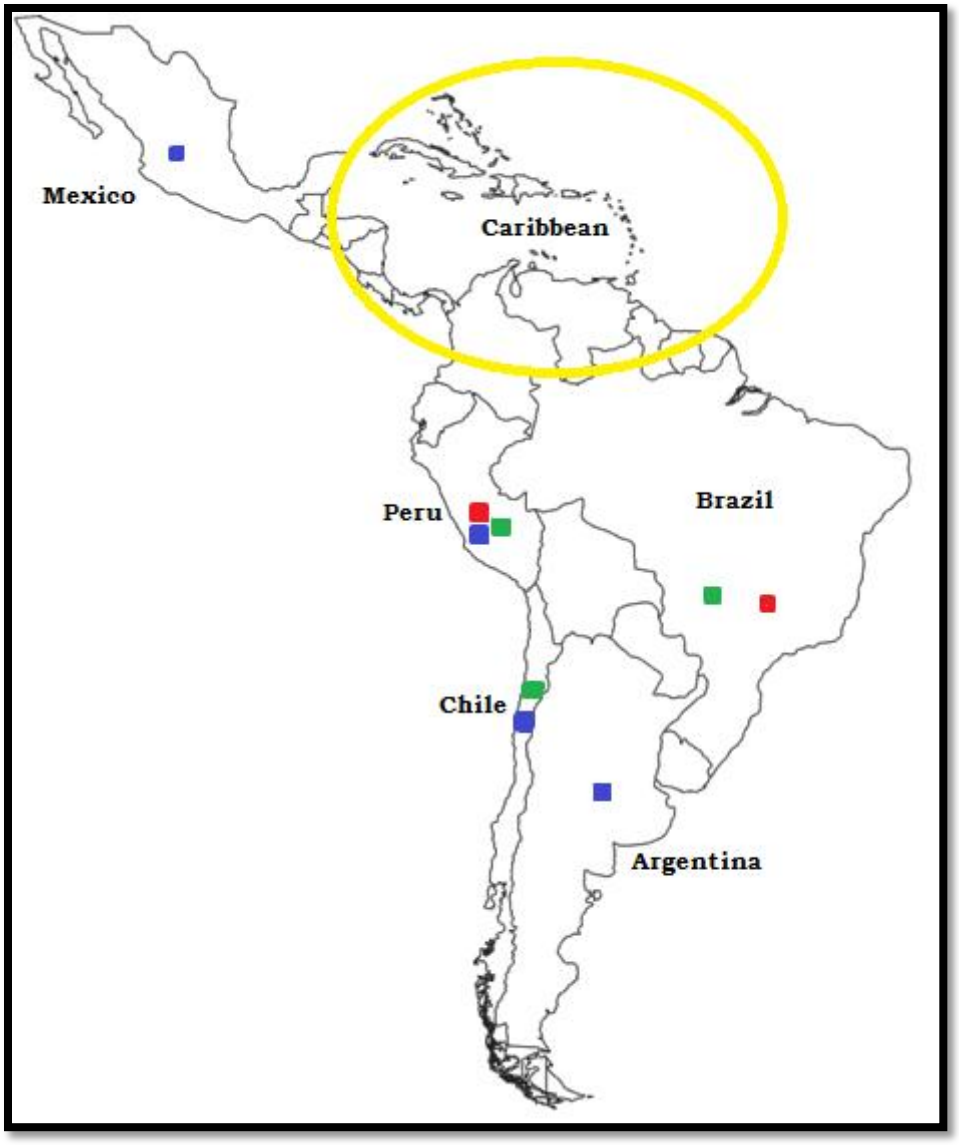

Figure 1.1.Distribution of important findings of *Ascaris sp.*, *Trichuris sp.* and *Enterobius sp.* eggs in ancient material. Red square denotes *Ascaris sp.*, green square *Trichuris sp.* and blue square *Enterobius sp.* eggs. The yellow circle demarks lack of paleoparasitological work on the upper region of South America, Central America and the Caribbean.

Saladoid Culture

The Saladoid culture also known as Igneri was first discovered in Saladero, Venezuela near the Orinoco River. They are described as primarily horticuralists native of South America that migrated to the Caribbean. This culture is characterized by their white on red pottery (Chanlatte and Narganes 2002, Drew 2009). It is believed that they arrived to Puerto Rico in 430 BC and disappeared by approximately 870 AD (Chanlatte and Narganes 2002). Saladoid sites have also been unearthed in all the Lesser Antilles and Dominican Republic and Haiti. In Puerto Rico, coprolites were recovered from this culture during excavations in Vieques and Guayanilla. Samples from Vieques were retrieved from the stratified deposits termed "YTA-2" and radiocarbon dated using charcoal, resulting in an approximate date of 385 to 230 BC. Samples from Guayanilla were retrieved from deposits termed "T-1" and radiocarbon dated using charcoal and shell, resulting in an approximate date of 430 BC to 490 AD (Sued-Badillo 2003).

Huecoid Culture

The Huecoid culture was first described in La Hueca, Vieques by Chanlatte and Narganes (1983). These findings were immediately controversial because they contradicted previous statements made by Rouse in 1999. Rouse believed Huecoids had diverged from Saladoids and named them Huecan Saladoids (Oliver 2009). The elliptic and asymmetric vessels and lithic artifacts found in the excavation clearly identified them as a different culture. Also very distinctive jade amulets resembling Andean condors were found, suggesting an origin for this culture. It is believed that this culture arrived in Puerto Rico around 550 BC and could have remained until 1545 AD according to radiocarbon dating (Chanlatte and Narganes 2005). Coprolites from this culture were recovered from excavations in Sorcé Estate from La Hueca, Vieques. Samples were retrieved from the stratified deposits termed "Z" and radiocarbon dated

using charcoal, shell and wood, indicating an approximate date of 5 AD to1545 AD (Sued-Badillo 2003).

Coprolites as ideal samples for paleoparasitological studies

Coprolites are mineralized or fossilized feces that can be preserved by natural conditions. They can be recovered from caves, archeological sites, latrine sediments and pelvic cavities of mummies. The term coprolite was first used by Buckland in 1829 but originally was used to describe dinosaur feces. Later, Harshberger (1896) pointed to the value of human coprolite analysis for diet reconstruction (Reinhard and Bryant 1992). First reports of parasites in coprolites were reported by Pizzi and Shenone in 1954 (Araújo et al. 1998). Depending on the number of samples analyzed many extrapolations from an individual or a community can be made. Entire diets can be deciphered and concrete epidemiological patterns can be also discovered if there are more than a hundred samples from the same area or archeological site (Reinhard and Bryant 1992). For the present study only 34 coprolites were analyzed, not enough for epidemiological analyses but concrete evidence of parasite presence can be shown.

This thesis will focus on microscopical analysis of coprolites found in two archeological sites in Puerto Rico. This manuscript has been divided into three chapters; the first chapter focuses general information on the cultures studied and the type of sample. The second chapter focuses on the general findings of the thesis research and comparison among the parasites found among the two cultures. The third chapter focuses on the importance of specific parasites in pre-Columbian cultures and how they can provide clues for understanding human migrations. Chapters two and three will be submitted for publication and have been written in article format according to the guidelines for authors of the desired journals.

CHAPTER 2

Archeological sites in Vieques and Guayanilla: Paleoparasitology of Huecoid and Saladoid coprolites from Puerto Rico

INTRODUCTION

The study of ancient parasites, paleoparasitology, seeks to understand what and how parasitic infections affected ancient humans. Parasites have been recovered from archeological sites in bones, latrine sediments, hair and coprolites which are fossilized or mineralized feces (Guichón et al. 2006, Johnson et al. 2008). Ruffer (1910) was the pioneer in the recovery of parasites when he successfully discovered *Schistosoma haematobium* in a mummy's kidney. By 1980 techniques were established for standardized analysis of ancient material (Araújo et al. 1998), including flotation (Reinhard et al. 1986), rehydration (Callen and Cameron 1960) and spontaneous sedimentation (Lutz 1919) of the material analyzed. Loreille et al. (2001) and more recently Gijón et al. (2010) took the analysis of ancient material to the molecular level, reinforcing previous identification and providing a more specific identification methodology. The development of this field gave paleoparasitologists tools to establish common activities of ancient cultures (Beltrame et al. 2010, Santoro et al. 2003, Sardella and Fugassa 2009). In the western hemisphere paleoparasitological studies have been carried out in the United States (Johnson et al. 2008), Mexico (Jiménez et al. 2012), and South America (Gonçalves et al. 2002). No such work has been done in the Caribbean. To our knowledge, this is the first paleoparasitological study in Puerto Rico using coprolites found in archeological sites.

The most common parasites found in archeological material are trematodes, nematodes and cestodes, but in rare cases some protozoa and ectoparasites could be found (Harter et al.

2003, Rick et al. 2002). Presence of parasites, particularly nematodes, could confirm agricultural activities or hunter-gatherer cultures. This study analyzes coprolite material from Huecoid (460 BC-870 AD) and Saladoid (550 BC-1545 AD) cultures collected during archeological excavations in Vieques and Guayanilla, Puerto Rico. These excavations were carried out by Chanlatte and Narganes (2005), archeologists from the University of Puerto Rico at Río Piedras, who made those samples available for this study. Archaeologists have described both of these groups primarily as farming cultures that as a secondary activity practiced hunting. Both of these practices were high risks activities that predisposed the cultures to different kinds of parasitic infections. Many nematodes need humid soils to developed and complete their cycles. Farming soils are ideal places to maintain continuous parasitic infections and re-infections.

The goal of this research is to identify parasites that affected ancient populations from two pre-Columbian cultures from Puerto Rico. Throughout this research the detection of ancient parasites could give clues on how was the lifestyle of ancient populations and their possible origin (Gonçalves et al. 2003). Knowing the parasites that affected humans in the past allows scientists to infer diets (Montenegro et al. 2006), calculate prevalence of parasites (Han et al. 2003), propose possible migrations (Dittmar 2009) and even establish evolutionary traces (Santoro et al. 2003).

MATERIALS AND METHODS

Sample acquisition. A sub-sample of 34 coprolites of probable human origin was provided by Centro de Investigaciones Arqueológicas from the University of Puerto Rico at Río Piedras (Figure 2.1). The samples were collected during excavations in Guayanilla and Sorcé Estate in La Hueca Vieques (Figure 2.2). Both excavations were directed by the archeologists Dr. Luis A. Chanlatte Baik and Yvonne M. Narganes Storde from 1975 to 1987. The archeological site from Guayanilla named "Tecla 1" was identified as the Saladoid culture. In Vieques two different cultures were identified, again the Saladoid culture and also the Huecoid culture was found (Figure 2.3). Dates were established by cultural context and by radiocarbon dating of material associated with the samples (Chanlatte and Narganes 2005, Sued-Badillo 2003).

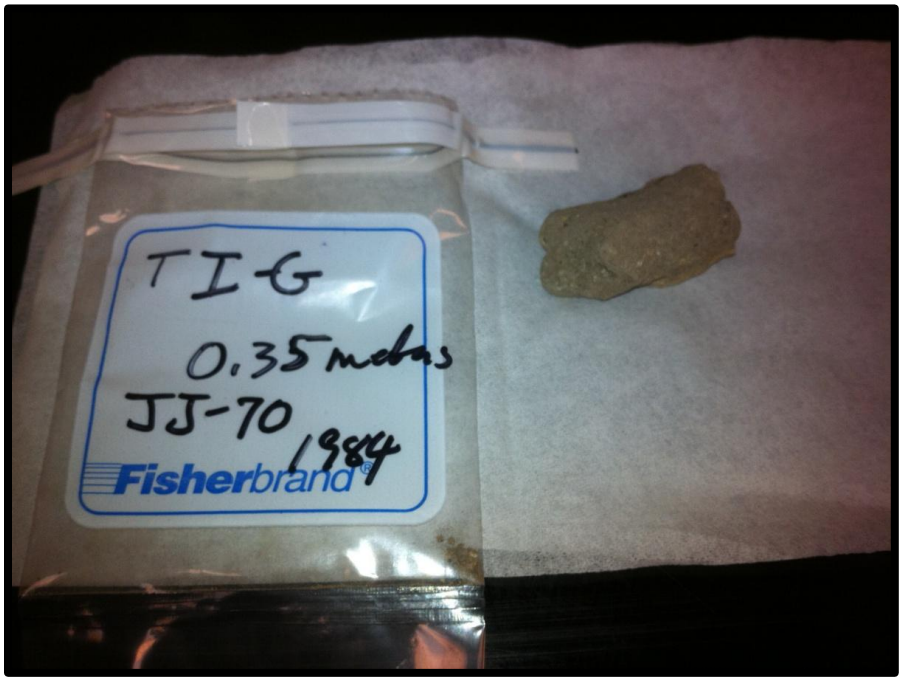

Figure 2.1.Coprolite sample from Saladoid culture from the site of Tecla 1, Guayanilla. Coprolite shape and diameter indicates possible human origin. Each bag containing coprolite was identified by archeological site, depth and plot number. In some cases year of recollection is included (Bag information: Top to bottom).

Figure 2.2.Excavation of the La Hueca site in Sorcé Estate, Vieques. Showing the square deposit plot and diagonal excavation. Used with permission (Chanlatte and Narganes 2005).

Figure 2.3.Map of the Caribbean showing the two sample sites in Puerto Rico. The gray square indicates the location of the archeological site of La Hueca in Vieques and the black square indicates the archeological site Tecla 1 in Guayanilla.

Microscopic analysis. One gram of each coprolite was rehydrated in 14ml of an aqueous solution of trisodium phosphate 0.5% for 72 hrs (Callen and Cameron 1960). Samples were then shaken vigorously, screened through a 1500μm mesh separating all macroscopic material from the sample. To the resultant filtrate, 1ml of 10% acetic formalin solution per 10g of filtrate was added (10:1) to avoid bacterial and fungal growth (Gonçalves et al. 2003). The filtered sample was allowed to settle for 72 hrs (Lutz 1919) after which ten microscope slides were prepared. Using a calibrated micropipette, 50μl of sediment from each sample was deposited on a slide and mixed with a drop of glycerin. A 20x20 cover slip was placed on top and the slide was scanned systematically from left to right (Han et al. 2003, Fugassa et al. 2006). Each parasite eggs and larvae found was photographed and measured at 40x and 60x with a calibrated ocular micrometer. For the lack of a taxonomic key to identify parasite eggs, morphological characters such as projections, shape, and presence of larva inside were used for identification. Other non-parasitic organisms were also photographed.

Statistical analysis. Abundance of parasites found from each culture, species richness, parasite prevalence, percentages of infection and comparisons among the cultures studied will be shown in graphs. Abundance will be defined as the total of parasitic eggs recovered and the richness as the total parasite species. Histograms were performed for the overall data per culture-site combination (Saladoid-Vieques, Saladoid-Guayanilla and Huecoid-Vieques) to evaluate their normality and then proceed with the pertinent analysis. A non-parametric ANOVA was performed in order to evaluate if there was a statistical significant difference (p-value <0.05) among the abundances and total parasite species found in the two cultures from Vieques and between the same cultures in the two different archeological sites (InfoStat 2010). A cluster using the qualitative biodiversity index of Jaccard (Villareal et al. 2006) was performed to

compare the three culture-site combinations from a community approach in terms of parasite species present among them using the Paleontological Statistic Software (PAST 2001). The β diversity Whittaker index (β_w) was used for the same data to evaluate the composition of the groups taking into account exclusive species (Villareal et al. 2006, Anderson et al. 2011). The Whittaker index output gives a result from 0 (maximum similarity) to 1 (minimum similarity) providing a numerical similarity approach (PAST 2001). Finally, a Principal Component Analysis (PCA) was performed using the Harpening's POPSTR variance program to examine the relationship between each of the coprolites positive for parasitic infection and the species present among them. Also the relationship among each culture site and the species among them were examined.

RESULTS

Of the 34 coprolites analyzed, 24 yielded parasite eggs (Table 2.1), 5 of 12 from Huecoid culture from Vieques, 5 of 7 from the Saladoid culture from Vieques and 14 of 15 from the Saladoid culture from Guayanilla. Intestinal parasites found were: *Ascaris lumbricoides* (Figure 2.4), *Trichuris trichiura* (Figure 2.5), *Enterobius vermicularis* (Figure 2.6), *Trichostrongylus sp.*(Figure 2.7), hookworms (Figure 2.8), *Diphyllobothrium sp.*(Figure 2.9), *Dipyllidium caninum* (Figure 2.10), cestodes (Figure 2.11), *Paragonimus westermani* (Figure 2.12), acanthocephalans (Figure 2.13) and four unknown trematodes (Figure 2.14). A mite, a non-parasitic organism was found (Figure 2.15).

Table 2.1.Identification and number of parasite eggs in each positive coprolite sample. Huecoid from Vieques (blue), Saladoid from Vieques (red) and Saladoid from Guayanilla (green).

Coprolite number	*Ascaris sp.*	*Trichuris sp.*	*Enterobius sp.*	*Trichostrongylus sp.*	Hookworm	*Dyphillobothrium sp.*	*Dipylidium sp.*	Cestode	*Paragonimus sp.*	Acanthocephala --	Trematode 1	Trematode 2	Trematode 3	Trematode 4	Total (parasites /each coprolite)
4	56	0	0	0	0	0	0	84	0	0	0	0	0	0	140
7	243	27	27	0	0	0	0	27	0	0	0	0	0	0	324
8	0	0	0	0	0	0	0	0	23	0	0	0	0	0	23
9	57	0	86	0	0	0	0	0	0	0	0	0	0	0	143
10	60	30	60	0	0	0	0	0	0	0	0	0	0	0	150
13	104	0	26	0	26	0	0	0	0	0	0	0	0	0	156
14	52	78	26	26	0	26	0	0	0	0	26	0	0	0	234
15	30	150	60	0	30	0	30	60	0	0	0	0	0	0	360
16	29	29	0	29	0	0	0	57	0	0	0	0	0	0	144
17	0	60	0	30	0	0	0	30	0	0	0	0	0	0	120
20	155	0	0	0	31	0	0	31	0	0	0	0	0	0	217
21	52	0	26	26	26	0	0	26	0	0	0	52	0	0	208
22	0	26	0	0	0	0	0	26	0	0	0	0	0	0	52
23	96	0	32	0	32	0	0	0	0	0	64	0	0	0	224
24	96	0	0	0	0	0	0	0	0	0	0	32	0	0	128
25	56	0	0	0	0	0	0	28	0	0	0	0	0	0	84
26	27	27	27	0	27	0	0	0	0	0	0	0	0	55	163
27	32	0	0	0	0	0	0	0	0	0	0	0	0	0	32
28	95	32	0	0	0	0	0	0	0	0	32	0	0	0	159
29	24	0	24	0	0	0	0	24	0	0	0	0	0	0	72
30	0	0	0	0	0	0	0	0	0	0	0	0	63	0	63
31	58	0	0	0	0	0	0	0	0	0	0	0	114	0	172
33	58	0	0	0	0	0	0	0	0	29	0	0	0	0	87
34	64	95	0	32	32	0	0	32	0	32	0	0	0	32	319
Total (Eggs/species)	1444	554	394	143	204	26	30	425	23	61	122	84	177	87	

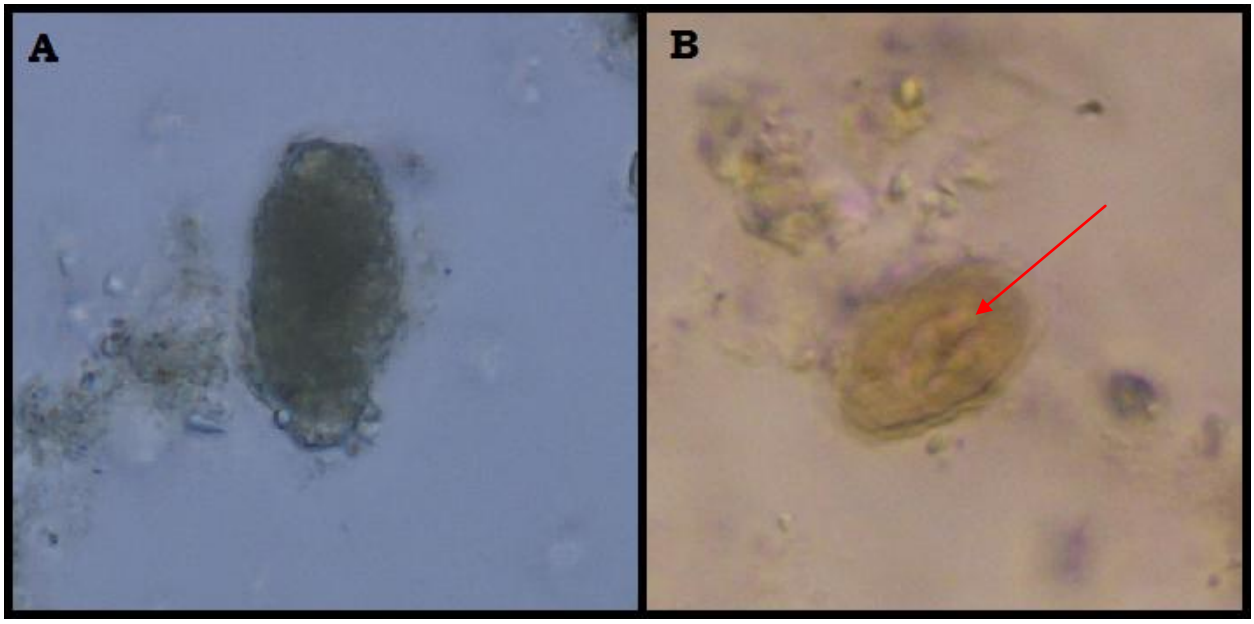

Figure 2.4.*Ascaris lumbricoides* eggs. (A) Unfertilized egg, notice elongated shape with no differentiated organism inside. (B) Fertilized eggs, notice development of a larva inside (red arrow).

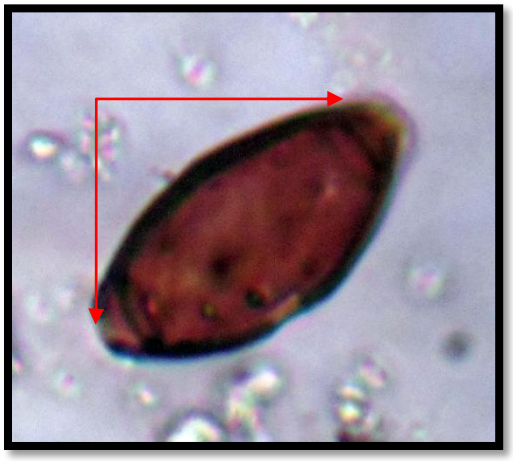

Figure 2.5.*Trichuris trichiura* egg. Well preserved egg, notice the two characteristic polar plugs or operculum (red arrow) of this species.

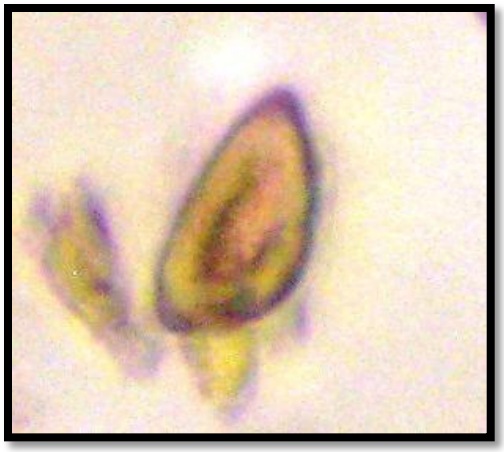

Figure 2.6.*Enterobius vermicularis* egg. Notice the characteristic shape, left side flat and right concave side (Forms like a letter D). Also notice thin walls of this egg.

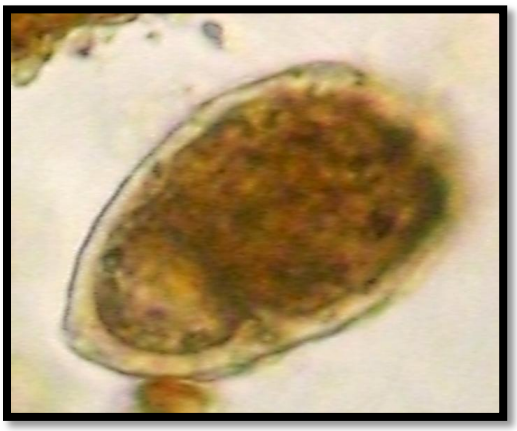

Figure 2.7.*Trichostrongylus sp.* egg. Notice clear thick wall and pointed end to the left, the oval right end characteristic of this egg is not visible due to scarce preservation of that part.

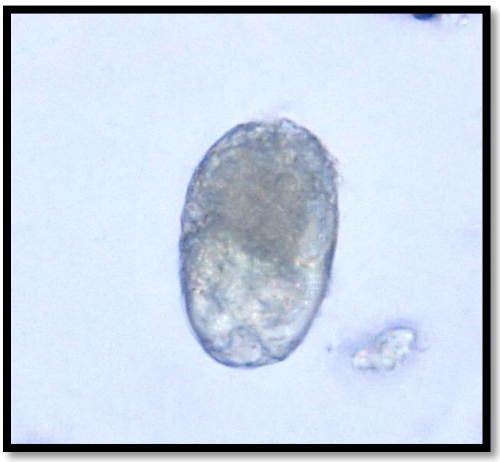

Figure 2.8.Hookworm egg. Notice well preserved wall and shape but with a ruptured bottom end indicating that already hatched.

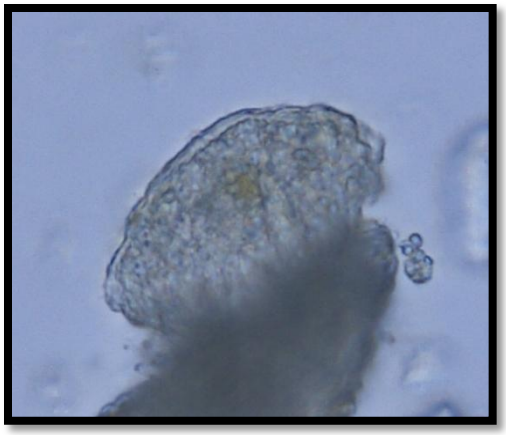

Figure 2.9.*Diphyllobothrium latum* egg. Notice not so well preserved egg with characteristic thin wall and form, top wider (right) than bottom (left).

Figure 3.10.*Dipylidium caninum* egg packet. Notice characteristic egg packet, the wall is not so well preserved but 10 eggs are visible inside.

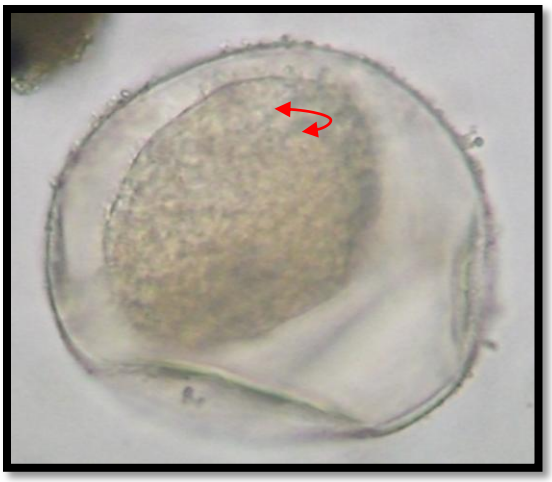

Figure 2.11.Cestode egg. Notice well preserved egg with round oncosphere. Two distinguishable hooks (red arrow) are visible, presumed to be a Hymenolepid egg.

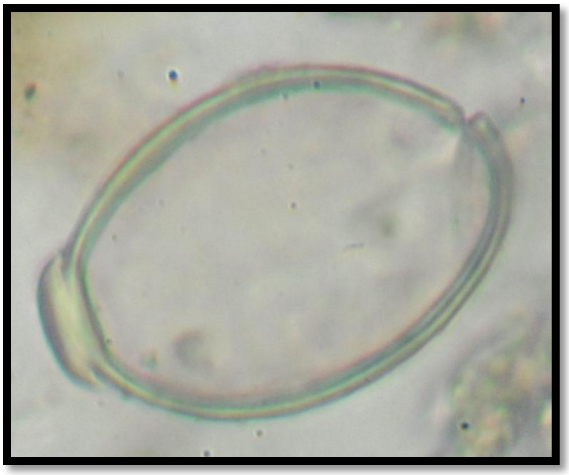

Figure 2.12.*Paragonimus westermani* egg. Notice well preserved empty egg with flattened operculum still attached and broken end.

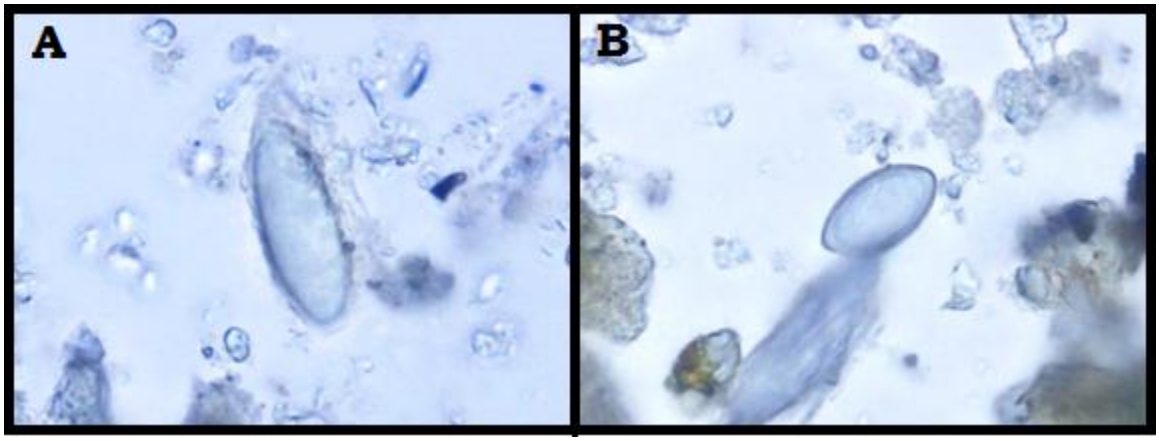

Figure 2.13.Acanthocephalan eggs. (A) Embrionated egg (B) Un-embrionated egg with presence of a vaguely visible acanthor in the inside.

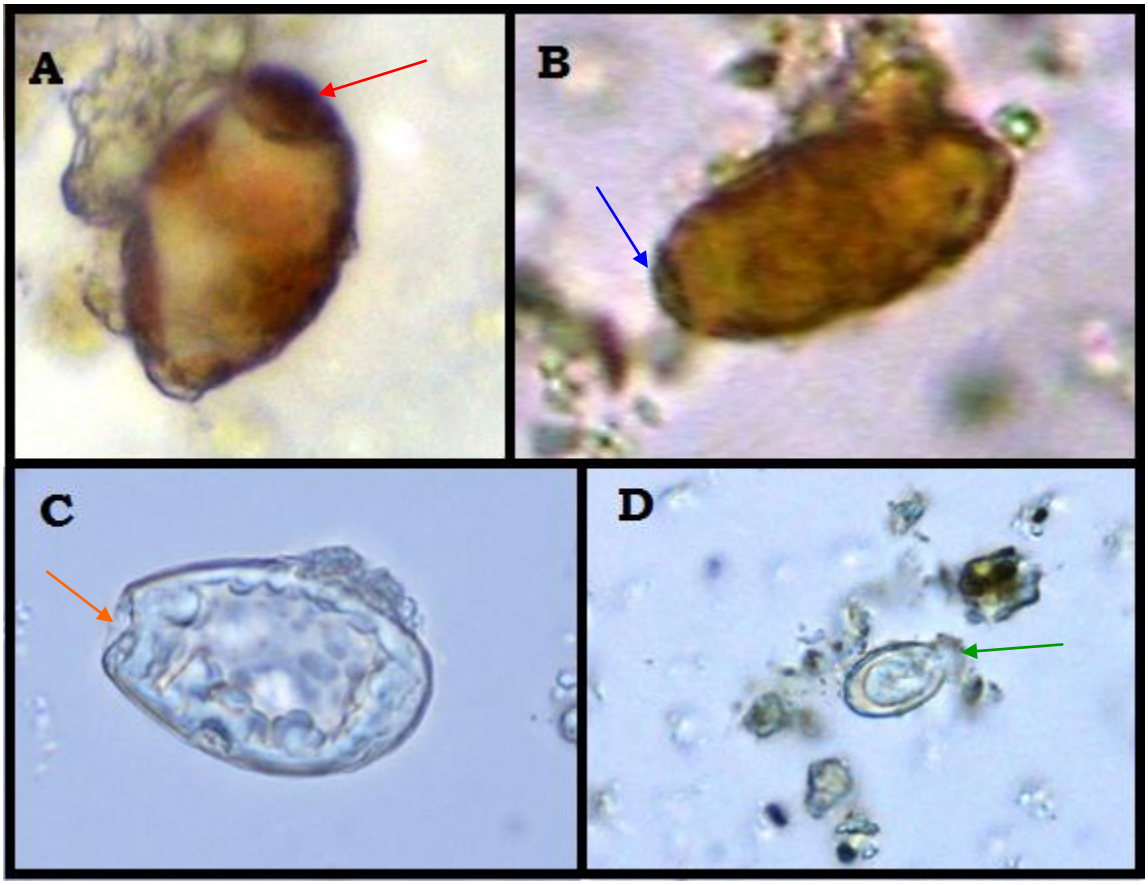

Figure2.14.Unknown trematode eggs. (A) Noticeable operculum (red arrow) and well defined walls. (B) Not so well preserved elongated egg with thin operculum (blue arrow). (C) Well preserved egg with thick walls, notice the absence of the operculum (orange arrow). (D) Oval egg hatching (green arrow).

Figure 2.15.Tarsonemidae mite found in Saladoid coprolite from Guayanilla. Notice most of the legs are still attached and visible.

The measurements for each species of parasite eggs are shown in Table 2.2. Figure 2.16 shows the total number of parasite eggs per gram (intensity) of rehydrated coprolite per culture and site. The prevalence and intensity of each parasite species from each culture and collection site are shown in Figure 2.17 and Table 2.3. The diversity of parasites in each culture and collection site is presented in Table 2.4. Combinations of parasite infections among positive coprolites are shown in Table 2.5.

Table 2.2.Average measurements for parasite eggs found in rehydrated coprolite samples. All measurements were made with a calibrated ocular micrometer.

Parasite	**Length (μm)**	**Width (μm)**
Ascaris lumbricoides (unfertilized)	52 (46-70)[a]	28 (23-45)
Ascaris lumbricoides (fertilized)	45 (33-47)	41 (32-45)
Trichuris trichiura	51 (44-65)	18 (16-27)
Enterobius vermicularis	41 (32-44)	25 (23-32)
Trichostrongylus sp.	57 (55-59)	30 (27-38)
Hookworms	42 (40-55)	28 (25-33)
Diphyllobothrium latum	36 (33-38)	20 (18-27)
Dipylidium caninum	73*	50*
Cestode	44 (32-49)	40 (30-43)
Paragonimus westermani	61*	33*
Acanthocephalans	55 (45-65)	20 (19-30)
Unknown Trematode 1	83 (79-85)	46 (42-56)
Unknown Trematode 2	38 (34-40)	18 (15-27)
Unknown Trematode 3	66 (61-69)	30 (25-33)
Unknown Trematode 4	28 (25-29)	18 (17-22)

(*) Only one egg found ([a]) Maximum and minimum lengths found

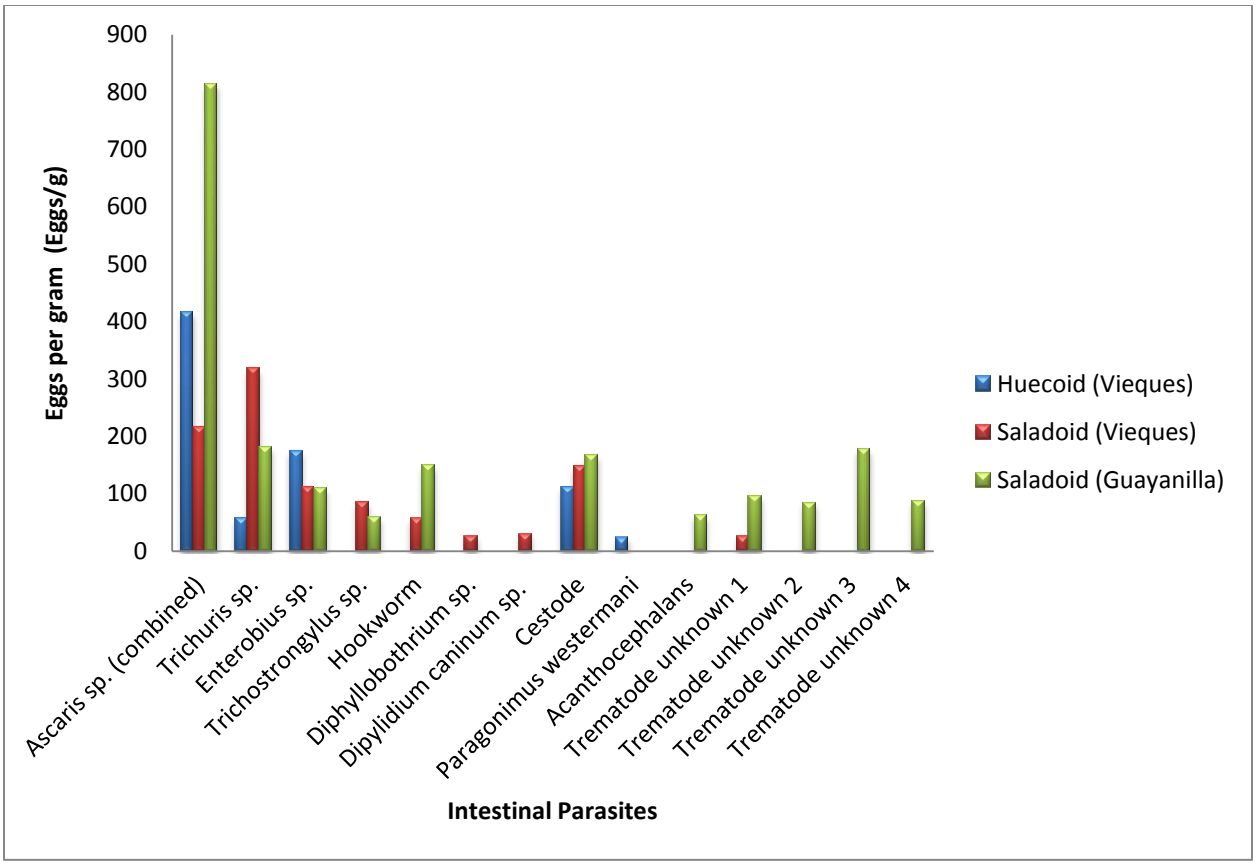

Figure 2.16.Total of parasite eggs estimated per gram of rehydrated coprolite for each species, culture and site.

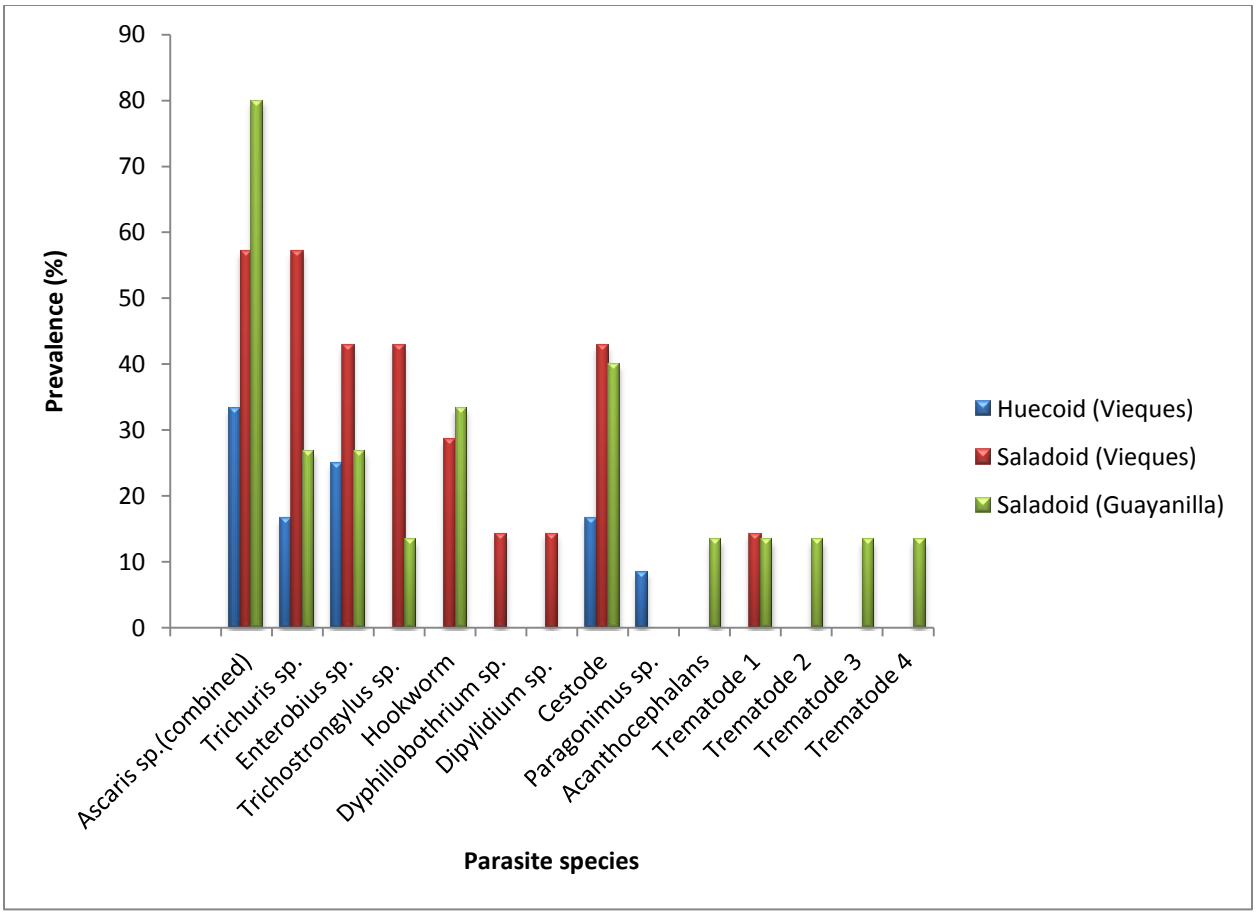

Figure 2.17.Prevalence of infection in each culture/site.

Table 2.3.Prevalence and intensity (expressed as number of eggs per gram) of each parasite species per culture and collection site.

Prevalence and intensity of Infection						
	Huecoid (Vieques) n=12		**Saladoid (Vieques) n=7**		**Saladoid (Guayanilla) n=15**	
Parasite species	**Number infected (%)**	**Average Intensity (EPG) (Range)**	**Number infected (%)**	**Average Intensity (EPG) (Range)**	**Number infected (%)**	**Average Intensity (EPG) (Range)**
***Ascaris lumbricoides* (unfertilized)**	2(17)	56.5 56-57	3(43)	27.0 26-29	9(60)	39.6 24-95
***Ascaris lumbricoides* (fertilized)**	2(17)	151.5 60-243	3(43)	44.7 26-78	8(53)	57.1 28-124
Trichuris trichiura	2(17)	28.5 27-30	4(57)	79.3 29-150	4(27)	48.0 26-95
Enterobius vermicularis	3(25)	57.7 27-86	3(43)	37.33 26-60	4(27)	27.3 24-32
Trichostrongylus sp.	0	0	3(43)	28.3 26-30	2(13)	29.0 26-32
Hookworms	0	0	2(29)	28.0 26-30	5(33)	29.6 26-32
Diphyllobothrium sp.	0	0	1(14)	26	0	0
Dipylidium caninum	0	0	1(14)	30	0	0
Cestode	2(17)	55.5 27-84	3(43)	49.3 30-60	6(40)	27.8 24-32
Paragonimus westermani	1(8)	23	0	0	0	0
Acanthocephalans	0	0	0	0	2(13)	30.5 29-32
Unknown Trematode 1	0	0	1(14)	26.0	2(13)	48.0 32-64
Unknown Trematode 2	0	0	0	0	2(13)	42.0 32-52
Unknown Trematode 3	0	0	0	0	2(13)	88.5 63-114
Unknown Trematode 4	0	0	0	0	2(13)	43.5 32-55
Total infections	12		24		49	
Richness	6		10		12	

Table 2.4.Diversity of parasite infections in each culture and site.

Culture	Number of parasite species per individual coprolite									
	1	2	3	4	5	6	7	8	Total infections	Richness Index
HV 5 of 12 infected (46.6%)	1	2	1	1					12	1.0
SV 5of 7 infected (71.4%)			1	2		1	1		24	3.4
SG 14 of 15 infected (93%)	2	3	4	1	2	1		1	48	3.2

HV-Huecoid Vieques SV-Saladoid Vieques SG-Saladoid Guayanilla

Table 2.5.Number of coprolites infected and combinations of parasite infections.

Num. of coprolites infected	**20**	**10**	**10**	***5***	**7**	**1**	**1**	**11**	**1**	**2**	**3**	**2**	**2**	**2**
Parasites species	***Ascaris sp.* (combined)**	***Trichuris sp.***	***Enterobius sp.***	***Trichostrongylus sp.***	**Hookworm**	***Dyphillobothrium sp.***	***Dipylidium sp.***	**Cestodes**	***Paragonimus sp.***	**Acanthocephalans**	**Trematode 1**	**Trematode 2**	**Trematode 3**	**Trematode 4**
***Ascaris sp.* (combined)**		8	10	4	7	1	1	9	0	2	3	2	1	2
Trichuris sp.			5	4	3	1	1	6	0	1	2	0	0	2
Enterobius sp.				2	5	1	1	4	0	0	2	1	0	1
Trichostrongylus sp.					2	1	0	4	0	1	1	1	0	1
Hookworm						0	1	4	0	1	1	1	0	2
Dyphillobothrium sp.							0	0	0	0	1	0	0	0
Dipylidum sp.								1	0	0	0	0	0	0
Cestodes									0	1	0	1	0	1
Paragonimus sp.										0	0	0	0	0
Acanthocephalans											0	0	0	1
Trematode 1												0	0	0
Trematode 2													0	0
Trematode 3														0
Trematode 4														

Parasite species richness was higher in the Saladoid culture with the Vieques samples showing a non-statistically significantly higher species richness than the Guayanilla material. The species richness of the Huecoid material was much less than the Saladoids. Of the 12 Huecoid samples only five were positive for any parasite infection (47%). The Saladoids had 71% and 93% infection rates from Vieques and Guayanilla respectively.

The most commonly found eggs were *Ascaris sp.* with 20 of the 34 samples, followed by cestodes with 12 of 34 and *Trichuris sp.* and *Enterobius sp.* with 10 infections each. Hookworm eggs were found in seven of the 34 samples. Only four of the parasites found were common to all three culture/sites including *Ascaris sp.*, *Trichuris sp.*, *Enterobius sp.* and cestodes. *Ascaris sp.* was the most prevalent in all culture/sites, however the prevalence in the Huecoid culture was much lower than in the other two and in Saladoids from Vieques were less prevalent than in Saladoids from Guayanilla. *Trichuris sp.* had the opposite relationship among the Saladoid sites with higher prevalence in the Huecoids from Vieques as compared with the Saladoids from Guayanilla but the Huecoid prevalence was again the lowest. *Enterobius sp.* had the same relative relationships as *Trichuris sp.* among the culture/sites. Saladoid infections by cestodes were similar among the two sites but again the Huecoid was much lower. *Trichostrongylus sp.* was present only in the Saladoid with a much higher prevalence in the Vieques site. Hookworms were again only found in the Saladoid with similar prevalence in both sites as was also seen with Trematode 1. Parasites that occurred in only a single site included *Diphyllobothrium sp.* and *Dipylidium sp.* in the Saladoids from Vieques; Acanthocephala, and trematodes 2,3 and 4 only in the Saladoids from Guayanilla. Only one single infection of *Paragonimus sp.* was found in the Huecoid.

Infection intensities were estimated by the number of eggs per gram (EPG) of coprolite material. The highest intensities were seen in *Ascaris sp.* with an overall EPG of 72.2 with a range of 24 to 243 EPG. The Huecoids had the highest intensities with an average EPG of 104.0 (range 56-243) followed by the Saladoids from Guayanilla with 67.7 EPG (24-155) with the Saladoids from Vieques having a slightly lower EPG of 53.7 (29-104). The next highest intensities were seen in *Trichuris sp.* with an average intensity of 55.4 EPG (range 26-150). The Saladoid of Vieques had the highest average intensity with 79.3 EPG (29-150) followed by the Saladoid of Guayanilla with average 48 EPG (26-95). The Huecoid had the lowest intensities of this parasite with average 28.5 EPG (27-30). Of the 10 samples infected with *Enterobius sp.* the average intensity was 39.4 EPG (24-86) with the Huecoid having the highest average intensity of 57.7 EPG (27-86) followed by the Saladoid from Vieques (average 37.7 EPG, range 26-60) and the Saladoid from Guayanilla (27.3 EPG range 24-32). The cestodes have the next highest infection intensity; the Huecoid with the highest intensity of 55.5 EPG (27-84) followed by the Saladoid from Vieques with 50.7 EPG (26-60) then the Saladoid from Guayanilla with average 27.8 EPG (24-32).

The two most common combinations of organisms occurred with *Ascaris sp.* and *Enterobius sp.* infections which occurred together in all of the 10 *Enterobius sp.* infections. The next most common association occurred with the combined cestodes where 10 of 12 cestode infections also occurred with *Ascaris sp.* infections. *Trichuris sp.* also had a strong combination with *Ascaris sp.* with eight of 10 *Trichuris sp.* infections occurring with *Ascaris sp.* Of the 12 cestode infections 10 occurred with *Ascaris sp.* but there was a negative association of trematodes with the combined cestodes with only two of 12 trematode infections found with cestodes. The *Paragonimus sp.* infection occurred with no other organism. All hookworm

infections occurred with *Ascaris sp.* and there was a strong relationship with *Enterobius sp.* infections where five of the seven hookworm infections occurred with this organism. Histograms for Saladoid culture in Guayanilla and in Vieques showed a normal distribution of parasite species present (Figure 2.17-2.18) but it was not the case for the Huecoid culture in Vieques (Figure 2.19). For this particular reason and the fact that the sample sizes were not equal for the three culture-site combinations (SG-SV-HV), a non-parametric ANOVA was performed to be more conservative. The non-parametric ANOVA for abundance between SG and SV was not statistically significant with a p-value of 0.8043 (Table 2.6). For the abundance comparison between SV and HV the result was again not statistically significant with a p-value of 0.1537 (Table 2.7). In terms of total parasite species richness, the comparison between SG and SV was not statistical significant with a p-value of 0.7215 (Table 2.8). Finally, the total parasite species richness comparison between SV and HV was statistically significant with a p-value of 0.0448 (Table 2.9). The cluster using Jaccard qualitative biodiversity index showed that the parasite community of the Saladoid culture in Vieques is approximately 58% more similar to the same culture in Guayanilla than to the Huecoid culture in the same site (Vieques) (Figure 2.21). The β diversity Whittaker index (β_W) showed a similarity of 0.2727 between SV and SG. Between SV and HV the similarity was 0.375 and for SG and HV was 0.4444. The PCA for positive coprolites and the species present, showed that most of them have a similar distribution pattern with the first two components representing 38% of the total variance. For the culture/site combination and the species present the patterns are very different from each other but a slight similarity can be observed between Huecoids and Saladoids from Vieques (Figure 2.22). All the variance for the culture/site combinations is represented by the first two components.

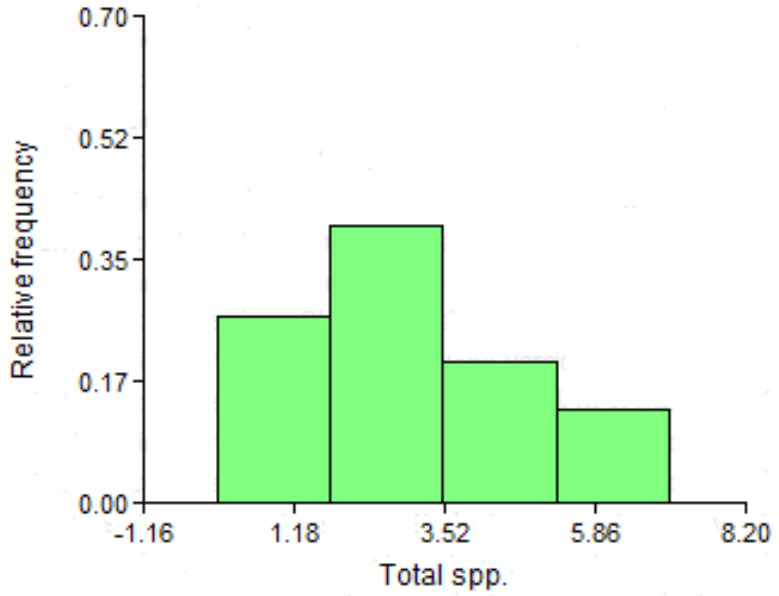

Figure 2.18.Histogram of total species per coprolite found from Saladoid culture in Guayanilla.

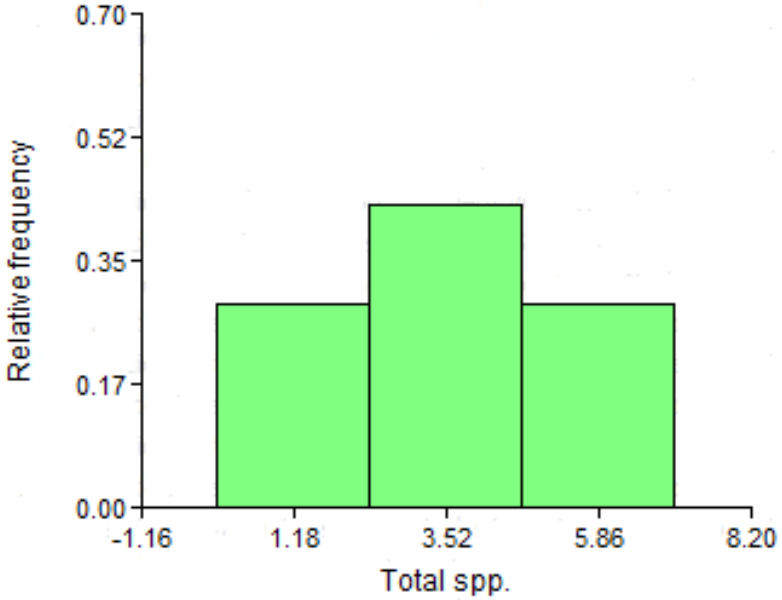

Figure 2.19 Histogram of total species per coprolite found from Saladoid culture in Vieques.

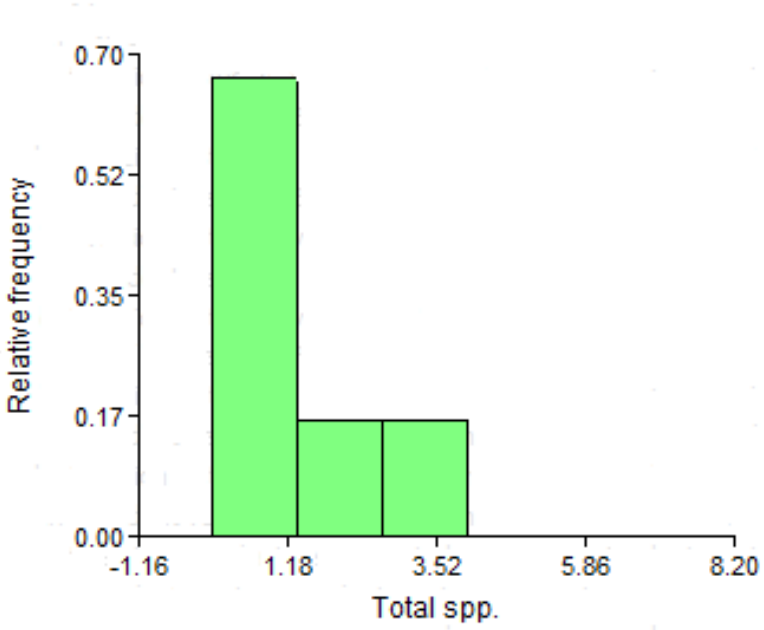

Figure 2.20. Histogram of total species per coprolite found from Huecoid culture in Vieques.

Table 2.6.Non-parametric ANOVA comparison of egg abundances between the Saladoid culture.

Variable	Site	N	Means	S.D.	Medians	H	p
Abundance	SG	15	8.67	5.49	8.00	0.08	0.7769
Abundance	SV	7	10.29	8.83	10.00		

Table 2.7.Non-parametric ANOVA comparison of egg abundances between the Saladoid and Huecoid cultures.

Variable	Site	N	Means	S.D.	Medians	H	p
Abundance	HV	12	4.67	7.50	0.00	1.83	0.1537
Abundance	SV	7	10.29	8.83	10.00		

Table 2.8.Non-parametric ANOVA comparison between species richness between the Saladoid culture.

Variable	Site	N	Means	S.D.	Medians	H	p
Total spp.	SG	15	3.07	2.02	3.00	0.15	0.6953
Total spp.	SV	7	3.43	2.70	4.00		

Table 2.9.Non-parametric ANOVA comparison between species richnessbetween the Saladoid and Huecoid cultures.

Variable	Site	N	Means	S.D.	Medians	H	p
Total spp.	HV	12	1.00	1.41	0.00	3.62	0.0448
Total spp.	SV	7	3.43	2.70	4.00		

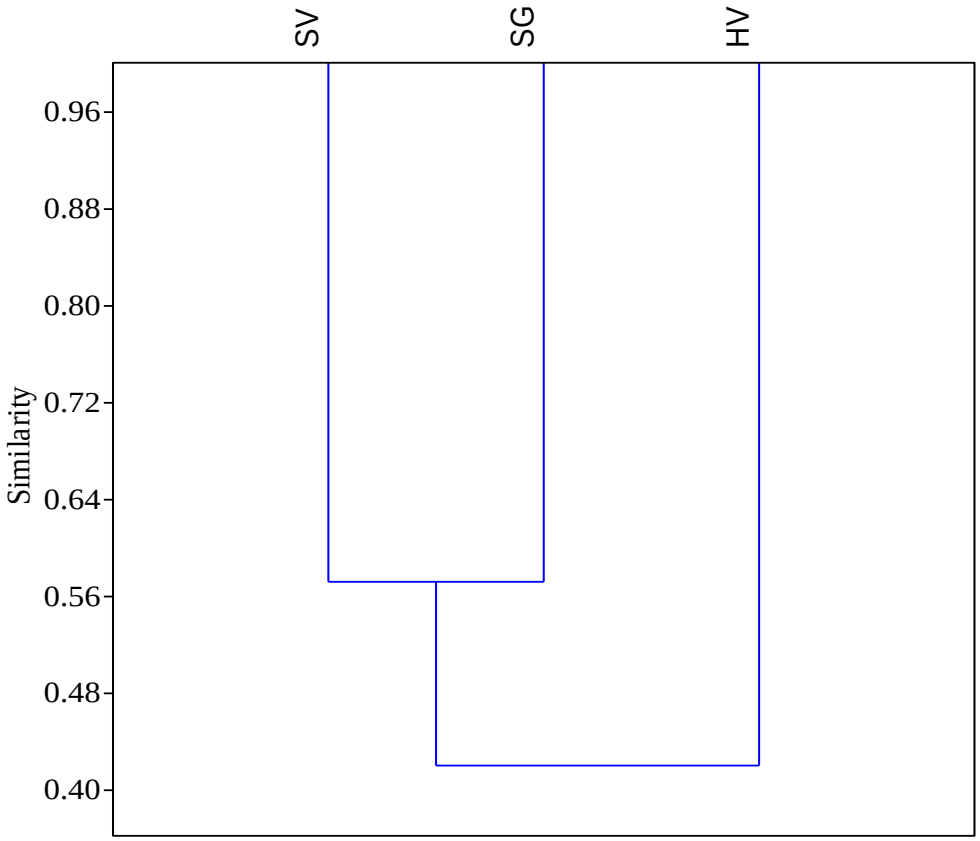

Figure 2.21.Similarity cluster with Jaccard index. Notice more similarity between SV and SG. A matrix of presence and absence of parasite species was performed in order to use this index. The used abbreviations are: SV-Saladoid culture in Vieques, SG-Saladoid culture in Guayanilla and HV-Huecoid culture in Vieques.

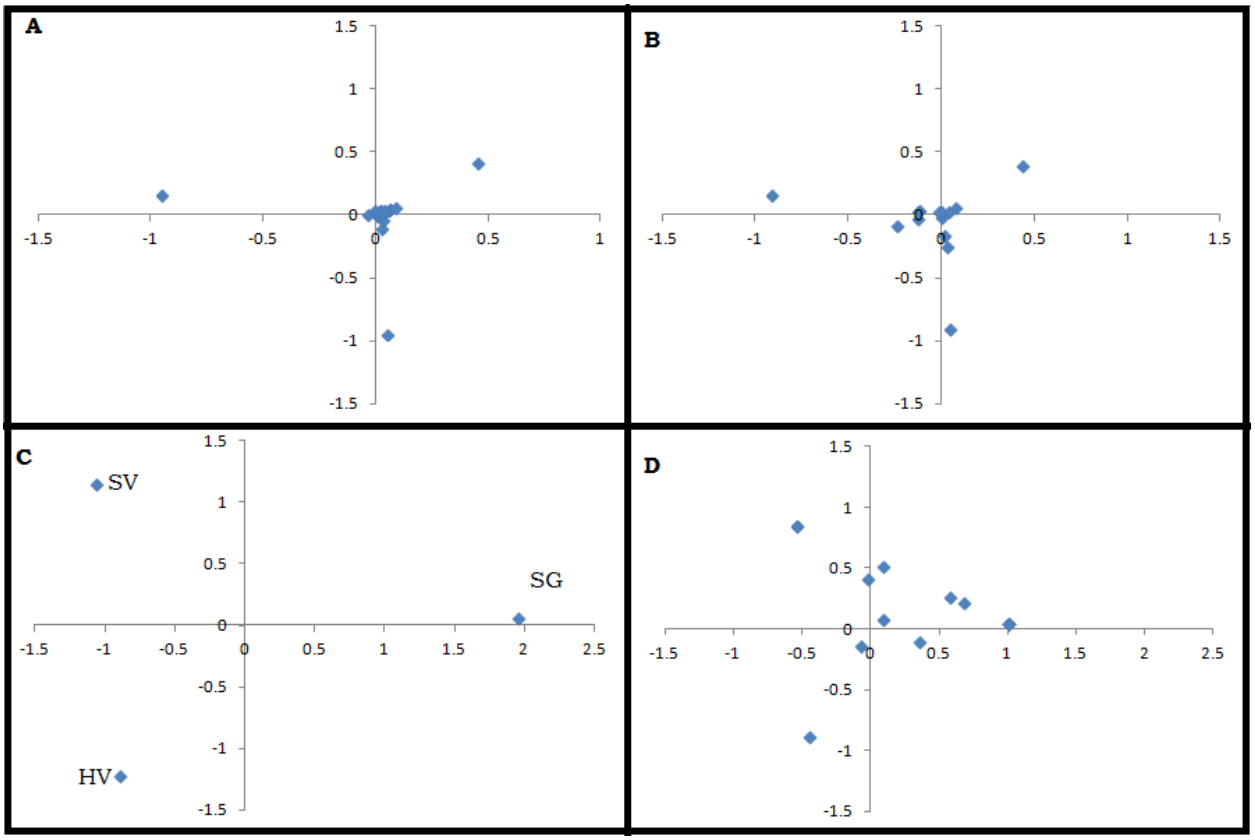

Figure 2.22.Principal Components Analysis (A) Coprolites positive for parasitic infections (B) Parasite species among positive coprolites (C) Culture/site combinations (D) Parasite species among culture/site combinations.

DISCUSSION

The parasites present in an organism reflect the activities, the lifestyle, environment where they developed and lived. Each parasite has different life cycle requirements in order to develop, infect a host and finally find another host. The relationship between parasites and their hosts has been studied through the use of paleoparasitology in many places with different techniques depending on the material analyzed. In the present study, coprolite material from Saladoid and Huecoid cultures was analyzed. These pre-Columbian cultures have been described by archeologists primarily as agriculturalists and hunters; with the aid of paleoparasitological techniques evidence for these and other theories may be better explained, supported or rejected.

It is very difficult to determine the origin of coprolites recovered from soil even if from identifiable latrines, if not from the body cavity of a corpse or mummy. Various authors have suggested methods of identification that include shape and diameter of the coprolite, color of the solution after rehydration and macroscopic findings among the samples such as charcoal and bone (Reinhard and Bryant 1992). In the samples used for this research the shape was consistent with a human coprolite. The color of the solution after the rehydration process varied from yellowish to dark brown and between the macroscopic parts of the sample after filtration bone was observed giving clues for the origin of the coprolites. But is the presence of parasites specific to hominids that allow a certain confirmation of coprolite origin.

Parasitic eggs may be recovered from coprolites in excellent condition but this may be difficult or impossible depending on the techniques used or the environmental conditions in which the samples were deposited. All the eggs recovered from coprolite rehydration in this research showed some shrinkage possibly due to desiccation and some level of distortion. Parasitic eggs could be poorly preserved when recovered from coprolites found in open sites. In

these settings feces could decompose before desiccation occurs. When this happens is common to find remains of scatophagous beetles, mites and/or fungus (Reinhard et al. 1986).

Microscopic analysis

The most abundant intestinal parasites found in this study were: *Ascaris lumbricoides*, *Trichuris trichiura*, *Enterobius vermicularis* and cestodes. These parasites have been found in other archeological sites throughout the world including South America (Araújo et al. 1985, Gonçalves et al. 2002 and Santoro et al. 2003), the presumed origin for the Saladoid and Huecoid cultures (Chanlatte and Narganes 2002, 2005). *Ascaris sp.* was found in human remains from Grande Grotte in France dating from 30,000 years BP, confirming its relation with humans through their various migrations (Bouchet et al. 1996, Leles et al. 2012). Gonçalves et al. (2003) reported cases of ascariasis in pre-Columbian America but pointed out that these infections were rare for unknown reasons. Well preserved eggs can be recovered due to the thick albuminous coat characteristic of fertilized and unfertilized eggs of this parasite. The *Ascaris sp.* life cycle requires humid soils with temperature around 25°C, eggs are not infective under ~16°C or over 38°C (Bogitsh and Cheng 1998). These soil conditions are easily found in agricultural settings. Archeologists have suggested that both Saladoid and Huecoid cultures practiced farming at some point and the finding of this parasite adds evidence for this activity (Reinhard and Araújo 2008).

The presence of *Trichuris trichiura* is consistent with previous studies that show a high persistence of this parasite in pre-Columbian populations (Araújo et al. 2011). This parasite is believed to have persisted from a non-human ancestor (Bundy et al. 1989 cited in Schmidt and Roberts 2009). The detection of *Trichuris sp.* cannot be mistaken because of their distinctive shape and presence of two polar plugs. Fresh trichurid eggs measure ~60x26 μm and recovered eggs measured ~51x18 μm, for an approximate shrinkage of 14%. The life cycle of this parasite

also requires humidity and damp soils characteristic of agricultural sites. This parasite and *Ascaris sp.* have been found together as reported by many authors (Moore 1981, Jones 1982, Fugassa et al. 2006).

Another parasite found within the rehydrated samples is *Enterobius vermicularis*. This parasite is the key to confirm that in fact the coprolites are of human origin since this parasite is host specific to humans. The oldest record of this parasite in human association is from North America, ~1075 BC (Fry and Moore 1969 cited in Araújo at al. 1985). *Enterobius sp.* does not necessarily need a humid soil to develop; its eggs are highly resistant to drying and can be viable in cool environments. Fresh eggs of this parasite measure ~62x28 μm and the ones recovered measured ~41x25 μm for an approximate shrinkage of 34%.

Cestode eggs were also found among the rehydrated coprolites. All cestode eggs found resemble the Hymenolepididae family, have round transparent onchosphere and embryphore without striations or filaments. This parasite parasitizes rats; humans can become infected by ingestion of food contaminated with an infected intermediate host, an insect. The finding of this parasite among the cultures denotes poor sanitation and probable rat infestation. Fresh cestode (Hymenolepid) eggs measure ~72x72 μm, but eggs found among the samples measure approximately 44x44 μm, presenting an approximate shrinkage of 39%.

Other parasitic eggs found in rehydrated coprolites but in less concentration were: *Trichostrongylus sp.*, Hookworms, *Diphyllobothrium latum, Dipilidium caninum, Paragonimus westermani* and Acanthocephalans. *Trichostrongylus sp.* has been known to parasitize humans and has been previously recovered in coprolites from Chile and Argentina (Gonçalves et al. 2003). Eggs from this genus are very similar to eggs of hookworms and *Strongyloides sp.* The difference lays in the size of the eggs and thickness of coat (Roberts and Janovy 2009). Fresh

eggs from this genus measure ~87x54 μm and recovered eggs measured ~57x30 μm, indicating an approximate shrinkage of 34%. Hookworm eggs were also found in the samples. Hookworms and *Trichostrongylus sp.*, were found among the Saladoid culture in both archeological sites of Guayanilla and Vieques. Fresh hookworm eggs are 68x48 μm and the recovered eggs measured 42x28 μm, shrinkage of ~38% occur.

Diphyllobothrium latum, commonly known as the broad fish tapeworm has been recovered from coprolites from pre-Columbian sites in South America (Kliks 1990 and Arriaza et al. 2010). Fresh eggs measure approximately 72x48 μm but recovered eggs measured ~ 36x20 μm. In this particular case shrinkage of ~50% occurred and is noticeable in the pictures taken were the eggs looked damaged and poorly preserved. *Dipylidium caninum* eggs were also found and with the same scarce abundance and distribution as *Diphyllobothrium sp.* Both were recovered only from the Saladoid culture in the archeological site of Vieques. The finding of this parasite shows a contamination with dog fleas, it does not mean that the coprolite has canid origin because *Enterobius vermicularis* was also found in the same coprolite. Jiménez et al. (2012) also found *Dipylidium caninum* in human coprolites from "Cueva de los muertos chiquitos" in Mexico. This parasite has a very characteristic packet with ~8-25 eggs inside and measures ~120x78 μm; recovered egg packets measured ~73x50 μm with ~10 visible eggs but the eggs reported in "Cueva de los muertos chiquitos" measured 23x18 μm.

A presumed *Paragonimus westermani* egg was found in the Huecoid culture from the archeological site in Vieques. The presence of this particular parasite coincides with the theory that this culture lived close to freshwater bodies and practice more fishing than the Saladoid culture. The infection with this parasite occurs when a human ingest raw or poorly cooked freshwater crustaceans containing metacercariae (Bogitsh and Cheng 1998). The last identified

parasite was the acanthocephalans, only found in the Saladoid culture from Guayanilla. Most common acanthocephalan infecting human are from the Moniliformidae family (Roberts and Janovy 2009). Eggs from this particular family measure ~90x65 μm in comparison with the ones recovered from the coprolites that measured ~55x20 μm, indicating an approximate shrinkage of 39%.

Four other egg morphologies were found but could not be identified and were called Unknown Trematodes (1-4). One non-parasitic organism, a mite from the Tarsonemidae family was found. Seabra Nogueira de Candanedo Guerra et al. (2003) also found this mite in coprolite samples and suggested that this organism invaded the coprolite after the defecation.

Statistical analyses

To evaluate how significant the abundance of eggs was and the species richness a non-parametric ANOVA was performed. There was no statistical significance among the abundances of SG-SV or SV-HV. There was only a statistical significance in the species richness of SV-HV. This result indicates that one of the combinations (SV and HV) has more parasite species than the other and that they are different. The difference in the intestinal parasites found for SV and HV could rest in two possibilities. First, that even when these two cultures are found relatively close to each other they did not live in same area at the same time or point in chronological history. Second, that they were very close cultures that did not interact enough with other groups to the point that a process of cross-contamination could not take place.

To analyze the intestinal parasites as a community a cluster using Jaccard biodiversity index was performed. This index takes into account the similarities between species among the communities using presence and absence of species, but does not recognize the replacement or exclusive species in a community (Villareal et al. 2006). According to the cluster the parasite

community of the Saladoids in Vieques is approximately 58% more similar to the Saladoids in Guayanilla than to the Huecoids in Vieques. This result also suggests that the Saladoids and the Huecoids were not in Vieques at the same time. The similarity between the parasite community of Saladoids could be explained by the fact that these cultures went through a process of migration in order to inhabit these particular areas. Also the similarity could be linked to similar lifestyles and common practices. They could have carried and maintained the parasites and with time or changing in conditions the parasites will remain, some will disappear and some could be added. In this particular case it seems that since the environmental conditions between Guayanilla and Vieques are not very different the parasite community remained relatively the same.

In order to see the similarities found among the culture-site combination from another perspective the β diversity Whittaker index was applied. This is a commonly used index that takes into account the replacement and exclusive species per community (Villareal et al. 2006). With this index 0 indicates maximum similarity and 1 minimum similarity. The results showed again a high similarity between SV and SG with 0.273 (73%). Also with the results from this index more similarity between SV and HV could be observed with 0.375 (62%). These results indicate that in fact the lack of similarity between the Saladoids and the Huecoids that was not determined with the Jaccard index, was the difference in the species present. To evaluate the variance and similarity among the coprolites studied and the culture/combinations a PCA was performed using POPSTR. PCA is a very common analysis used to see how different variables interact with each other. Most of the coprolites positive for parasitic infection are similar to each other, the differences lay in coprolites with unique infections. All three culture/site combinations are different from each other but Huecoid and Saladoid cultures in Vieques are more similar to

each other, parasite species that describe this relationship are very similar again with the exception of points that represent unique species.

This is the first paleoparasitological study done in Puerto Rico and the Caribbean. Detection of parasites from these two different cultures is very important because there is no previous record on parasitic infections of Puerto Rico's native peoples. The findings of this study represent evidence of parasitic infections prior to European colonization. To reinforce the process of identification molecular techniques could be applied. To see the pattern more clearly in terms of parasite abundance, richness and distribution the analysis of more samples is recommended. For future work an ecological approach to the coprolite analysis may give rich information in terms of culture description.

CHAPTER 3

HOOKWORMS IN PRE-COLUMBIAN HUMAN COPROLITES FROM SALADOID CULTURE IN PUERTO RICO

INTRODUCTION

Parasites have accompanied humans since the beginning of the species and through each of their migrations around the world. Paleoparasitology has helped evaluate the development of the evolutionary arms race between humans and parasites through time. Some parasites are very host-specific; this is the case of hookworms, intestinal parasitic worms that feed on human blood. These organisms are called inherited parasites (Barrett et al. 1998, Brooks and Ferrao 2005), parasites that have infected the same host through its process of evolution. The presence of these parasites could help theorize human migrations.

In 1921, Darling pointed to an apparent hookworm infestation in South America prior to Columbus's arrival. This was not conclusive until Allison et al. (1974) officially identified the parasites in the intestine of a Peruvian mummy dated 890-950 years BP. Other scientists confirmed these findings (Ferreira et al. 1980, 1983, 1987, Araújo et al. 1988). The original theory of hookworm infection in the New World stated that hookworms came via the slave trade after the European conquest (Smillie 1922, Manter 1967 cited in Araújo et al. 1988). Many theories on human migrations have been extrapolated from finding these specific parasites (Araújo, Ferreira and Confalonieri 1981) as well migration models have been developed that eliminate the Beringian land bridge as the only human migration for the peopling of the New World, since is not a viable route for parasitic success (Montenegro et al. 2006).

In Puerto Rico, paleoparasitological techniques could be applied to identify intestinal parasites such as hookworms. The finding of this parasite in the island could add another piece of the puzzle of migrations to the Americas. Coprolites recovered from archeological sites corresponding to Huecoid and Saladoid cultures in Puerto Rico were studied. Archaeologists have described both of these groups primarily as farming cultures that as a secondary activity practiced hunting. Both of these practices were high risks activities that predisposed the cultures to parasitic infections. Many parasites specifically nematodes need humid soils to developed and complete their cycles. Hookworms are an exceptional example of parasites with complex cycles that involve temperature and humidity requisites. Farming soils are ideal places to maintain continuous parasitic infections and re-infections. Parasites recovered from this study could be compared to investigations performed in Venezuela and the Andean region, places according to archeologists Chanlatte and Narganes are the origin of these cultures (Anonymous 2002, 2005). The findings of this type of study could provide another clue that will bring us closer to answer the old question of how the peopling of the Americas occurred and specifically how this process took place in Puerto Rico.

MATERIALS AND METHODS

Subsamples of 34 coprolites from to archeological sites in Puerto Rico were provided by Centro de Investigaciones Arqueológicas from the University of Puerto Rico at Río Piedras. One gram of each specimen was rehydrated in 14ml of an aqueous solution of trisodium phosphate 0.5% for 72 hrs (Callen and Cameron 1960). The rehydrated samples were mixed approximately 10:1 in acetic formalin solution 10% to avoid bacterial and fungal growth (Gonçalves et al. 2003). The samples were allowed to sediment as proposed by Lutz (1919). Ten preparations

were made from each sample to estimate the amount of eggs per sample (Han et al. 2003). Each preparation was made with 50µl of sediment mixed with a drop of glycerin on a microscope slide (Fugassa et al. 2006). Parasite eggs found were photographed and measured at 40x and 60x with a calibrated ocular micrometer. For the lack of a taxonomic key to identify parasite eggs, morphological characters such as projections, shape, presence of larvae and others were used in the process of identification.

RESULTS

From the 34 coprolites analyzed only 7 were positive for hookworm infection. Only coprolites from the Saladoid culture in the two archeological sites were positive for the infection. The estimate of eggs per gram for the Saladoid culture in the archeological site of Sorcé Estate in Vieques was 56 eggs/g of rehydrated coprolite. For the Saladoid culture in the archeological site of Tecla 1 in Guayanilla the estimate was 148 eggs/g of rehydrated coprolite (Figure 3.1). The confirmation of the eggs found among the samples was made by morphology and measurements (length and width) of the eggs (Figure 3.2).

In addition, a well preserved rhabditiform larva or juvenile stage 1 (J1) was found in coprolite #24 from the Saladoid culture also in the archeological site of Tecla 1 in Guayanilla (Figure 3.3). The larva was identified using morphological characteristics such as the size of the bucal cavity in order differentiated from other similar juveniles. Finally, an unusual and unexpected finding, an adult male hookworm was found in coprolite #27 from the Saladoid culture in Guayanilla, the worm was identified for the presence of the still well preserved copulatory bursa (Figure 3.4).

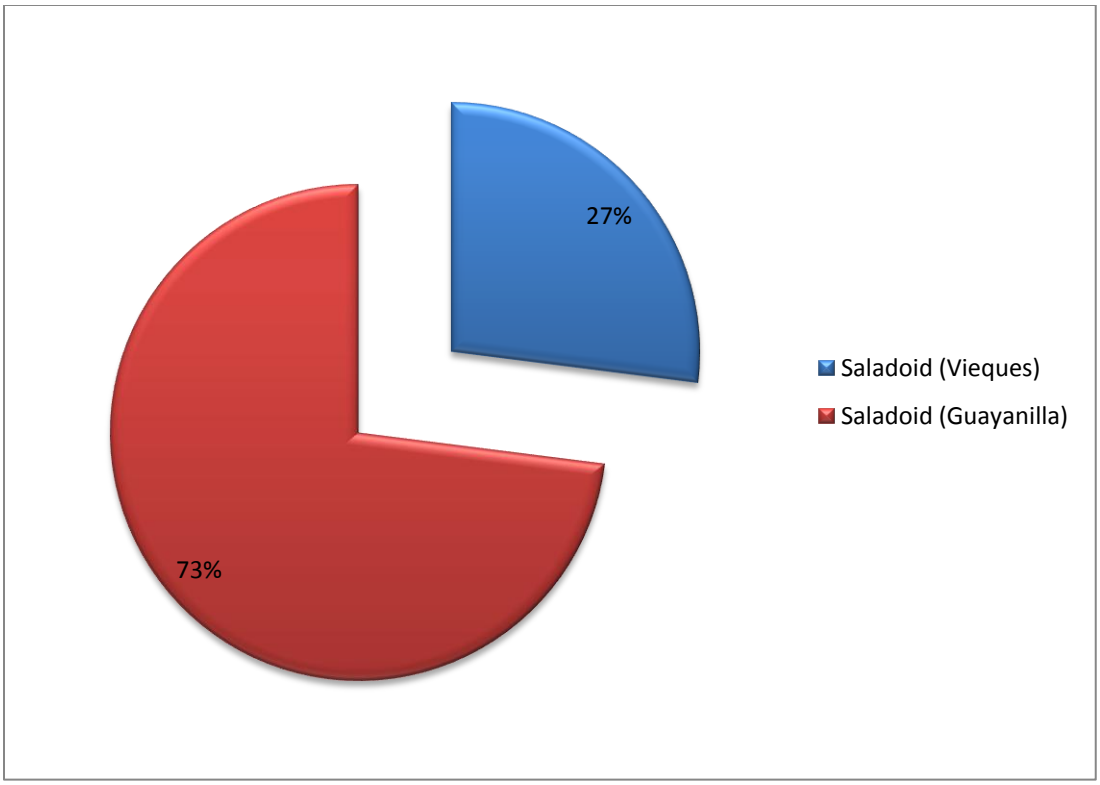

Figure3.1. Hookworm eggs per gram in the Saladoid culture**.** The archeological site of Tecla 1 in Guayanilla had 5 of 14 coprolites infected with this parasite and 2 of 5 in Vieques.

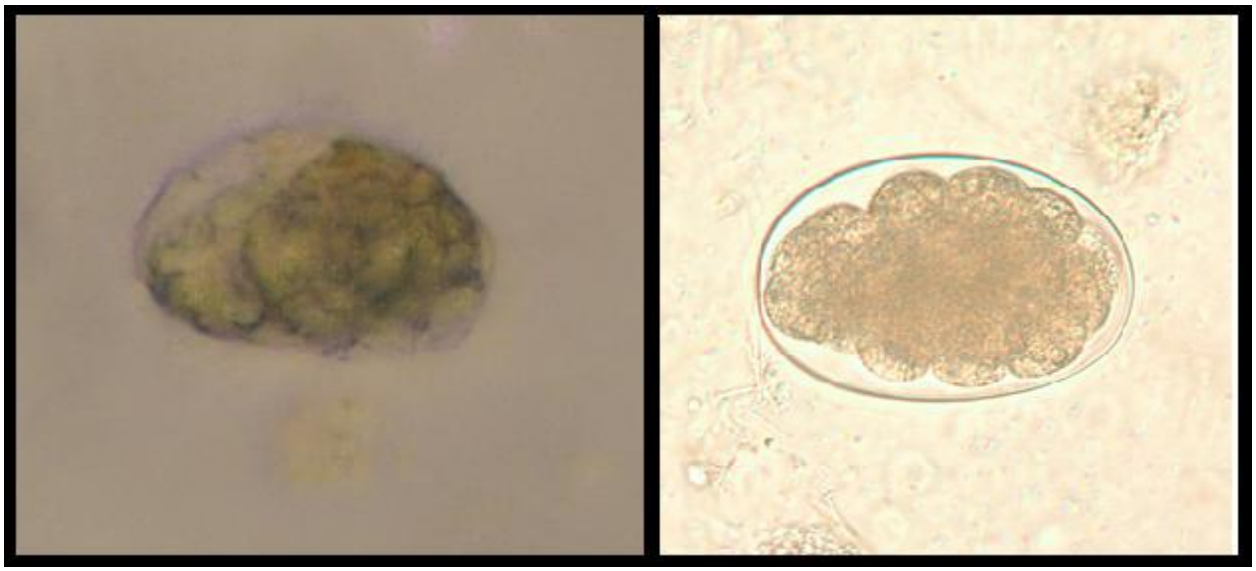

Figure 3.2.Hookworm egg comparison. (A) Hookworm egg found in a coprolite sample, notice damaged but visible outside layer and cell development in the inside. (B) Fresh hookworm egg from the Center for Disease Control (CDC) parasite image library. Measurements (length and width): 50x25 μm (A), 60x35 μm (B).

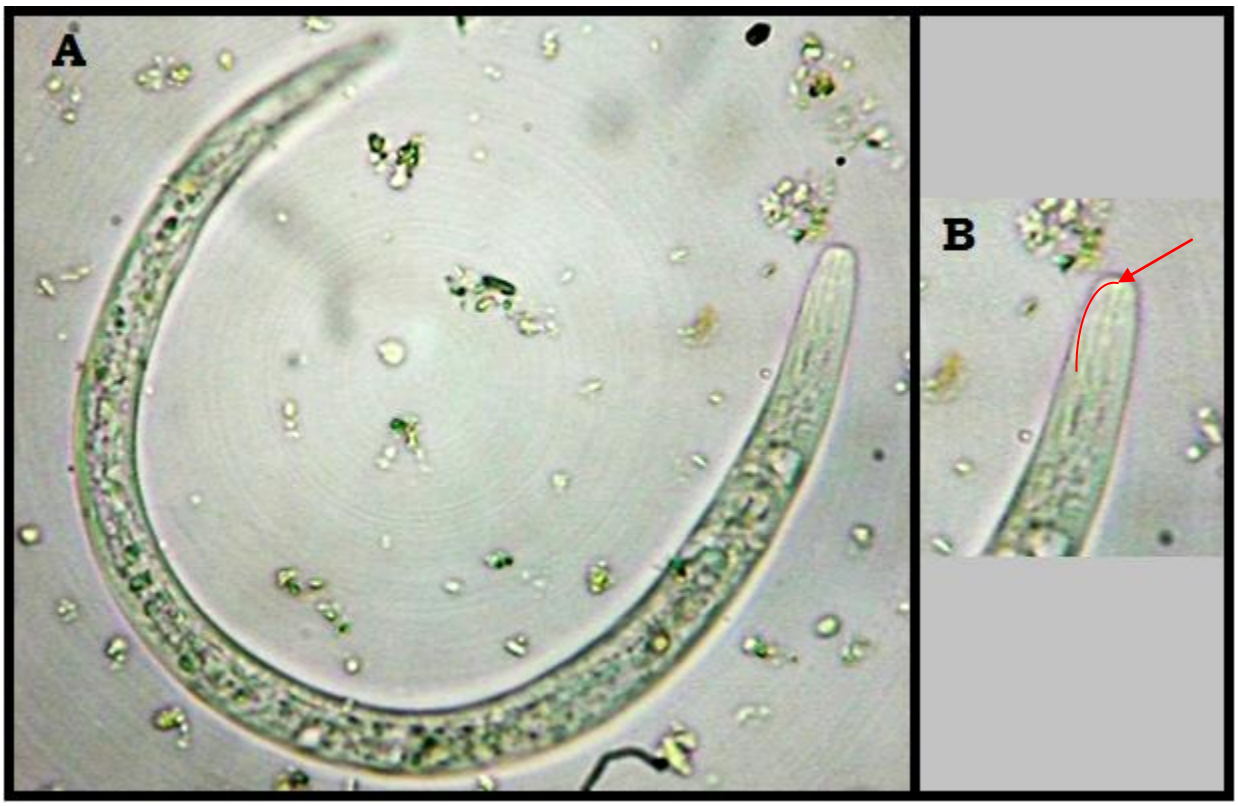

Figure 3.3.Hookworm rhabditiform larva from the Saladoid culture. (A) Notice complete juvenile, posterior part not completely visible. (B) Close-up of anterior part of the juvenile, red arrow points to the long bucal cavity distinctive of this stage. Measures: 288µm.

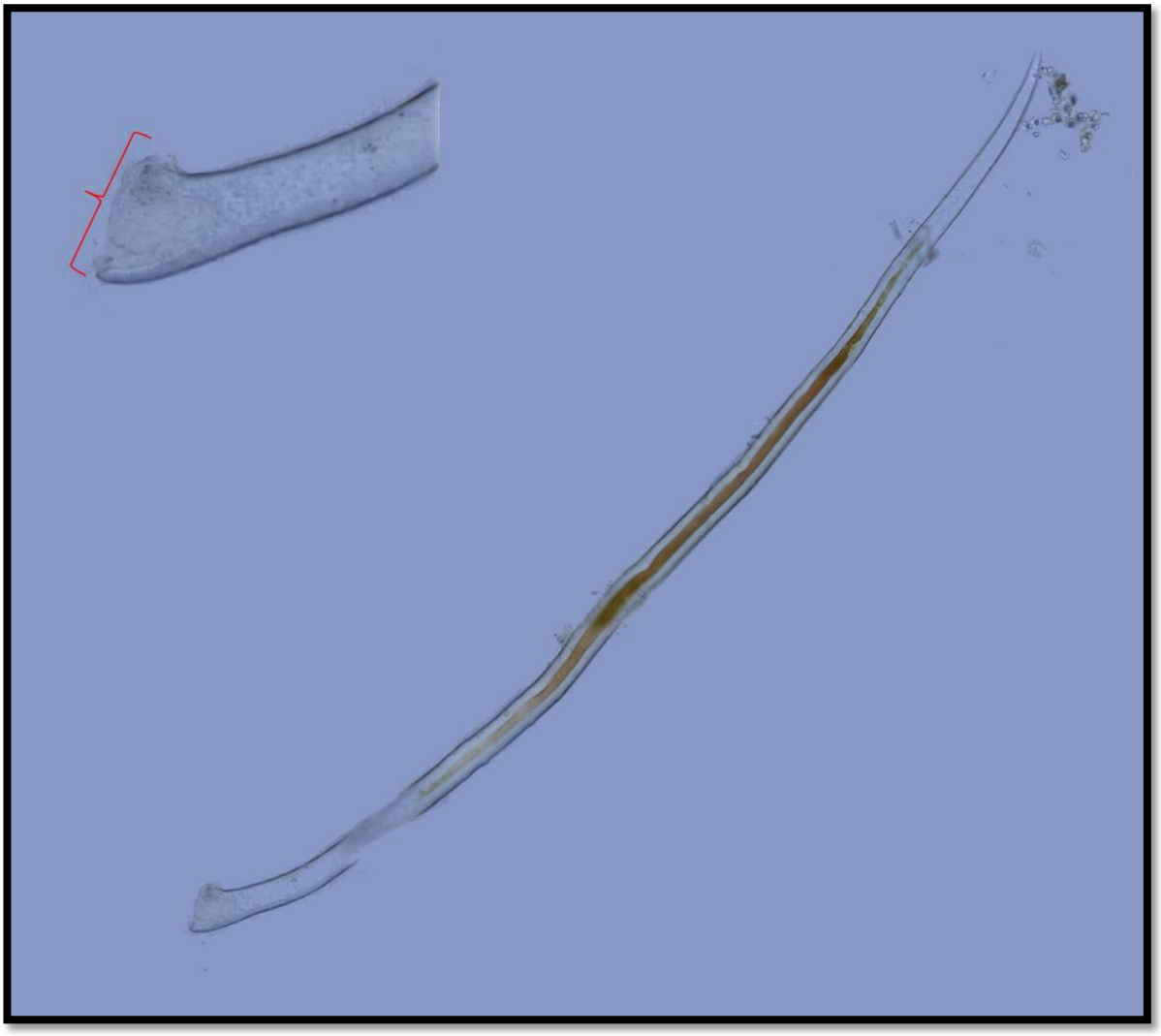

Figure3.4.Adult male hookworm from Saladoid culture. Notice red line pointing to the close up of distinctive copulatory bursa of a male hookworm with poorly preserved rays. Anterior part or cephalic area could not be observed.

DISCUSSION

Hookworms are intestinal nematodes important not only for their pathology to the infected host but also for their presence among ancient pre-Columbian remains that can shed light on human migrations. Since the first official confirmation of hookworm infection in South America by Allison et al. (1974) in a Peruvian mummy, the field of paleoparasitology has been trying to explain how this parasite established itself in the Americas. This question has been approached from a parasitological perspective more than simple migration routes for the complexity and requirements of their cycle and the biological history of the two most common hookworm species infecting humans, *Ancylostoma duodenale* and *Necator americanus*. The dispersion of human infection with *Ancylostoma duodenale* has been considered to occur from northern Africa and the southern part of Europe and for *Necator americanus* from the southeastern part of the Sahara and southern Asia (Manter 1967 cited in Araújo et al. 1988, Montenegro et al. 2006).

Ancylostoma sp. and *Necator sp*. share many characteristic in their life cycles and morphology, which makes very difficult to distinguish one from another especially in ancient material. The most common species found in the Americas is *N. americanus* but this particular species is not an exclusive human parasite because is also known to parasitize dogs. In the case of *A. duodenale,* humans act as an almost exclusive definitive host (Bogitsh and Cheng 1998). Both of these parasites need at least three conditions in order for the infection to be successful: warm humid soils (for adequate juvenile development), poor sanitation and the presence of a population in constant contact with contaminated area. All the mentioned factors were present among the Saladoid culture in Puerto Rico. This culture has been primarily described by

archeologists as a farming culture, activity that requires humid soils. During the excavations performed in the archeological sites of Tecla 1 and La Hueca in Sorcé estate Vieques some plots were defined as common latrines. This was the main point of origin of the human coprolites analyzed and key points were the continuous infection could be maintained.

A total 21% (7 of 34) of hookworm infection was found among the analyzed samples. None of the coprolites from the Huecoid culture in Vieques was positive for the infection. This does not mean the infection was restricted to the Saladoid culture but could suggest that hookworm eggs from this particular culture could have been more damaged and were not easily identified by morphological characteristics. A more asserted reason for the absence of hookworms in the Huecoid culture could be that they come from the Andean region and with altitude the temperatures are lower; these parasites do not succeed in this environment. From the two Saladoid archeological sites, Guayanilla was responsible for the major abundance with a percentage of positive coprolite infection of 33% (5 from 15 coprolites). All the eggs recovered were measured and compared with previous hookworm findings. The average size of recovered eggs was 42x28 μm which is relatively similar to those reported by Sianto et al. (2005) from a Brazilian mummified body, 57x35 μm. Minimum differences between the previously reported measurements and the ones from this study could rest on the fact that the coprolite samples were recovered from the soil and in this setting desiccation could happen faster due to environmental conditions.

Not only the presence of hookworm eggs in feces could indicate an infection with this organism, in other cases the presence of identifiable rhabditiform larvae would make the identification more efficient and certain. This was the case of a single rhabditiform larva (J1)

found in a coprolite from the Saladoid culture in Guayanilla. The well preserved J1 was identified as a hookworm larva, mainly for the elongated bucal cavity, the presence of the rhabditoid esophagus (circular shaped) and a total length of 288 μm. Rhabditiform larvae are very similar to those from the genus *Strongyloides sp.* but this one has a very short bucal cavity (less than half of its width) typical of hookworms.

A totally unexpected and unusual finding occurred; an adult male hookworm was recovered also from the Saladoid culture in Guayanilla. The adult was identified by the presence of a well preserved copulatory bursa, distinctive reproductive structures of these parasitic organisms. The adult worm was very delicate and the attempts to rotate it in order to properly see the bucal capsule failed. The part of the worm clearly appreciated was the dorsal part; the bursal rays were visible but not the spicule which is located in the ventral part of the body. In the inside of the worm poorly defined masses resembling the intestine for the particular dark red-brownish color was noted. These parasites feed on blood and need a multi-protease cascade to digest host hemoglobin (Williamson et al. 2003 cited in Schmidt and Roberts 2009.). This could give the particular color to the digestive tract. Findings of adult hookworms in feces are very rare and even more in coprolite samples. This is the first record of an adult hookworm from ancient material outside the pelvic cavity of a preserved corpse or mummy. There are two possible hypotheses to explain this finding. (a) The infection among the Saladoid culture particularly in the archeological site of Tecla 1 in Guayanilla could have been very high and prevalent, in this case unviable adults were eventually excreted. (b) Many cultures and specifically ancient groups tended to purge themselves from time to time as part of rituals or to alleviate discomforts and worms could have voided with feces, Reinhard and Araújo (2008) called this anthelminths. The second hypothesis could be the more asserted one because this would produce the same result. A

medication would induce adult parasites to be excreted with the feces or to be absorbed by the body. The major abundance of hookworm eggs and the findings of both the rhabditiform larva and the adult male hookworm in the Saladoid culture from the archeological site of Tecla 1 in Guayanilla, could suggest a pattern of epidemiological infection in this culture, but more samples are needed to support this statement.

The findings of this research contribute to the question of human migrations through the Americas and even more to Puerto Rico. Coprolites positive in this research belonged to the Saladoid culture, their origin has been traced from Venezuela near the Orinoco River were the first settlements of this culture have been found (Chanlatte and Narganes 2002). Paleoparasitological analyses have been made in Venezuela but none from the particular region of Saladero, point of origin of the Saladoid culture. Still the big question not answered is how this parasite arrived in the New World. Definitely the environmental conditions hookworms need were not available through the Beringian land bridge; the clear evidence of this parasite in pre-Columbian archeological remains gives additional support to the theories of coastal and transpacific migrations (Araújo et al. 1988). The best way to address this answer will be complementing researches like this one at paleoparasitological level paired with molecular analysis to trace the parasites footprint from their point of origin in the Old World to the Americas. Concrete statements on this matter could then be finally made.

CONCLUSIONS

1. Detection of intestinal parasite eggs with rehydration and sedimentation techniques was successful.

2. A total of 15 different intestinal parasites were found among coprolites from Saladoid and Huecoid pre-Columbian cultures. The most common parasite species found were: *Ascaris lumbricoides*, *Trichuris trichiura*, *Enterobius vermicularis* and cestodes.

3. No statistical significant difference was found between the abundances of parasitic eggs between the Saladoid cultures in the two archeological sites. Same results were found between the Saladoid and the Huecoid cultures in Vieques.

4. A statistical significant difference was found in the parasite species richness between Saladoid and Huecoid cultures in Vieques but no for Saladoids in the two archeological sites.

5. As a community, the parasites present in Saladoid culture from Guayanilla are more similar to those of the same culture in Vieques than to those of the Huecoid culture in Vieques, using the Jaccard index.

6. Results suggest that the differences between the cultures in Vieques lays on a chronological difference in settlements rather than the presence of social barriers blocking the transmission of infection from one culture to another.

7. Clear evidence of hookworm presence in different stages from the Saladoid culture suggests an epidemiological pattern but more samples should be analyzed to make a strong statement.

8. Hookworm findings in pre-Columbian settlements or archeological sites add evidence of this parasitic presence before European colonization as suggested by previous researchers.

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