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PREVALENCIA, DISTRIBUCIÓN, DIVERSIDAD GENÉTICA DE *Blastocystis* Y SU IMPLICACIÓN CLÍNICA EN UNA POBLACIÓN RURAL DEL ESTADO DE MORELOS, MÉXICO

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Mi Alma mater

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"Todo Lo Que Puedes Imaginar

Es Real"

Pablo Picasso

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PRESENTACIÓN

Blastocystis es un microorganismo protista con una alta distribución mundial, siendo, mayormente identificado en estudios epidemiológicos de parásitos. En las dos últimas décadas, el interés de la comunidad científica por este microorganismo ha incrementado debido al poco conocimiento entorno a su papel en la salud y enfermedad humana. El carácter patogénico de *Blastocystis* continúa sin esclarecerse: lo mismo surgen reportes que identifican a *Blastocystis* como un factor etiológico de sintomatología gastrointestinal, como los que lo documentan como un comensal del tracto digestivo en el huésped humano al ser identificado en individuos asintomáticos, aparentemente sanos.

El huésped humano puede ser colonizado con múltiples subtipos o haplotipos diferentes de este protista. Se ha estimado que las infecciones mixtas por *Blastocystis* en humanos tienen un rango de 1.1% y 14.3 %, con un promedio mundial del 6%, sin embargo, existe pocos datos sobre estas infecciones, ya que la metodología empleada en la mayoría de los estudios reportados solo permite identificar un solo subtipo, subestimando así la prevalencia real de las infecciones mixtas. Este último punto es de gran importancia, ya que estudios previos han abierto la posibilidad de competencia o cooperación entre los subtipos de *Blastocystis* para una colonización intestinal exitosa.

Por tal motivo, el objetivo de este proyecto fue el estudiar a *Blastocystis* en la comunidad rural de Xoxocotla en el estado de Morelos, México, para identificar los subtipos de *Blastocystis* prevalentes en la población, evaluar su estado de salud a través de la aplicación del cuestionario ROMA III para el diagnóstico de trastornos y síndromes gastrointestinales funcionales, y establecer una asociación con variables

sociodemográficas y socioeconómicas de importancia para la presentación de la blastocistosis.

Los resultados obtenidos en este estudio son un importante aporte en el estudio de *Blastocystis*, que pueden ayudar aclarar el papel de este organismo en el tracto digestivo humano en su carácter patógeno, comensal o mutualista.

Para cumplir con este objetivo, este proyecto ha sido dividido en cuatro capítulos y una sección de anexos. El primer capítulo es una panorámica del estado actual de *Blastocystis*, incluye una introducción, taxonomía, formas morfológicas y ciclo de vida de *Blastocystis*, su estatus patógeno, comensal o mutualista en el tracto intestinal, distribución y diversidad genética de subtipos, una breve descripción de área de la población estudiada y finalmente la justificación y objetivo para la realización del presente proyecto.

El segundo capítulo muestra el primer uso de secuenciación de amplicones (NGS) usando secuenciación paralela masiva como una herramienta poderosa para investigar infecciones mixtas y detectar subtipos de *Blastocystis* de baja abundancia en población humana, y la asociación de los subtipos únicos e infecciones mixtas con variables socioeconómicas y sociodemográficas de importancia para la presentación de la blastocistosis. Los resultados obtenidos nos mostraron que en nuestra población de estudio se encontró un 13.7% de infecciones mixtas. Para esta población en la edad adulta existe una mayor propensión a ser infectado por *Blastocystis*. El estatus de comensal o mutualista de *Blastocystis* en esta población se reafirmó al ser una población con alta prevalencia de infección por *Blastocystis* y por el estatus asintomático de los individuos estudiados. Los

resultados fueron publicados en la revista internacional *Parasites & Vector* con un factor de imparto de 3.34 (Rojas-Velázquez L., et al. 2019).

En el tercer capítulo se abordó la distribución y diversidad genética del subtipo 3 (ST3) de *Blastocystis* en la población de Xoxocotla, así como su comparación con otras poblaciones a nivel nacional e internacional; la metodología empleada fue mediante un análisis microscópico para la determinación de la frecuencia de parásitos, y del uso de la técnica de la reacción en cadena de la polimerasa (PCR) para la amplificación de un fragmento de 600 pb de el gen de la subunidad pequeña del RNA ribosomal (SSU-rDNA). Los resultados obtenidos nos permitieron identificar a los subtipos más prevalentes en la población, encontrando al ST3 como el de mayor frecuencia, datos utilizados para la elaboración de una red de haplotipos del subtipo 3, en donde observamos que el haplotipo 1 de ST3 estaba ampliamente distribuido a nivel mundial y que pudiera estar asociado al fenómeno de migración y al parecer es el ancestro común del cual el resto de los haplotipos derivan. Los resultados obtenidos en este capítulo fueron publicados en la revista internacional *Biomed Research International*, con un factor de impacto de 2.58 (Rojas-Velazquez L., et al. 2018).

El cuarto capítulo incluye la discusión general y conclusiones de la tesis. Se estableció el comportamiento de *Blastocystis* en esta población, su carácter comensal o mutualista y la distribución geográfica del subtipo 3 en la población estudiada en comparación con poblaciones nacionales e internacionales, y se aborda la diversidad intra-subtipo 3 y su probable asociación con el fenómeno de migración en relación con su distribución mundial. El análisis constituye un ejemplo del potencial de estudio que tiene

Blastocystis en proyectos futuros para el mejor entendimiento de este microorganismo en el ser humano.

Y por último, se incluye una sección de anexos que consta de otras publicaciones generadas durante el desarrollo del doctorado, las cuales fueron fundamentales en la formación de conocimiento relevante en las parasitosis, y que aportaron una fuente importante de investigación para la realización de esta tesis.

RESUMEN

Blastocystis es un protista que pertenece al grupo de los Stramenopiles y en los últimos años su prevalencia a nivel mundial ha aumentado considerablemente, desplazando a parásitos como *Entamoeba histolytica* y *Giardia lambia*. Recientemente, el estudio de *Blastocystis* ha cobrado interés por parte de la comunidad científica, ya que su papel en la salud y enfermedad continúa sin esclarecerse. Existe poca información en relación con su epidemiología, distribución geográfica, diversidad genética y su papel como patógeno, comensal o mutualista en el tracto intestinal del humano, razón por la cual, en la comunidad de Xoxocotla en el estado de Morelos fue el marco para el desarrollo del presente trabajo. Nuestra población de estudio estuvo conformada por 182 voluntarios, con un intervalo de edad de 2-51 años, 96 personas del sexo femenino y 86 masculino. De todos los participantes en el estudio se recolectaron muestras de heces, y se les realizó dos encuestas, una fue para la recolección de variables socieconómicas y sociodemogáficas y la segunda fue la aplicación del cuestionario ROMA III para el diagnóstico de trastornos gastrointestinales funcionales, con el cual se estableció el estatus de salud de los participantes. Asimismo, se obtuvo una carta de consentimiento informado y durante todo el estudio la participación fue voluntaria. Para la determinación de la parasitosis en la población de Xoxocotla se realizó el análisis microscópico de las heces y posteriormente se procesaron para la extracción de DNA que se utilizó para la determinación de subtipos en la población. Se encontró una alta prevalencia de *Blastocystis* (74%), los subtipos prevalentes fueron el ST3 (81.5 %), seguido del ST2 (17.7%), y el ST1 (16.9%), en el 13.57% se presentaron infecciones mixtas de subtipos de *Blastocystis*. No existió asociación entre la infección por *Blastocystis* y sintomatología gastrointestinal, pese a la alta prevalencia de *Blastocystis* los individuos presentaron un

estatus asintomático. En cuanto a la diversidad y distribución geográfica de los subtipos de *Blastocystis* se encontró que la distribución del subtipo 3 está ampliamente distribuido a nivel mundial y que dicha distribución pueda estar asociada al fenómeno de migración. En cuanto a la diversidad intra-subtipo de ST3, con la red de haplotipos se determinó que el haplotipo 1 pueda ser el ancestro común del cual el resto de los haplotipos se hayan originado.

Los datos obtenidos en este trabajo contribuyen a la comprensión de la epidemiología de la infección por *Blastocystis* en humanos y pueden utilizarse para diseñar futuros estudios de este controvertido microorganismo.

ABSTRACT

Blastocystis is a protist that belongs to the stramenopiles group, and in recent years its prevalence worldwide has increased considerably, displacing parasites such as *Entamoeba histolytica* and *Giardia lambia*. Recently, the study of *Blastocystis* has adquired interest from the scientific community, since its role in health and disease continues without being clarified. There is a great lack of information regarding its epidemiology, geographical distribution, genetic diversity and its role as a pathogen, commensal or mutualist in the human intestinal tract. Present study was performed in the community of Xoxocotla, in the state of Morelos, our study population consisted of 182 volunteers, in an age range of 2-51 years, and a gender distribution of 96 female and 86 male. Stool samples were collected from all participants in the study, and two surveys were conducted, one was for the collection of socio-economic and sociodemographic variables and the second was the application of the ROMA III questionnaire for the diagnosis of functional gastrointestinal disorders to established the health status of the participants, likewise an informed consent letter was obtained and during the entire study the participation was voluntary. For the determination of parasitosis in the population of Xoxocotla, a microscopic analysis of the stool samples was carried out and subsequently processed for DNA extraction, this material was used for the determination of subtypes in the population. The results obtained allowed us to know the high prevalence of *Blastocystis* in the community (74%), the prevalents subtypes in the community were ST3 (81.5%), followed by ST2 (17.7%), and ST1 (16.9%), in 13.57% there were mixed infections of subtypes of *Blastocystis*. There was no association between *Blastocystis* and gastrointestinal symptomatology, despite the high prevalence of *Blastocystis* individuals had an asymptomatic status. Regarding, the diversity and geographical distribution of the subtypes of *Blastocystis* it was found that the

distribution of subtype 3 is widely distributed worldwide and that such distribution can be associated with the phenomenon of migration. Regarding, the intrasubtype diversity of ST3 with the haplotype network, it was determined that haplotype 1 may be the common ancestor from which the rest of the haplotypes have originated.

The data obtained in this work contribute to the understanding of the epidemiology of *Blastocystis* infection in humans and can be used to design future studies on this controversial microorganism.

CAPÍTULO I

Estado actual de *Blastocystis*: un panorama general

Introducción

Blastocystis es un protista unicelular entérico de los seres humanos y de algunos animales, con una distribución mundial (Tan, 2004; Stenzel y Boreham, 1996). *Blastocystis* ha sido propuesto como un patógeno emergente para el humano, desplazando a los parásitos tradicionalmente endémicos en países en vías de desarrollo, como *Entamoeba histolytica* y *Giardia duodenalis* (Rodríguez et al., 2008).

El examen microscópico de muestras fecales frescas es el método diagnóstico de rutina para la detección de *Blastocystis*, pero su similitud con otros protozoarios intestinales y la variación en su morfología pueden llevar a un diagnóstico erróneo, y a una subestimación de la prevalencia de este protista (Baldo et al., 2004). Esto ha dificultado entender su modo de reproducción, ciclo de vida, prevalencia y patogénesis, por lo que en la actualidad su carácter como patógeno intestinal es controvertido, lo que a su vez ha permitido que desde el punto de vista médico no sea considerado un organismo causante de enfermedad, ni de la necesidad de tratamiento médico para su eliminación.

Sin embargo, en las últimas dos décadas, a nivel mundial se ha observado un aumento en la frecuencia de *Blastocystis*, dando pie al incremento de estudios epidemiológicos en los cuales *Blastocystis* ha sido el protista intestinal más reportado (Stensvold y Clark, 2016; Ramirez et al., 2016; Jiménez et al.,2019), con un mayor predominio en los países subdesarrollados, en los cuales las condiciones de higiene, la exposición a los animales domésticos y peridomésticos, así como al consumo de alimentos y/o agua contaminada, representan un ambiente idóneo para la de presentación de Blastocystosis (Clark et al., 2013).

Blastocystis no es exclusivo de estos países en vías de desarrollo, encontrándose también presente en países desarrollados (Beyhan, 2015). La prevalencia de *Blastocystis* a

nivel mundial varía entre un 0.5% hasta un 62% (Clark et al., 2013), dicho intervalo dificulta establecer la prevalencia real de *Blastocystis*, la cual está condicionada principalmente por dos factores: la predisposición del individuo a ser colonizado y la herramienta de diagnóstico utilizada: análisis microscópico o biología molecular a través de la reacción en cadena de la polimerasa (PCR), ambos estudios no comparables ni en sensibilidad o especificidad dando como resultado datos inconsistentes en la prevalencia de *Blastocystis* (Clark et al., 2013).

En la práctica médica, *Blastocystis* no ha sido aceptado completamente como un factor etiológico causante de enfermedad, aunque su presencia se ha asociado con una gran variedad de síntomas, principalmente síntomas gastrointestinales, como diarrea y dolor abdominal, que en la mayoría de los casos son autolimitados. Ademas, la infección por *Bastocystis* también ha sido asociada con el Síndrome de Intestino Irritable (SII). (Yakoob et al., 2004; Giacometti et al., 1999). Recientemente, en México se encontró una asociación entre la infección por *Blastocystis* y la presentación de diarrea, así como con el desarrollo de SII (Ramirez-Miranda et al., 2010; Jimenez-Gonzalez et al., 2012). Algunos estudios han informado que la capacidad patogénica de *Blastocystis* de producir o no sintomatología depende del subtipo (ST) colonizante de *Blastocystis* (Clark, 1997; Alfellani et al., 2013).

A nivel mundial se ha descrito la gran diversidad de *Blastocystis.* La evidencia molecular basada en el gen de la subunidad pequeña del RNA ribosomal (rRNA SSU), sugiere que se pueden reconocer al menos 17 subtipos (STs) genéticos dentro de *Blastocystis* y nueve de estos subtipos se encuentran en humanos (Silberman JD, 1996 Stenzel y Boreham, 1996). Al momento, los subtipos ST1 a ST9 y ST12 han sido identificados en humanos, siendo el subtipo 3 el más frecuente en los pacientes

sintomáticos, seguido por el subtipo 1 y el 2 (Dograman-Al et al., 2009; Moosavi et al., 2012; Yoshikawa et al., 2004, Ramírez JD et al., 2016; Jimenez et al., 2019).

Los estudios sobre la clasificación taxonómica de *Blastocystis* han ayudado a conocer mejor a este microorganismo. Sin embargo, aún falta conocimiento sobre su diversidad genética, su distribución geográfica, la especificidad del huésped y su papel en el intestino humano. Por lo tanto, los estudios de epidemiología molecular de la infección por este protista nos ayudarían a comprender mejor el papel de *Blastocystis* en el proceso de salud y enfermedad.

Taxonomía, Morfología y Ciclo de Vida de *Blastocystis*

Desde el año1900 se tiene conocimiento de la existencia de *Blastocystis,* época en la cual se le consideraba como una levadura saprófita del tracto digestivo (1911-1912) (Alexeieff, 1991; Brumpt, 1912). Posteriormente, en 1967 se clasificó dentro del grupo de los protistas, pero no es hasta 1996, con el advenimiento de la biología molecular cuando taxonómicamente se clasificó dentro del grupo de los Stremenopiles, a través de la caracterización molecular de genes conservados (RNA ribosomal 18S, proteína HSP 70 y el factor de elongación alfa) (Stenzel et al., 1991; Silberman et al., 1996).

En un análisis microscópico, *Blastocystis* puede ser confundido con otros protistas intestinales, dada la variación morfológica de este organismo, el cual presenta cuatro formas morfológicas:

1) Forma vacuolar, tiene un tamaño de 2-200µm, y cuenta con 1 a 4 núcleos, posee una vacuola central que ocupa el 70 al 90% de su tamaño (Stenzel y Boreham, 1996).

2) Forma granular, tiene un tamaño de 6.5-8µm, con 1 a 4 núcleos, además contiene gránulos en la vacuola central los cuales pueden ser metabólicos y lípídicos (Boreham y Stenzel, 1993; Stenzel y Boreham, 1996).

3) Forma ameboide, mide entre 2.6-7.8µm, tiene 1 a 2 núcleos, esta forma raramente se reporta en el examen coproparasitoscópico debido a la dificultad de diferenciación con otros parásitos en la microscopía. Es la forma patogénica de *Blastocystis* (Tan y Suresh, 2006).

4) Forma quística de este parásito es la forma infectante que iniciará nuevamente el ciclo de vida de *Blastocystis*. Los quistes son de un tamaño entre 3-10 µm, y contiene 1-4 núcleos. El quiste tiene una pared, que puede estar rodeada por una capa fibrilar (Stenzel y Boreham, 1996).

En relación al ciclo de vida *de Blastocystis*, como otros microorganismos se adquiere por la de la ingestión de alimentos y/o agua contaminada con los quistes de *Blastocystis*, los cuales serán ingeridos y pasarán por el tracto digestivo del hospedero, al instalarse en el intestino *Blastocystis* iniciará su colonización, dando origen a sus diferentes formas morfológicas (Tan, 2004). Figura 1

Una particularidad de *Blastocystis* es el ser un microorganismo que infecta a diversos hospederos, por lo cual aumenta su grado de trasmisión, ya que los diferentes hospederos pueden estar colonizados por los mismos o diferentes subtipos *Blastocystis* (Alfellani et al., 2013; Alfellani et al., 2013a; Clark et al., 2013).

Ciclo de vida y morfología de *Blastocystis*
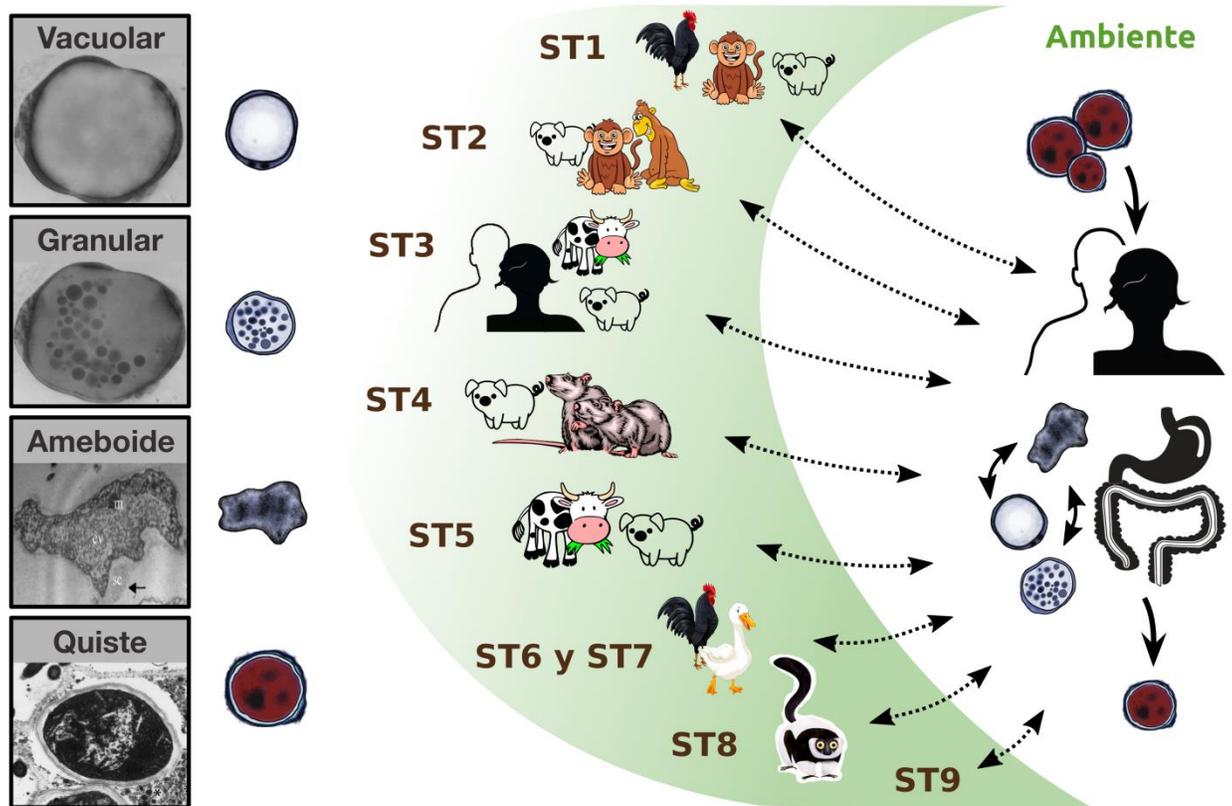
Figura 1 Adaptada de Tan KSW, 2004

Blastocystis: asociación clínica y su comportamiento como patógeno, comensal o mutualista en el tracto digestivo humano

Blastocystis tiene una distribución mundial, mostrando una alta frecuencia de infección en población humana en países subdesarrollados y desarrollados (Clark et al., 2013; Alfellani et al., 2013). Se estima que mil millones de humanos se encuentran colonizados por _Blastocystis_ (Tan, 2008). _Blastocystis_, al ser considerado como un parásito oportunista solo se reportaba en pacientes inmunocomprometidos e inmunosuprimidos, como los pacientes infectados con el VIH, quienes, debido a la inmunodeficiencia, podrían ser más susceptibles a la infección por parásitos que la población general (Zali et al, 2004; Hailemariam et al, 2004; Gassama et al, 2001).

Durante muchos años, en la práctica médica, _Blastocystis_ ha sido considerado como un agente infeccioso no patógeno, que carecía de importancia clínica. Sin embargo, varias publicaciones han referido la asociación _Blastocystis_ con el síndrome del intestino irritable (SII) (Jimenez-Gonzalez et al., 2012; Ramirez-Miranda et al., 2010; Yakoob et al., 2004), y también se ha detectado a _Blastocystis_ en pacientes con síntomas gastrointestinales como diarrea, dolor y distensión abdominal (Poirier et al., 2012; Beatty et al., 2014; Clark et al., 2013, Stensvold y Clark, 2016). Sin embargo, también existe otro gran número de reportes en donde _Blastocystis_ se comporta como un comensal del tracto digestivo en el huésped humano, al identificarse una alta prevalencia de esta infección en individuos asintomáticos aparentemente sanos (Pandey et al., 2015; Stensvold y Clark, 2016; Andersen y Stensvold, 2016).

Un punto poco abordado es que el humano puede ser colonizado con múltiples subtipos o haplotipos diferentes de _Blastocysts_, dada la existencia de diversas fuentes genéticas de infección. Estudios previos han abierto la posibilidad de competencia o

cooperación entre los subtipos de *Blastocystis*, que pueden ser un factor relevante para una colonización intestinal exitosa (Scanlan et al., 2015). Con base en estudios previos de la diversidad de subtipos de *Blastocystis* en humanos se ha estimado que la prevalencia mundial de infecciones mixtas por *Blastocystis* es del 6% (Scanlan et al., 2015, Maloney et al., 2019). Actualmente sólo el grupo de Scanlan (2015) ha analizado directamente las infecciones mixtas de subtipos de *Blastocystis* en humanos a través de un ensayo de reacción en cadena de la polimerasa (PCR) anidado para la identificar a los subtipos más comunes de *Blastocystis* (ST1 a ST4) en una cohorte humana sana (n = 50). Ellos encontraron que el 22% de las muestras positivas para *Blastocystis* que previamente habían caracterizado con un solo subtipo contenían múltiples subtipos, lo que nos indica que las infecciones mixtas de subtipo están subrrepresentadas (Scanlan et al., 2015). Por lo tanto, es necesario contar con más estudios que tengan como objetivo caracterizar las infecciones mixtas de subtipos y la variación dentro de los subtipos para comprender la dinámica de transmisión, la epidemiología y la patogenicidad de *Blastocystis* en humanos y animales, que nos ayude a comprender mejor su papel de *Blastocystis*, en particular si es realmente un patógeno, o sólo un comensal o incluso un mutualista en el intestino humano.

Diversidad genética y distribución geográfica de subtipos

Debido a las mejoras en biología molecular y al uso de la PCR ha sido posible estudiar la diversidad genética de *Blastocystis*. Se han identificado 17 subtipos (STs) usando como blanco el gen de la subunidad pequeña del RNA ribosomal (SSUrRNA). Dentro de los 17 subtipos descritos, solo 9 subtipos son capaces de infectar a los humanos. En relación con la variación genética entre los subtipos de *Blastocystis*, se considera entre un 3%-5% de divergencia entre un subtipo y otro para ser considerado como un nuevo subtipo (Silberman et al., 1996; Kukoschke y Müller, 1991; Boreham et al., 1992).

A nivel mundial, los subtipos con mayor frecuencia son ST1, ST2, ST3 y ST4 (Alfellani et al., 2013; Engsbro et al., 2014; Forsell et al., 2012). Sin embargo, la distribución geográfica no es homogénea: los subtipos 1, 2 y 3 presentan una distribución mundial, mientras que el subtipo 4 que parece ser más frecuente en Europa y Asia (Alfellani et al., 2013; Ramírez et al., 2016). Recientemente, Ramírez y colaboradores (2019) reportaron la distribución de subtipos en el sur y norte del continente Americano, identificando los 9 subtipos capaces de infectar al humano, los cuales también fueron encontrados en otros vertebrados, lo que nos habla de la gran capacidad zoonótica de transmisión de ese microorganismo (Jiménez et al., 2019). Los STs 1-3 fueron los de mayor frecuencia, al igual que en el resto del mundo. Y detectaron otros subtipos en muy bajas proporciones como es el caso de los STs 4, 7 y 8. Se ha hablado de que estos subtipos son de predomino en Europa, sin embargo, estos subtipos pueden deberse a migración o turismo, haciendo que estos subtipos sean introducidos en el continente Americano (Bühler et al., 2014; Hagmann et al., 2014; Cheong et al., 2009). Figura 2

Distribución geográfica de subtipos de *Blastocystis*

Europa

Alemania, Dinamarca, España, Francia, Grecia, Holanda, Irlanda, Italia, Polonia, Reino Unido, Suecia

Subtipos
1, 2, 3, 4, 6, 7, 8 y 9

Asia

Bangladesh, China, filipinas, Irán, Japón, Nepal, Malasia, Tailandia, Turquía, Singapur

Subtipos
1, 2, 3, 4, 6, 7

América

Estados Unidos, México, Colombia, Brasil, Ecuador, Perú, Bolivia, Chile, Argentina

Subtipos
1, 2, 3, 4, 5, 6, 7, 8, 12, Novel ST

África

Egipto, Libia, Nigeria, Tanzania

Subtipos
1, 2, 3, 6 y 7

Adaptado de: Alfellani MA et al., 2013; Ramírez JD et al., 2016; Jimenez et al., 2019.

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En México se cuentan con datos de la distribución de subtipos en cuatro estados (Ciudad de México, Michoacán, Sonora y Estado de México), en donde ST3 es el que ocupa el mayor porcentaje (42.14%) seguido de los subtipos 1 (38.01%), 2 (18.18%) y 7 (1.65%) (Villalobos et al., 2014, Villegas-Gómez et al., 2015; Vargas-Sanchez et al., 2015; Alarcon-Valdes et al., 2018). Figura 3

Para mejorar la información sobre la distribución de subtipos en México es necesario un muestreo de más estados y hospederos que nos permitan evaluar la distribución real de los subtipos de *Blastocystis* ya reportados y la probable existencia de nuevos subtipos para poder entender mejor su papel y epidemiología.

Distribución geográfica de subtipos de *Blastocystis* en México
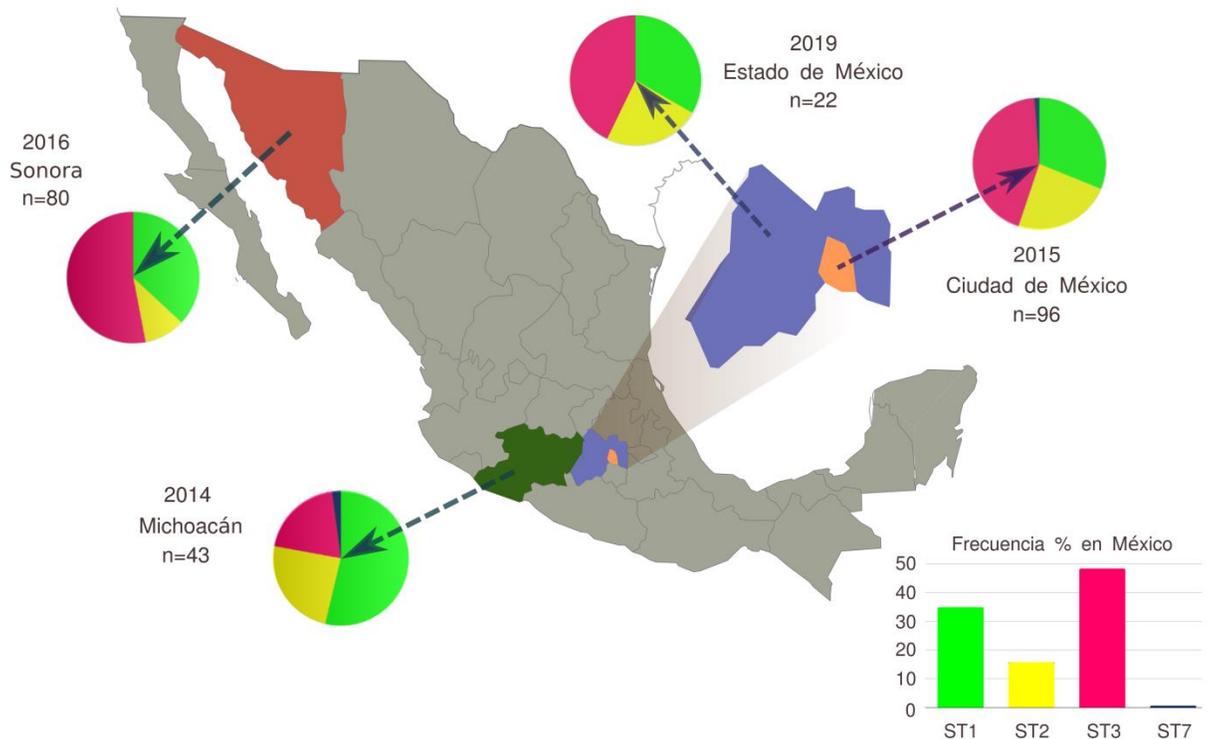
Villalobos G et al., 2014 ; Vargas GB et al., 2015; Villegas I et al., 2016, Alarcon-Valdes P et al., 2018.

Area de estudio: Comunidad de Xoxocotla

Xoxocotla pertenece al municipio de Puente de Ixtla en Morelos, en las coordenadas geográficas latitud 18°41′06″N y longitud 99°14′38″O, con una elevación promedio de 700 m sobre el nivel del mar. Se encuentra a 120 Km de la Ciudad de México y 70 kilómetros la ciudad de Cuernavaca. Su territorio comprende un área de 29,917 Km^2. Tiene un clima tropical (cálidos-subhúmedo).

En Xoxocotla viven 21,074 personas de las cuales 10,668 son del género masculino y 10,606 del femenino. Hay 11,635 ciudadanos mayores de 18 años, 1187 con 60 años o más. Sus habitantes tiene un promedio de 4 años de escuela y 2678 personas mayores de

15 años tienen educación post-básica. Entre las personas de 15 años o más se encuentran unos 2123 analfabetas. Hay un total de 4069 hogares en Xoxocotla. De estos hogares 1945 hogares tienen piso de tierra y 563 consisten en un cuarto solo, SEDESOL en el 2013 considero que el 58.6% de la población se encontraba en pobreza extrema (SEDESOL, INEGI).

Justificación

Blastocystis es un parásito intestinal común que se encuentra en humanos y muchos otros vertebrados. Se le ha asociado con la presencia de síntomas gastrointestinales como diarrea, dolor y distensión abdominal, sin embargo, la alta prevalencia de esta infección en individuos asintomáticos aparentemente sanos, dificulta establecer el verdadero comportamiento de *Blastocystis* en el tracto digestivo en el humano. La prevalencia y distribución de sus subtipos se han descrito en poblaciones humanas del mundo, sin embargo, aún faltan estudios moleculares de *Blastocystis* en América del Norte y en particular para México. Es necesario contar con mas estudios que tengan como objetivo el estudio de este microorganismo para comprender su dinámica de transmisión, su epidemiología y patogenicidad de *Blastocystis* que nos ayuden a comprender si es un patógeno, un comensal o incluso un mutualista en el intestino humano.

Objetivo general

Determinar la prevalencia, distribución y diversidad genética de *Blastocystis* y su asociación con sintomatología grastrointestinal, en la población rural de la comunidad de Xococotla en el estado de Morelos, México.

Objetivos particulares

- Determinar la frecuencia de *Blastocystis* e identificar los subtipos prevalentes e infecciones mixtas de subtipos de *Blastocystis* en la comunidad de Xoxocotla.

- Analizar la posible asociación entre los diferentes subtipos de *Blastocystis* y la presentación de trastornos gastrointetinales en la población estudiada.

- Analizar la distribución y diversidad genética de los subtipos identificados en la población de Xoxocotla.

Use of next generation amplicon sequencing to study *Blastocystis* genetic diversity in a rural human population from Mexico

Liliana Rojas-Velázquez, Jenny G. Maloney, Aleksey Molokin, Patricia Morán, Angélica Serrano-Vázquez, Enrique González, Horacio Pérez-Juárez, Cecilia Ximénez, Monica Santin. Use of next generation amplicon sequencing to study Blastocystis genetic diversity in a rural human population from Mexico. Parasites Vectors 12, 566 (2019) https://doi.org/doi:10.1186/s13071-019-3814-z

Resumen

El parásito intestinal *Blastocystis* se encuentra en humanos y animales de todo el mundo. Se propaga a través del consumo de alimentos y/o agua contaminadas, y se ha asociado con una variedad de síntomas intestinales. *Blastocystis* es uno de los parásitos intestinales más comunes en humanos, sin embargo, su prevalencia y distribución en humanos en América del Norte no está bien caracterizada. Para este estudio se analizó el DNA extraído de muestras fecales obtenidas de 182 habitantes de una población rural en México y se aplicó la secuenciación de amplicones de próxima generación de una región del gen de la subunidad pequeño del RNA ribosomal (rRNA SSU) de *Blastocystis*, para caracterizar la prevalencia, distribución de subtipos y diversidad de subtipos en humanos. De las 182 muestras analizadas en este estudio, el 68.1% (124) contenía uno o más subtipos de *Blastocystis*. El subtipo 3 fue el subtipo más común observado, encontrándose en el 81.5% de las muestras positivas en esta población, seguido de el subtipo 1 (16.9%) y subtipo 2 (17.7%) y se observaron infecciones mixtas en el 13,7% de las muestras positivas. En cuanto a los resultados obtenidos, las probabilidades de tener *Blastocystis* aumentaron en la edad adulta (> 15 años; OR: 1.72, P <0.0001), y las probabilidades de tener el subtipo 1 aumentaron en presencia de animales de granja (OR: 1.51, P = 0.03). Las probabilidades de tener subtipo 1, subtipo 2 o una infección mixta disminuyeron en presencia de pisos de cemento (OR: -1.61, P = 0.005; OR: -1.14, P = 0.03; OR: -1.48, P = 0.02), posiblemente indicando que los factores socioeconómicos están involucrados en el riesgo de adquirir uno de estos subtipos. En conclusión estos datos contribuyen a nuestra comprensión de la epidemiología de la infección por *Blastocystis* en humanos y pueden utilizarse para diseñar futuros estudios que tengan como objetivo caracterizar mejor las vías de transmisión y los resultados de salud de las infecciones por *Blastocystis*.

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Use of next-generation amplicon sequencing to study *Blastocystis* genetic diversity in a rural human population from Mexico

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Abstract

Background: The intestinal parasite *Blastocystis* is found in humans and animals around the world. It is spread through the consumption of contaminated food and water and has been associated with a variety of intestinal symptoms. *Blastocystis* is one of the most common intestinal parasites in humans, yet its prevalence and distribution in humans in North America is not well characterized.

Methods: Next-generation amplicon sequencing of a region of the *Blastocystis SSU* rRNA gene was applied to DNA extracted from fecal specimens obtained from 182 inhabitants of a rural population in Mexico to characterize *Blastocystis* prevalence, subtype distribution, and intra-host subtype diversity in humans.

Results: Of the 182 samples tested in this study, 68.1% (124) contained one or more *Blastocystis* subtypes. Subtype 3 was the most common subtype observed and was found in 81.5% of the positive samples. Subtype 1, 16.9% of the positive samples, and subtype 2, 17.7% of the positive samples, were also found in this population. Mixed infections were observed in 13.7% of the positive samples. In this population, the odds of having *Blastocystis* increased in adulthood (> 15 years; OR: 1.72, *P* < 0.0001), and the odds of having subtype 1 increased in the presence of farm animals (OR: 1.51, *P* = 0.03). The odds of having subtype 1, subtype 2, or a mixed infection decreased in the presence of cement flooring (OR: − 1.61, *P* = 0.005; OR: − 1.14, *P* = 0.03; OR: − 1.48, *P* = 0.02) possibly indicating socioeconomic factors are involved in the risk of acquiring one of these subtypes.

Conclusions: These data contribute to our understanding of the epidemiology of *Blastocystis* infection in humans and can be used to shape future studies which aim to better characterize the transmission pathways and health outcomes of *Blastocystis* infections.

Keywords: *Blastocystis*, Human, Mexico, Mixed infections, Next-generation sequencing, Risk factors, Subtypes

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Background

Blastocystis is a cosmopolitan enteric parasite found in humans and a wide range of animals across the world. Currently, *Blastocystis* is the most common intestinal parasite in humans in developing and developed countries [1]. However, the role of *Blastocystis* as a pathogen is still controversial, mainly because it is found in both patients suffering with intestinal symptomatology, such as diarrhea, flatulence, bloating or abdominal discomfort, as well as in healthy people [2]. *Blastocystis* has also been associated with irritable bowel syndrome and with cutaneous symptoms (urticaria) [2, 3]. *Blastocystis* is transmitted *via* the fecal-oral route either indirectly through ingestion of food or water contaminated with cysts or directly by contact with infected persons or animals [4].

A high degree of genetic diversity has been found among *Blastocystis* isolates based on nucleotide differences in the small subunit (*SSU*) of the ribosomal RNA (rRNA) gene. So far, at least 26 subtypes (STs) have been proposed [5–7]. Ten subtypes, ST1-ST9 and ST12, have been reported in humans, and of these subtypes all but ST9 are also found in other mammalian and avian hosts indicating the potential for zoonotic transmission [8, 9]. Of the ten subtypes reported in humans, ST1 to ST4 are most commonly found in humans worldwide [10]. *Blastocystis* in humans in North America is not well characterized at the molecular level. In fact, in the USA, only one molecular survey of *Blastocystis* has been performed in humans and found ST1, ST2 and ST3 were all present in humans from Colorado [11]. In Mexico, most molecular studies have been conducted in patient populations to understand the association between *Blastocystis* and irritable bowel syndrome and have reported ST1, ST2, ST3 and ST7 in humans [12, 13].

Molecular characterization to identify subtypes present in samples is critical to unravel *Blastocystis* epidemiology and to characterize subtype level differences in host specificity, transmission, public health significance, and pathogenicity. Mixed subtype infections are often overlooked in molecular studies of *Blastocystis*, and a better characterization of these infections is needed to fully understand the epidemiology of *Blastocystis*. Recently, it was demonstrated that next-generation amplicon sequencing is a powerful tool to investigate mixed infections and detect low abundance subtypes of *Blastocystis* [14]. The aim of the present study was to investigate *Blastocystis* in a rural population from Mexico using next-generation amplicon sequencing to better characterize *Blastocystis* prevalence, subtype distribution, and intra-host subtype diversity in humans and to evaluate potential association of socioeconomic factors with *Blastocystis* infection in this population.

Methods

Study population

One hundred and eighty-two volunteers living in the community of Xoxocotla, State of Morelos (Mexico) participated in the study conducted between May and November 2014 that included 86 males, 96 females, 66 children ($\leq$ 15 years-old), and 116 adults (> 15 years-old) with age ranging from 2 to 51 years (median age of 23 years). Each participant provided three fecal samples that were collected on three consecutive days. The samples were maintained at 4 °C and transported to the laboratory in Mexico City. The sample size was calculated considering the total number of inhabitants in Xoxotla (21,074). The minimum required sample size was calculated to be 96 individuals based on an expected frequency of intestinal parasitic infection of 50%, the worst acceptable level was 10%, the confidence level was 95%, and the results were considered statistically significant when $P < 0.05$.

DNA extraction

A similar volume of the three samples provided by each participant was combined and mixed thoroughly. Then, an aliquot of 250 mg from the mixture was used to extract genomic DNA using the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) per manufacturer's instructions. DNA was stored at -20 °C until further molecular analysis.

Molecular detection, NGS amplicon library preparation and bioinformatic analysis

Next-generation amplicon sequencing libraries were prepared as previously described [6]. Briefly, all samples were screened by PCR using primers ILMN_Blast505_532F and ILMN_Blast998_1017R. These primers amplify a region of the *SSU* rRNA gene and are identical to Blast505_532F/Blast998_1017R [15], with the exception of containing the Illumina overhang adapter sequences on the 5′-end. Final libraries were quantified using the Quant-iT dsDNA Broad-Range Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) on a SpectraMax iD5 (Molecular devices, San Jose, CA, USA) prior to normalization. A final pooled library concentration of 8 pM with 20% PhiX control was sequenced using Illumina MiSeq 600 cycle v3 chemistry (Illumina, San Diego, CA, USA). Paired end reads were processed and analyzed with an in-house pipeline that uses the BBTools package v38.22 [16], VSEARCH v2.8.0 [17], and BLAST+ 2.7.1. After removing singletons, clustering and the assignment of centroid sequences to operational taxonomic units (OTU) was performed within each sample at a 98% identity threshold. Only those OTUs with a minimum of 100 sequences were retained. All raw fastq files

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were deposited to the NCBI sequence read archive under the accession number PRJNA523857. The nucleotide sequences for unique OTUs obtained in this study have been deposited in GenBank under the accession numbers MK874780-MK874822.

Sociodemographic variables

At the time of providing the samples, a questionnaire was administered to collect information on the following variables: age (child $\leq$ 15 years-old or adult > 15 years-old), gender (male or female), presence of symptoms (present or absent), type of flooring (dirt or cement), water source (city or other source), sewage disposal (in-house or other disposal), presence of animals (livestock, poultry, or companion), and presence of house pests (present or absent). The status of symptomatic was defined per the ROME III criteria commonly used by clinicians to classify gastrointestinal disorders.

Data analysis

Logistic regression analysis was used to identify factors associated with *Blastocystis* infection. The following demographic and socioeconomic variables were included: age (child or adult), presence of symptoms defined as answering yes to one or more Rome III criteria (asymptomatic or symptomatic), type of flooring (dirt or cement), water source (city water or other source), sewage disposal (in-house or other source), presence of domestic animals (yes or no), presence of farm animals (yes or no), presence of chickens (yes or no), and presence of house pests (yes or no). Collected sociodemographic information for this population is presented in Table 1. P-values < 0.05 were considered statistically significant. Statistical analyses were performed using R version 3.5.1 (R Core Team, 2018).

Results

Blastocystis prevalence

Of the 182 samples screened 68.1% (124) were found to contain *Blastocystis* by PCR. A higher prevalence was observed in adults (81.9%; 95/116) than in children (43.9%; 29/66), and in females (62%; 54/87) than in males (74%; 70/95) (Table 1). A similar prevalence was found for asymptomatic (68%; 98/144) and symptomatic (68%; 26/38) participants (Table 1 and Fig. 1).

Blastocystis subtypes identified using next-generation amplicon sequencing

All 124 PCR-positive samples were sequenced using the MiSeq platform. A total of 17,514,676 read pairs were generated from the samples included in this study with an average of 141,247 reads per sample. Following trimming, pair merging, and quality filtering there were a total of 4,968,142 merged reads. After chimera filtering 4,784,056 remained. Clustering yielded 176 *Blastocystis* OTUs across the 124 *Blastocystis*-positive samples of which 43 (24.4%) OTUs were unique (Table 2).

Three *Blastocystis* subtypes (ST1, ST2 and ST3) were detected in this study. Mono-subtype infections were more common than mixed infections (more than one subtype present in a single sample) representing 86.3% ($n = 107$) and 13.7% ($n = 17$) of the positive samples, respectively (Table 3; Additional file 1: Table S1). Subtype 3 was the most frequently observed subtype in this population and was found in 81.5% ($n = 101$) of the positive samples either as mono-infection ($n = 84$) or a mixed infection ($n = 17$) (Table 3). Subtypes 1 and 2 were observed in 16.9% ($n = 21$) and 17.7% ($n = 22$) positive samples, respectively, as either mono-infections (9 ST1 and 14 ST2) or mixed (12 ST1 and 8 ST2). A mix of ST1 and ST3 was the most common subtype combination and was found in 52.9% ($n = 9$) of the mixed infection samples. A mix of ST2 and ST3 was observed in 29.4% ($n = 5$) of mixed infections, and a mix of ST1, ST2 and ST3 was observed in 17.6% ($n = 3$) of the mixed infection samples. No ST1 and ST2 mixed infections were detected (Table 3).

Intra-subtype variability

Forty-three unique OTUs were detected among the three *Blastocystis* subtypes present in this study. Subtype 1 and 2 had similar intra-subtype diversity in this study with 15 unique OTUs among 21 ST1-positive samples and 16 unique OTUs among the 22 ST2-positive samples (Table 2). Subtype 3 displayed the least intra-subtype diversity with only 12 unique OTUs among 101 ST3-positive samples. Samples frequently contained multiple unique OTUs of ST1 and ST2, and up to three unique OTUs of ST1 or ST2 were detected in individual samples (Table 3). However, multiple OTUs of ST3 were not observed in the same sample. Furthermore, while unique OTUs of ST1 and ST2 were relatively evenly distributed among individual samples, two unique OTUs of ST3 were dominant in this population and were observed in 81 of 101 *Blastocystis* ST3-positive samples (Table 2).
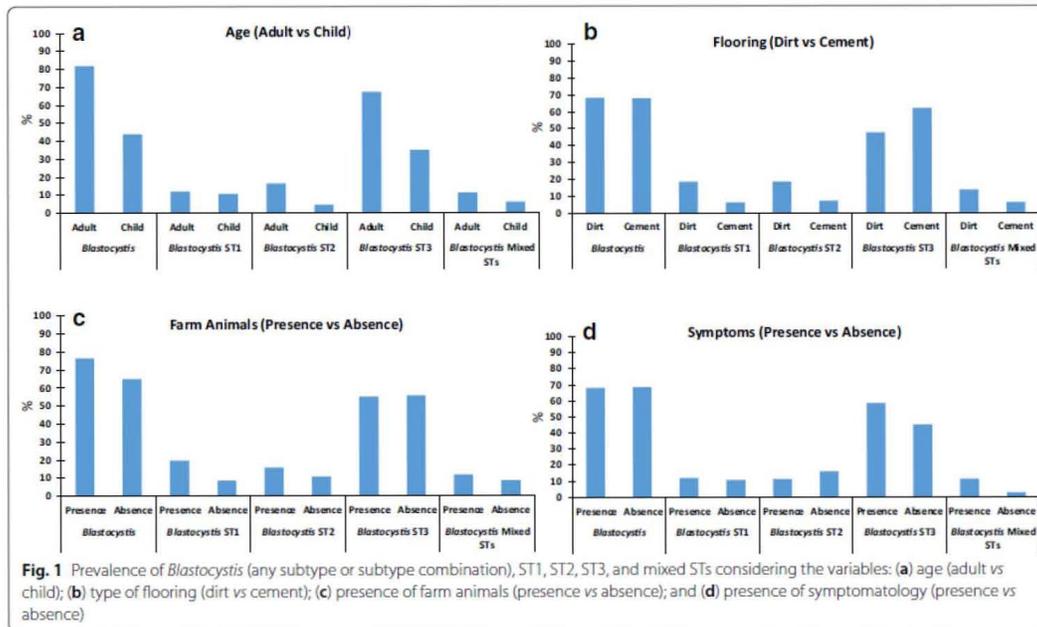
Association between sociodemographic variables and presence of *Blastocystis*

Logistic regression analyses were performed to determine if any associations existed between *Blastocystis* infection and gender, adulthood, presence of symptoms, type of flooring, water source, sewage disposal, presence of domestic animals, presence of farm animals, presence

Table 1 Sociodemographic variables studied by logistic regression analysis

Variable	Blastocystis			Blastocystis ST1			Blastocystis ST2			Blastocystis ST3			Blastocystis Mixed STs		
	Pos/Neg	P-value	Log odds	Pos/Neg	P-value	Log odds	Pos/Neg	P-value	Log odds	Pos/Neg	P-value	Log odds	Pos/Neg	P-value	Log odds
Age															
≤ 15 years	95/21	**<0.0001**	**1.72**	14/103	0.9	−0.09	19/97	**0.02**	**1.60**	78/38	**0.0003**	**1.36**	13/103	0.4	0.52
> 15 years	29/37			7/59			3/63			23/43			4/62		
Gender															
Male	54/33	0.07	0.66	9/78	0.7	0.23	12/75	0.5	−0.36	45/42	0.2	0.39	10/77	0.3	−0.62
Female	70/25			12/83			10/85			39/56			7/88		
Symptoms															
Asymptomatic	98/46	0.2	−0.58	17/127	0.3	−0.70	16/128	0.9	0.07	84/60	**0.04**	**−0.88**	16/128	0.08	−2.00
Symptomatic	26/12			4/34			6/32			17/21			1/37		
Flooring															
Dirt	56/26	1	−0.02	15/67	**0.005**	**−1.61**	15/67	**0.03**	**−1.14**	39/43	0.08	0.61	11/71	**0.02**	**−1.48**
Cement	68/32			6/94			7/93			62/38			6/94		
Water															
City	101/44	0.6	−0.26	16/129	0.7	−0.24	17/128	0.7	−0.18	85/60	0.4	−0.39	15/130	0.1	−1.45
Other	23/14			5/32			5/32			16/21			2/35		
Sewer system															
In-house	26/12	0.9	0.05	2/36	0.1	1.73	4/34	0.6	0.42	22/16	0.9	−0.04	2/36	0.1	1.42
Other	98/46			19/125			18/126			79/65			15/129		
Domestic animals															
Presence	75/30	0.4	0.68	14/91	0.2	2.19	16/89	0.2	2.39	56/49	0.9	−0.14	9/96	0.2	2.08
Absence	49/28			7/70			6/71			45/32			8/69		
Farm animals															
Presence	39/12	0.2	0.57	10/41	**0.03**	**1.51**	8/43	1	−0.03	28/23	0.8	0.13	6/45	0.3	0.77
Absence	85/46			11/120			14/117			73/58			11/120		
Poultry															
Presence	10/3	0.7	0.27	2/11	0.6	0.57	3/10	0.4	0.89	6/7	0.6	−0.38	1/12	0.9	0.25
Absence	114/55			19/150			19/169			95/74			16/153		
House pests															
Presence	80/33	0.5	−0.53	14/99	0.1	−2.55	16/97	0.2	−2.02	61/52	1	0	9/104	0.1	−2.60
Absence	44/25			7/62			6/63			40/29			8/61		

Abbreviations: Pos/Neg, Positive/Negative

P < 0.05 are in bold

Fig. 1 Prevalence of *Blastocystis* (any subtype or subtype combination), ST1, ST2, ST3, and mixed STs considering the variables: (**a**) age (adult *vs* child); (**b**) type of flooring (dirt *vs* cement); (**c**) presence of farm animals (presence *vs* absence); and (**d**) presence of symptomatology (presence *vs* absence)

of chickens, or presence of house pests (Table 1). Adulthood was the only variable with a statistically significant association with *Blastocystis* infection (any subtype or subtype combination) (Fig. 1a). The odds of having *Blastocystis* was greater in the adult category (OR: 1.72, 95% CI: 0.95–2.49, $P < 0.0001$), and adulthood also had a statistically significant association with being ST2- and ST3-positive when these outcomes were considered individually (OR: 1.6, 95% CI: 0.23–2.97, $P = 0.02$; OR: 1.36, 95% CI: 0.62–2.09, $P = 0.0003$) (Fig. 1a). Flooring type was also statistically significant for the outcomes of mixed STs, ST1- and ST2-positive (OR: -1.48, 95% CI: -2.70 to -0.27, $P = 0.02$; OR: -1.61, 95% CI: -2.74 to -0.48, $P = 0.005$; OR: -1.14, 95% CI: -2.17 to -0.11, $P = 0.03$) (Fig. 1b). The odds of having mixed STs, ST1 or ST2 infection were all found to be lower if a sample came from a person living in a home with a cement floor. This effect was not observed for *Blastocystis* infection (any subtype or subtype combination) or ST3 infections. The presence of farm animals increased the odds of being ST1-positive (OR: 1.51, 95% CI: 0.18–2.84, $P = 0.03$) (Fig. 1c). Being symptomatic decreased the odds of being ST3 positive, although the association was weak (OR: -0.88, 95% CI: -1.73 to -0.03, $P = 0.04$) (Fig. 1d). No other statistically significant associations between *Blastocystis* infection status or individual subtypes were found.

Discussion

Blastocystis is a common intestinal parasite found in humans and many other animals, and its prevalence and subtype distribution have been described in human populations from around the world [10]. However, molecular studies of *Blastocystis* in North America remain lacking. This study describes the prevalence and subtypes of *Blastocystis* present in a rural population in Mexico for which data on socioeconomic and demographic variables was also collected. This study also represents the first application of next-generation amplicon sequencing (NGS) of the *Blastocystis SSU* rRNA gene to a human population to characterize intra-host subtype diversity.

Fecal samples from 182 humans ranging in age from 2 to 51 years from a single rural community in Mexico were screened for the presence of *Blastocystis* by PCR of the *SSU* rRNA gene follow by NGS to determine subtypes. One or more *Blastocystis* subtype was found in 68.1% of the samples. While this prevalence is on par with other studies of *Blastocystis* prevalence in rural populations from both the Americas and other regions of the world, it is on the higher end of the reported prevalence of *Blastocystis* in humans. Diagnostic methods used in different studies could account for prevalence differences. Indeed, a lower prevalence was reported for *Blastocystis* for this same study population

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Table 2 Unique operational taxonomic units (OTUs) obtained for *Blastocystis* subtypes by next generation amplicon sequencing

ST	No. of unique OTUs per subtype	Unique OTU ID# (GenBank ID)	No. of samples containing OTU
ST1	15	1a (MK874787)	5
		1b (MK874795)	4
		1c (MK874813)	4
		1d (MK874786)	3
		1e (MK874810)	3
		1f (MK874789)	2
		1g (MK874797)	2
		1h (MK874807)	2
		1i (MK874796)	1
		1j (MK874798)	1
		1k (MK874802)	1
		1l (MK874816)	1
		1m (MK874817)	1
		1n (MK874819)	1
		1o (MK874822)	1
ST2	16	2a (MK874794)	7
		2b (MK874785)	6
		2c (MK874792)	5
		2d (MK874793)	4
		2e (MK874806)	4
		2f (MK874790)	3
		2g (MK874803)	3
		2h (MK874814)	2
		2i (MK874815)	2
		2j (MK874804)	1
		2k (MK874805)	1
		2l (MK874808)	1
		2m (MK874809)	1
		2n (MK874811)	1
		2o (MK874818)	1
		2p (MK874821)	1
ST3	12	3a (MK874780)	60
		3b (MK874781)	21
		3c (MK874782)	4
		3d (MK874783)	4
		3e (MK874784)	4
		3f (MK874801)	2
		3g (MK874788)	1
		3h (MK874791)	1
		3i (MK874799)	1
		3j (MK874800)	1
		3k (MK874812)	1
		3l (MK874820)	1

using microscopy (59.9%;109/182) with molecular confirmation in only 66% of those 109 microscopy positives [18].

From the 124 positive samples in this study, subtypes 1, 2 and 3 were found. These three subtypes are frequently reported in humans in the Americas and around the world [10]. Subtype 3 was the dominant subtype in this population, and was observed in 81.5% of *Blastocystis*-positive samples and 55.5% of the study population. Subtypes 1 and 2 were observed much less frequently in 16.9% and 17.7% of the positive samples, respectively. These results are similar to observations of *Blastocystis* subtypes from a multi-country study in South America and a study in Brazil where ST3 was the most prevalent subtype found among *Blastocystis*-positive human samples followed by ST1 and ST2 [8, 19]. Both studies reported other subtypes in low numbers, ST4-ST8, ST12, and novel subtypes in the multi-country study and ST4, ST6 and ST8 in the study from Brazil. However, no other *Blastocystis* subtypes were observed in the samples in this study. Similarly, in a study in the USA that examined 50 family units (101 adults and 38 children/adolescents) from Colorado only subtypes ST1 (20%), ST2 (30%), and ST3 (50%) were identified [11]. In a survey of intestinal parasites in members of the Tapirapé ethnic group from the Brazilian Amazon region, only ST1, ST2 and ST3 were detected, but in their study ST1 was the most frequently identified subtype [20]. These differences between studies could be due to the geographical restrictions of some studies and may reflect population level or climatic influences on risk of infection with different *Blastocystis* subtypes.

The worldwide prevalence of *Blastocystis* mixed infections has been estimated to be 6% from previous studies on *Blastocystis* subtype diversity in humans [21]. This topic is not well explored, and only one study has directly addressed *Blastocystis* mixed subtype infections in humans [22]. By using a nested PCR assay that can identify subtypes 1 through 4, Scanlan et al. [22] demonstrated that 22% of *Blastocystis*-positive samples previously shown to contain a single subtype contained multiple *Blastocystis* subtypes. An advantage of NGS is its ability to assess intra-host subtype diversity [6]. The present study identified 17 mixed infections representing 13.7% of all *Blastocystis* infections in the population. Mixed subtype infections are underrepresented compared to expectations from subtype prevalence. However, mixed infections in our study were within the range of observations from other human studies [21,

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Table 3 *Blastocystis* prevalence for each subtype in mono-infections and for the different subtype combinations in mixed subtype infections

	Mono subtype infections				Mixed subtype infections			
	ST1 only	ST2 only	ST3 only	Total mono-infections	ST1/ST3 mix	ST2/ST3 mix	ST1/ST2/ST3 mix	Total mixed infections
Total positive samples	9	14	84	107	9	5	3	17
Percentage of all samples	4.9	7.7	46.2	58.8	4.9	2.7	1.6	9.2
Percentage of positive samples	7.3	11.3	67.7	86.3	7.3	4.0	2.4	13.7
Percentage of mixed samples	na	na	na	na	52.9	29.4	17.6	na

Abbreviations: na, not applicable

22]. Combinations of ST1 + ST3, ST2 + ST3, and ST1 + ST2 + ST3 were all observed. ST3 was observed in all mixed *Blastocystis* infections, ST1 in 12 mixed infections, and ST2 in eight mixed infections. Although the population studied here was limited in its *Blastocystis* subtype diversity, these data support the use of NGS for exploring mixed subtype infections in humans.

Intra-subtype variability could play a role in understanding *Blastocystis* transmission and pathogenicity [6, 14, 23–25]. In the present study, intra-subtype variability varied widely between the subtypes. While ST1 and ST2 had high proportions of unique sequences, ST3 was surprisingly homogeneous. Unique sequences represented 71.4% of ST1 sequences and 72.7% of ST2 sequences, but only 11.9% of ST3 sequences were unique. Two OTUs of ST3 also dominated in this population representing 80.2% of all ST3 observations. This lower level of intra-subtype variability has been reported before for ST3 both in humans and cattle [6, 26]. There was also no within-host variation in ST3 despite multiple OTUs of ST1 or ST2 being frequently observed in the same host. The homogeneity of ST3 in this population may indicate that ST3 is highly endemic in this community and may be acquired from a common source or may pass more easily between humans than the other subtypes observed in this study.

In previous studies *Blastocystis* infection has been associated with factors such as age, animal contact, and sanitation practices [27–29]. To better understand what factors might influence *Blastocystis* infection risk in this population, demographic and socioeconomic data was collected and logistic regression analysis was used to determine if any associations exist between having *Blastocystis*, mixed STs, ST1, ST2 or ST3 infections and any of these factors. Because only two of the 43 unique OTUs detected in this study were found in more than 10 samples, statistical analyses attempting to link unique OTUs with risk factors were not performed.

In this population, the only factor associated with having *Blastocystis* was adulthood, with the odds of being *Blastocystis*-positive being greater in the adult (> 15 years-old) category (OR: 1.72, $P \leq 0.0001$). In the present study, 116 of the 182 samples were adults (> 15 years-old) and 81.9% were *Blastocystis*-positive while 43.9% of the 66 children were *Blastocystis*-positive. Age has been indicated as a risk factor for *Blastocystis* in other studies, and human infants and young animals tend to have lower infection rates than adults [6, 29–31]. Adulthood was also associated with increased odds of having an ST2 or an ST3 infection (OR: 1.6, $P = 0.02$; OR: 1.36, $P = 0.03$), but this association was not observed for mixed or ST1 infections. These results could indicate that behavioral or physiological differences between adults and children may be important in determining infection risk for specific subtypes of *Blastocystis*.

Flooring material was significantly associated with mixed, ST1, and ST2 infections, and the odds of having one of these types of infections was decreased in homes with cement floors (OR: − 1.48, $P = 0.02$; OR: − 1.61, $P = 0.005$; OR: − 1.14, $P = 0.03$). This outcome may capture some socioeconomic effect such as improved hygiene not directly measured in this study as socioeconomic status has been associated with infection risk previously [30]. Having farm animals significantly increased the odds of having ST1 (OR: 1.51, $P = 0.03$). No other subtypes were associated with animals in this study. These results may indicate that zoonotic transmission of ST1 occurs in this population although data on the subtypes circulating in the animals owned or handled by ST1-positive individuals would be necessary to confirm this finding. Notably, artiodactyls such as pigs and cattle are the second most common host reported for ST1 after humans, further supporting the potential for zoonotic transmission of this subtype [10].

Being symptomatic, defined as answering yes to one or more Rome III criteria, was significantly associated

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with ST3 infection, although the association was negative (OR: -0.88, $P = 0.04$). Being symptomatic decreased the odds of having ST3. This association while significant was not strong and caution should be taken in overinterpretation of this result as associations between subtypes and symptomatology is still unclear. Some studies have indicated that there is no association between diarrhea and ST1, ST2 or ST3, but ST4 is associated with diarrhea and irritable bowel syndrome [32–34]. However, ST1 and ST3 have been associated with intestinal symptoms in other studies [35, 36].

Subtype 1 has been found in drinking water in Thailand and river water in Nepal, and waterborne transmission of *Blastocystis* to humans was suspected in these studies [28]. In the present study, no statistically significant association was found between infection and water source indicating that transmission in this community may occur through other routes. No statistically significant associations were found between any infection outcomes and sewage disposal, presence of domestic animals, presence of chickens, or presence of house pests.

Conclusions

This study provides important information about the epidemiology of *Blastocystis* and represents the first application of a *Blastocystis*-specific NGS protocol to study *Blastocystis* in humans. Although the study population described had a relatively homogenous *Blastocystis* subtype community, infection status and individual subtypes could still be linked to specific risk factors. More studies which aim to characterize mixed subtype infections and intra-subtype variation are needed to understand the transmission dynamics, epidemiology, and pathogenicity of *Blastocystis* in humans and animals. NGS provides a valuable tool for achieving this goal.

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s13071-019-3814-z.

Additional file 1: Table S1. Blastocystis subtypes relative abundance in positive samples identified by next generation amplicon sequencing.

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Table 3. Unique operational taxonomic units (OTUs) obtained for *Blastocystis* subtypes by next generation amplicon sequencing.

ST	No. of unique OTUs per subtype	Unique OTU ID# (GenBank Accession Number)	No. of samples containing OTU
ST1	15	1a (MK874787)	5
		1b (MK874795)	4
		1c (MK874813)	4
		1d (MK874786)	3
		1e (MK874810)	3
		1f (MK874789)	2
		1g (MK874797)	2
		1h (MK874807)	2
		1i (MK874796)	1
		1j (MK874798)	1
		1k (MK874802)	1
		1l (MK874816)	1
		1m (MK874817)	1
		1n (MK874819)	1
		1o (MK874822)	1
ST2	16	2a (MK874794)	7
		2b (MK874785)	6
		2c (MK874792)	5
		2d (MK874793)	4
		2e (MK874806)	4
		2f (MK874790)	3
		2g (MK874803)	3
		2h (MK874814)	2
		2i (MK874815)	2
		2j (MK874804)	1

		2k (MK874805)	1
		2l (MK874808)	1
		2m (MK874809)	1
		2n (MK874811)	1
		2o (MK874818)	1
		2p (MK874821)	1
ST3	12	3a (MK874780)	60
		3b (MK874781)	21
		3c (MK874782)	4
		3d (MK874783)	4
		3e (MK874784)	4
		3f (MK874801)	2
		3g (MK874788)	1
		3h (MK874791)	1
		3i (MK874799)	1
		3j (MK874800)	1
		3k (MK874812)	1
		3l (MK874820)	1

Genetic Diversity and Distribution of *Blastocystis* Subtype 3 in Human Populations, with Special Reference to a Rural Population in Central Mexico

Resumen

Blastocystis subtipo 3 (ST3) es un protista parasitario que se encuentra en el tracto digestivo de humanos sintomáticos y asintomáticos en todo el mundo. Si bien este parásito exhibe una alta prevalencia en la población humana, su distribución geográfica y diversidad genética global aún se desconocen. Esta brecha en el conocimiento limita la comprensión de los mecanismos de propagación, la epidemiología y el impacto que este parásito tiene en las poblaciones humanas. Aquí proporcionamos nuevos datos sobre la distribución geográfica y la diversidad genética de *Blastocystis* ST3 de una población rural de México. Para hacerlo recolectamos muestras fecales de esta población y amplificamos una parte de la región del gene de la subunidad pequeña del rRNA ribosomal (SSU-DNAr) y comparamos su diversidad genética y estructura poblacional con la observada previamente en poblaciones de *Blastocystis* ST3 de otras regiones del mundo. Los resultados de la diversidad genética de *Blastocystis* ST3 mostraron una gran diversidad de haplotipos a nivel mundial, sin embargo, a nivel de la población de Morelos esta diversidad de haplotipos fue baja. La red de haplotipos reveló que el haplotipo 1 es el ancestro común del cual los otros haplotipos se han generaron recientemente. Finalmente, los resultados sugirieron una expansión reciente de la diversidad genética de *Blastocystis* ST3 en todo el mundo, asociada probablemente al fenómeno de migración.

Research Article

Genetic Diversity and Distribution of *Blastocystis* Subtype 3 in Human Populations, with Special Reference to a Rural Population in Central Mexico

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Blastocystis subtype 3 (ST3) is a parasitic protist found in the digestive tract of symptomatic and asymptomatic humans around the world. While this parasite exhibits a high prevalence in the human population, its true geographic distribution and global genetic diversity are still unknown. This gap in knowledge limits the understanding of the spread mechanisms, epidemiology, and impact that this parasite has on human populations. Herein, we provided new data on the geographical distribution and genetic diversity of *Blastocystis* ST3 from a rural human population in Mexico. To do so, we collected and targeted the SSU-rDNA region in fecal samples from this population and further compared its genetic diversity and structure with that previously observed in populations of *Blastocystis* ST3 from other regions of the planet. Our analyses reveled that diversity of *Blastocysiis* ST3 showed a high haplotype diversity and genetic structure to the world level; however, they were low in the Morelos population. The haplotype network revealed a common widespread haplotype from which the others were generated recently. Finally, our results suggested a recent expansion of the diversity of *Blastocystis* ST3 worldwide.

1. Introduction

Blastocystis (Heterokonta, Stramenopiles) is a genus comprising parasitic protists that inhabit the digestive tract of several metazoans, such as fishes, amphibians, birds, reptiles, rodents, and humans [1–3]. *Blastocystis* is globally distributed, showing a high rate of infection from underdeveloped to developed countries [4, 5].

This parasite is often transmitted via the oral-fecal route to people who work directly with animals, such as those involved in intensive animal farming or industrial livestock production [6]. In humans, the signs and symptoms associated with *Blastocystis* infection range from diarrhea to flatulence, bloating, and abdominal discomfort [7, 8], with the "irritable bowel syndrome" (IBS) being the most frequent clinical manifestation [8–11].

Molecular evidence based on the small subunit ribosomal RNA (SSU-rDNA) gene suggests that, at least, 17 genetic subtypes can be recognized within *Blastocystis* [12]. Nine of these subtypes are found in humans, with subtype 3 (ST3 hereafter) being the most common in epidemiological studies worldwide [12–18]. ST3 has been regarded to trigger IBS in humans [8], and recent research also suggests an association between this subtype and colorectal cancer [19]. Other studies, however, suggest a lack of association between the ST3 and some type of symptomatology in humans [7, 20]. While *Blastocystis* ST3 has medical importance and high prevalence in humans, the real magnitude of its genetic diversity and geographical distribution remains so far unknown [5, 17]. Apparently, the ST3 exhibits a broader geographical distribution and higher genetic diversity than other genetic subtypes of *Blastocystis* [16, 21], but this hypothesis still needs to be tested using genetic data and clinical cases from both well-studied and undersampled geographical areas. In this context, there are very few geographical and genetic data on *Blastocystis* ST3 from Mexico.

Herein, we aimed to provide new data on the geographical distribution and genetic diversity of *Blastocystis* ST3 from a rural and asymptomatic human population in Mexico. To do so, we collected and targeted the SSU-rDNA region in fecal samples from this population and further compared its genetic diversity and structure with those previously observed in populations of *Blastocystis* ST3 from other regions of the planet.

2. Materials and Methods

2.1. Ethical Considerations. The protocol used in this study was conducted under the ethical principles and approval of both the Mexican Commission on Ethics and Research of the Health Ministry of the State of Morelos (Comisiones de Ética y de Investigación del Ministerio de Salud del Estado de Morelos) and the Commission on Ethics in Research of the Facultad de Medicina of the Universidad Nacional Autónoma de México (UNAM) (Comité de Ética de Investigación de la Facultad de Medicina de la Universidad Nacional Autónoma de México). The guidelines of the committees are based on the Mexican Official Norm (Norma Oficial Mexicana

NOM-012-SSA3-2007), which regulates the ethical principles of every research on humans and on laboratory animals, as well as on the Declaration of Helsinki, which set ethical principles regarding human experimentation developed by the World Health Organization (WHO).

Based on the abovementioned guidelines, our study only used samples from volunteers, who were respectively informed about the objectives of this research, the potential risks (if any), and the sampling procedures. We obtained an informed consent letter from all the participants.

2.2. Sampling and Analysis. Between May and November 2015, fecal samples were collected from 182 volunteers (86 males and 96 females) from Puente de Ixtla in the community of Xoxocotla, State of Morelos (Mexico), ranging in age from 2 to 51 years old. The asymptomatic status was defined according to the ROME III criteria. Three fecal samples were collected from each volunteer on three consecutive days. The samples were maintained at 4°C and transported to the laboratory in Mexico City on the same day of collection. A subsample of each fecal sample was smeared, stained with 4% Lugol's iodine solution, and examined under a light microscope at 10x and 40x magnifications [22].

2.3. Amplification and Sequencing of SSU-rDNA. DNA was extracted from fresh fecal samples using QIAamp DNA stool kit (QIAGEN, Hilden, Germany) and following the manufacturer's instructions. PCR protocol targeting the SSU-rDNA was conducted according to Scicluna et al. [23]. In brief, we used a total mixture of 20 μl : 20 μM of primers RD5 (5′-ATC TGG TTG ATC CTG CCAG T-3′) and BhRDr (5′-GAG CTT TTT AAC TGC AAC AAC G-3′) [23], as well as 0.025 U of polymerase (AmpliTaq Platinum Polymerase, Invitrogen). To verify the presence of a single band and the size of the amplified products (approximately 600 bp), the PCR products were separated by electrophoresis in agarose gel (1.5%) in the presence of ethidium bromide, visualized by ultraviolet transillumination, and photographed. The amplification product of a 600 bp fragment of the *Blastocystis* SSU-rDNA was purified and sequenced using a dideoxynucleotide-terminal method. Sequencing was carried out in a capillary sequencer (ABI-Avant 100, University of Washington). The sequences obtained were edited and/or analyzed with BioEdit, MEGA 5.0 software [24, 25], and ad hoc scripts from Python. These sequences were compared to sequences available in GenBank, employing BLAST to establish their identity. The final sequences were deposited in GenBank under accession numbers MF539962–MF540015.

2.4. Global Genetic Diversity and Haplotype Network for Blastocystis ST3. We investigated the global genetic diversity (i.e., Latin America, Europe, and Asia) within *Blastocystis* ST3 using the novel SSU-rDNA sequences reported in the present study and those previously reported within the literature. We provided an exhaustive list of the latter sequences ($n = 169$) and sources in the Supplementary Material (available here). We investigated the following descriptive statistics of genetic
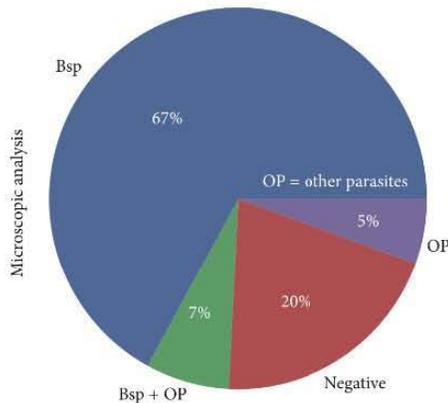
FIGURE 1: Frequency of intestinal parasites based on microscopic analysis of the fecal samples taken in Morelos, Mexico. *Blastocystis* was the only parasitic infection found in 67% of individuals and in 7% in coinfection with other parasites. Bsp: *Blastocystis*; OP: parasites other than *Blastocystis*; BSP + OP: coinfection of *Blastocystis* and other parasites; Negative: no parasite found. Among OP: Chm, *Chilomastix mesnili*; Ec, *Entamoeba coli*; En, *Endolimax nana*; Hn, *Hymenolepis nana*; Gl, *Giardia lamblia*; Ib, *Iodamoeba bütschlii*.

FIGURE 2: Frequency of *Blastocystis* subtypes in the study population. Targeting the SSU-rDNA according to DNA-barcoding, Three *Blastocystis* subtypes (ST) were recorded according to the following frequencies: *Blastocystis* ST1, 9.7% ($n = 7$ samples); ST2, 15.3% ($n = 11$ samples); and ST3, 75% ($n = 54$ samples).

diversity using the software DnaSP ver. 5.10.01 [26]: number of segregating sites (S), number of haplotypes (h), haplotype diversity (Hd), and nucleotide diversity (π) for each set of sequences according to their geographical region of origin. We built a global haplotype network using TCS network inference method [27] implemented in the PopART program ver. 1.7 (http://popart.otago.ac.nz/downloads.shtml) to investigate the global genealogical relationship between the different haplotypes of *Blastocystis* ST3. We also ran Tajima's D test in the software DnaSP [26] to investigate possible events of global population expansion on *Blastocystis* ST3. We finally estimated pairwise F_{ST} statistics in the software Arlequin ver. 3.11 (http://cmpg.unibe.ch/software/arlequin3) to investigate whether the geographical populations of *Blastocystis* ST3 were genetically structured.

3. Results

3.1. Frequency of Blastocystis ST3 in Morelos, Mexico. A microscopic analysis revealed that 148 (81.32%) of the 182 fecal samples collected in Morelos (Mexico) exhibited at least some type of intestinal parasite. These 148 samples (positive samples hereafter) harbored different parasites, including representatives of *Blastocystis*, *Chilomastix mesnili*, *Entamoeba coli*, *Hymenolepis nana*, *Iodamoeba bütschlii*, *Endolimax nana*, the *Entamoeba histolytica/Entamoeba dispar* complex, and *Giardia lamblia*. Among the abovementioned parasites, *Blastocystis* had the greatest frequency, occurring in 109 (74%) out of 148 positive samples. It was also the unique parasite in 99 (67%) out of 148 positive samples, and 7% of the samples (10/148) were coinfected with other parasites (Figure 1).

Further PCR and sequencing procedures successfully confirmed the presence of three different *Blastocystis* subtypes in 72 of the 148 positive samples collected in Morelos. These three *Blastocystis* subtypes (ST) were recorded according to the following frequencies: *Blastocystis* ST1, 9.7% ($n = 7$ samples); ST2, 15.3% ($n = 11$ samples); and ST3, 75% ($n = 54$ samples) (Figure 2).

3.2. Genetic Diversity of ST3 and Haplotype Network. Genetic diversity indices revealed a total of 44 segregating sites (S) and 20 haplotypes (h), as well as a total haplotype diversity (Hd) of 0.563 and nucleotide diversity (π) of 0.019. Tajima's D test provided values ranging between -1.303 and -2.363 (Table 1). A pairwise F_{st} analysis revealed that there is very low genetic differentiation between all geographical populations of *Blastocystis* ST3 (Table 2).

The number of haplotypes ranged from 3 to 15 between human populations, the number of segregating sites ranged between 1 and 35, haplotype diversity ranged between 0.142 and 0.740, and nucleotide diversity ranged between 0.001 and 0.045 (Table 1). The ST3 genetic diversity of Latin American populations (except Morelos's population) and Eurasia exhibited the highest values of genetic diversity indices in contrast to Morelos's population, where low haplotype diversity (three haplotypes) was detected (Table 1).

The haplotype network showed the haplotype distribution of the ST3 (Figure 3). In general, the worldwide haplotype network evidenced large levels of diversity, with a total of 20 haplotypes, and haplotype 1 was the dominant. The network showed a star topology radial distribution (Figure 3). Also, haplotype 1 was the most frequently found in Morelos's population and this haplotype is commonly distributed in American populations.

4. Discussion

In the present study, we analyzed the frequency and distribution of *Blastocystis* subtypes in an asymptomatic rural population. The results revealed a great frequency of *Blastocystis*

TABLE 1: Statistics data of genetic diversity observed within different geographical populations of *Blastocystis* ST3 around the world.

Populations	N	S	h	Hd	π	±SD	Tajima's D
Morelos	54	1	3	0.14186	0.00107	0.068	−1.68258[ns]
Latin America	69	25	15	0.67775	0.01334	0.058	−2.36261[**]
Eurasia	46	35	11	0.74010	0.04473	0.052	−1.30307[ns]
All populations	169	44	20	0.56276	0.01886	0.044	−2.20000[**]

N: number of sequences; S: number of segregating sites; h: number of haplotypes; Hd: haplotype diversity; π: nucleotide diversity; ns: not significant. [**] $p < 0.01$. Latin America: *Blastocystis* populations of North and South America (i.e., Mexico, Colombia, Brazil, Ecuador, Bolivia, Peru, and Argentina), except that of Morelos. Eurasia: *Blastocystis* populations of Europa and Asia (i.e., Nepal, Switzerland, Iraq, Italy, and France).

TABLE 2: Estimates of F_{ST} based on the SSU-rDNA variation observed between different geographical populations of the parasite *Blastocystis* ST3.

Population	Morelos	Latin America	Eurasia
Morelos	------		
Latin America	0.04165[ns]	------	
Eurasia	0.09164[ns]	0.05975[ns]	------

Latin America: *Blastocystis* populations of North and South America (i.e., Mexico, Colombia, Brazil, Ecuador, Bolivia, Peru, and Argentina), except that of Morelos. Eurasia: *Blastocystis* populations of Europe and Asia (i.e., Nepal, Switzerland, Iraq, Italy, and France). Probability obtained by a permutation test with 50,000 replicates. ns: not significant.

FIGURE 3: Haplotype network of *Blastocystis* ST3 of human populations at different regions from Latin America, Europe, and Asia. Each circle represents a haplotype, and each color represents the place where it was obtained. The size of each circle is proportional to the frequency of the haplotype in each population, where it was found. The circles in black stand for missing haplotypes and the short lines show the mutational steps.

from 74%, above the national average, as the frequency of this parasite varies from 23% to 61% in Mexico [20, 28, 29]. Recent studies in South America have described similar frequency of *Blastocystis* in the human population (21% to 67%) [30–32]. Around the world, *Blastocystis* exhibits a frequency range of 0.5% to 62% [4]. The higher prevalence of *Blastocystis* has been linked to hygiene factors including the consumption of food/water contaminated with *Blastocystis* and exposure to domestic and peridomestic animals infected too with this parasite [4, 33].

Many years ago, *Blastocystis* was considered as saprophytic yeast of the digestive tract, innocuous for the host [34]. Nowadays, we can observe that this parasite is widely distributed in the human population in the world and it is similarly distributed in symptomatic and asymptomatic individuals. For instance, Morelos's population (Mexico) showed a high infection frequency of *Blastocystis* although the participants were asymptomatic, suggesting tolerance to this parasite, as reported elsewhere [20, 35, 36].

Blastocystis has a high worldwide genetic diversity, represented by 17 subtypes (ST1–ST17) [5]. It is possible that there are other subtypes capable of infecting humans and other vertebrates [4, 5]. Regarding the distribution of subtypes in the present study, ST1 (9.7%), ST2 (15.3%), and ST3 (75%) were identified among 72 human isolates successfully genotyped. Globally, *Blastocystis* ST3 is the most prevalent subtype in humans found in different geographic areas [5, 37]. In our study, the frequency of ST3 was 75%, high compared to other populations of Mexico as the state of Michoacan, where the frequency of this subtype was 21%, and in Mexico City it was 42% [38, 39].

In Latin America, the frequency of ST3 is high [4, 37], with the following frequencies reported for *Blastocystis* ST3 in
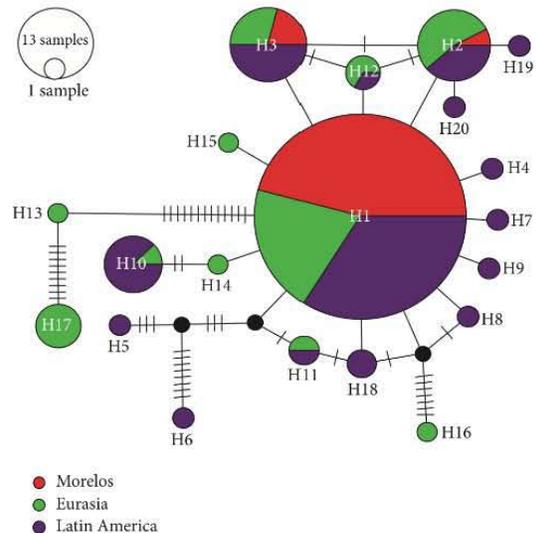
this political region: 14% in Colombia, 36% in Brazil, 84% in Ecuador, 30% in Bolivia, 92% in Peru, and 63% in Argentina [40]. While ST3 is amply distributed worldwide, it is more prevalent in Latin America [37], which opens the possibility that this subtype was generated in this geographic area and spread to the rest of the continents.

To improve our knowledge on the magnitude of the genetic diversity of *Blastocystis* ST3 on the planet, we analyzed sequences of Mexico City, South America, France, Switzerland, Italy, Nepal, and Iraq and the sequences obtained in the present study (169 total sequences, 54 from Morelos and 115 from the NCBI database). The genetic parameters calculated for these sequences suggested a recent population increase or directional/purifying selection. These results are supported by the haplotype network, which showed a star topology with the haplotypes distributed in a radial way supporting inferences of a recent geographical expansion in *Blastocystis*

ST3. This behavior is similar to reporting by other parasites, such as *Plasmodium falciparum* [41].

A total of 20 haplotypes were found on all sequences analyzed. Haplotype 1 was the most abundant and widely distributed, being detected in the majority of the studied countries, mainly in Latin America. These results are showing that haplotype 1 is perhaps an ancestral type from which all the other haplotypes have been generated recently. Haplotype 1 probably originated in Latin America and has recently colonized other regions of the world, probably via human migration [42].

In some studies, it has been observed that this subtype has been strongly related to rural populations [8, 43]. It is possible to hypothesize that the migration phenomenon [42] occurs mainly from the displacement of these rural communities infected with *Blastocystis* ST3 haplotype 1 spreading this parasite into the cities. Already in large cities, the transmission of *Blastocystis* ST3 haplotype 1 could happen from one person to another [43]. In addition, due to the opening of political borders between developed countries, this process of travel-associated infection is common, because travelers may act as carriers of this parasite [44].

Also, it is possible that the other haplotypes of *Blastocystis* ST3 could be colonizing different areas promoted by the human migratory phenomenon. This idea would explain the high representation of certain haplotypes throughout the American continent and others in European countries. For example, seven sequences of haplotype 9 correspond to South America and only one corresponds to Europe (Switzerland).

However, to test both hypotheses, it will be necessary to have a larger number of sequences and complete genomes from around the world. It is known that migration causes mobility of parasites and it is important to have information of other sociodemographic parameters of the host as the nationality or antecedents of previous travels to endemic areas [45]. In addition, the distribution of *Blastocystis* could be related to the lack of symptoms that occurs in many cases, because asymptomatic *Blastocystis* infections are not treated and therefore there are a large number of carriers of this parasite. It is known that these factors can influence the distribution of parasites [42, 46].

With respect to Morelos, all the sequences of *Blastocystis* ST3 were grouped into three haplotypes, which means that there is a low genetic diversity, a reduced rate of mutations, and little genetic differentiation; this suggests isolation and homogeneity in the population.

5. Conclusions

To our knowledge, this is the first study analyzing the haplotype diversity and distribution of *Blastocystis* ST3 subtypes in different human populations. In addition, our work facilitates the vision of a global distribution in *Blastocystis* ST3. We provide evidence of a recent expansion of this subtype that may be related to the migration of humans to other regions of the world. However, it is necessary to continue studying this parasite, in order to generate a more complete knowledge that allows us to know the course of *Blastocystis* infection, its epidemiology, and the causal factors that contribute to its dispersion dynamics and distribution.

Disclosure

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Acknowledgments

Supplementary Materials

List of the sequences reported in the present study and those previously reported within the literature. *(Supplementary Materials)*

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Material suplementario

Access Number	Country	Haplotype
MF539962	Mexico, Morelos	1
MF539963	Mexico, Morelos	1
MF539964	Mexico, Morelos	1
MF539965	Mexico, Morelos	1
MF539966	Mexico, Morelos	1
MF539967	Mexico, Morelos	1
MF539968	Mexico, Morelos	1
MF539969	Mexico, Morelos	1
MF539970	Mexico, Morelos	1
MF539971	Mexico, Morelos	1
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MF539989	Mexico, Morelos	1

MF539990	Mexico, Morelos	1
MF539991	Mexico, Morelos	2
MF539992	Mexico, Morelos	1
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KP055726.1	Mexico, Mexico City	1
KP055724.1	Mexico, Mexico City	1
KP055716.1	Mexico, Mexico City	1
KP055714.1	Mexico, Mexico City	1
KP055711.1	Mexico, Mexico City	1
KP055709.1	Mexico, Mexico City	3
KP055705.1	Mexico, Mexico City	1
KP055704.1	Mexico, Mexico City	1
KP055701.1	Mexico, Mexico City	5
KP055700.1	Mexico, Mexico City	6
KP055699.1	Mexico, Mexico City	1
KP055698.1	Mexico, Mexico City	7
KP055697.1	Mexico, Mexico City	1
KP055693.1	Mexico, Mexico City	1
KP055691.1	Mexico, Mexico City	1
KP055690.1	Mexico, Mexico City	8
KP055686.1	Mexico, Mexico City	3
KP055682.1	Mexico, Mexico City	1
KP055681.1	Mexico, Mexico City	1
KP055680.1	Mexico, Mexico City	1
KP055679.1	Mexico, Mexico City	1
KP055677.1	Mexico, Mexico City	1
KP055676.1	Mexico, Mexico City	9
KP055675.1	Mexico, Mexico City	1
KP055673.1	Mexico, Mexico City	1
KP055671.1	Mexico, Mexico City	1
KP055668.1	Mexico, Mexico City	1
KP055667.1	Mexico, Mexico City	1

KP055666.1	Mexico, Mexico City	3
KP055665.1	Mexico, Mexico City	1
KP055662.1	Mexico, Mexico City	1
KM438213.1	Nepal, Qatar	1
JN587549.1	Sweden	10
JN587548.1	Sweden	1
JN587547.1	Sweden	2
JN587546.1	Sweden	11
LT222067.1	Iraq	12
LT222066.1	Iraq	12
KP284172.1	Italy	3
KP284171.1	Italy	1
FJ666896.1	France	1
FJ666895.1	France	2
FJ666894.1	France	1
FJ666893.1	France	1
FJ666892.1	France	1
FJ666890.1	France	3
FJ666889.1	France	1
FJ666888.1	France	1
FJ666883.1	France	1
FJ666881.1	France	1
FJ666878.1	France	13
FJ666872.1	France	2
FJ666871.1	France	2
FJ666870.1	France	2
FJ666867.1	France	1
FJ666866.1	France	2
FJ666864.1	France	1
FJ666862.1	France	14
FJ666859.1	France	1
FJ666853.1	France	2

FJ666850.1	France	1
FJ666849.1	France	15
FJ666848.1	France	1
FJ666841.1	France	1
FJ666891.1	France	3
FJ666882.1	France	1
FJ666877.1	France	1
FJ666873.1	France	16
FJ666851.1	France	1
FJ666842.1	France	1
JN942529.1	France	1
FJ666887.1	France	17
FJ666857.1	France	17
FJ666854.1	France	17
FJ666847.1	France	17
FJ666844.1	France	17
FJ666897.1	France	3
KU928117.1	SurAmerica	1
KU928116.1	SurAmerica	2
KU928115.1	SurAmerica	3
KU928114.1	SurAmerica	18
KU928112.1	SurAmerica	18
KU928111.1	SurAmerica	1
KU928110.1	SurAmerica	2
KU928109.1	SurAmerica	19
KU928108.1	SurAmerica	2
KU928107.1	SurAmerica	1
KU928106.1	SurAmerica	1
KU928105.1	SurAmerica	2
KU928104.1	SurAmerica	10
KU928103.1	SurAmerica	20
KU928102.1	SurAmerica	1

KU928101.1	SurAmerica	12
KU928100.1	SurAmerica	11
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KU928098.1	SurAmerica	10
KU928097.1	SurAmerica	10
KU928096.1	SurAmerica	10
KU928095.1	SurAmerica	10
KU928094.1	SurAmerica	1
KU928093.1	SurAmerica	10
KU928092.1	SurAmerica	3
KU928091.1	SurAmerica	2
KM374618.1	SurAmerica	3
KM374617.1	SurAmerica	1

CAPÍTULO IV

DISCUSIÓN GENERAL

En el presente proyecto se estudiaron muestras fecales de 182 individuos, de edades comprendidas entre 2 y 51 años provenientes de una comunidad rural (Xoxocotla) del estado de Morelos en México. Las muestras se examinaron para detectar la presencia de *Blastocystis* por PCR del gen SSU rRNA. Seguido por NGS para determinar los subtipos. Se encontró en el 68.1% (124/182) de las muestras uno o más subtipos de *Blastocystis,* que previamente habían sido analizadas con confirmación molecular en el 59.9% (109/182) (Rojas-Velázquez et al., 2018). Los diferentes métodos de diagnóstico utilizados en esta población podrían explicar las diferencias de la prevalencia estimada, dado que las distintas tecnologías moleculares empleadas distan en sensibilidad y especifidad. En particular, una ventaja del uso de NGS es la detección de infecciones mixtas por diferentes subtipos o variabilidad intra-subtipo de *Blastocystis* (Maloney et al., 2019).

Con la aplicación de NGS se identificaron los subtipos 1, 2 y 3 en las 124 muestras positivas para *Blastocystis*. Estos tres subtipos se reportan con frecuencia en humanos en las Américas y en todo el mundo (Stensvold, 2015). El subtipo 3 fue el subtipo dominante en esta población, y se observó en el 81.5% de las muestras positivas para *Blastocystis* y en el 55.5% de la población total estudiada. Los subtipos 1 y 2 se observaron con mucho menor frecuencia, en 16.9% y 17.7% de las muestras positivas para *Blastocystis*, respectivamente. Estos resultados son similares a los reportados en varios países en América del Sur y en Estados Unidos, en donde ST3 es el subtipo más frecuente encontrado en muestras humanas positivas a *Blastocystis* seguido de ST1 y ST2 (Ramírez

et al., 2016; Seguí et al., 2018; Scanlan et al.,2016). Nuestros resultados contrastan por ejemplo con los obtenidos en el grupo étnico Tapirapé de la región amazónica brasileña, en el cual se detectaron ST1, ST2 y ST3, pero ST1 fue el subtipo identificado con mayor frecuencia (Malheiros et al., 2011). Estas diferencias entre los estudios podrían deberse a las restricciones geográficas y pueden reflejar el nivel de la población o las influencias climáticas sobre el riesgo de infección con diferentes subtipos de *Blastocystis*.

La prevalencia de infecciones mixtas por *Blastocystis* en humanos se ha estimado en un rango de 1.1% y 14.3 %, con un promedio mundial del 6% (Scanlan et al., 2015, Maloney et al., 2019). Al momento sólo se cuentan con datos de infecciones mixtas por diferentes subtipos reportados por Scanlan et al. (2015), mediante un ensayo de PCR anidado identificaron los subtipos 1 a 4 (Scanlan et al., 2015), demostraron que el 22% de las muestras positivas para *Blastocystis* que previamente habían identificado con un solo subtipo (Scanlan et al., 2014) contenían múltiples subtipos de *Blastocystis*.

En el presente estudio empleando NGS se identificaron 17 infecciones mixtas que representaron el 13.7% de todas las infecciones por *Blastocystis* en la población. Se observaron combinaciones de ST1+ST3, ST2+ST3 y ST1+ST2+ST3. El subtipo 3 (ST3) se observó en todas las infecciones mixtas de *Blastocystis*, ST1 en doce infecciones mixtas y ST2 en ocho infecciones mixtas. Aunque la población estudiada fue limitada en su diversidad de subtipos de *Blastocystis*, los resultados respaldan el uso de NGS para explorar infecciones mixtas de subtipos en humanos.

La variabilidad intra-subtipo podría desempeñar un papel importante en la comprensión de la transmisión y la patogenicidad de *Blastocystis* (Maloney et al., 2019; Clark, 1997; Fayer et al., 2012; Fayer et al., 2014). En el presente estudio, la variabilidad

intra-subtipo varió ampliamente entre los subtipos. Mientras que ST1 y ST2 tuvieron altas proporciones de secuencias únicas, ST3 fue sorprendentemente homogéneo. Las secuencias únicas representaron el 71.4% de las secuencias ST1 y el 72.7% de las secuencias ST2, pero solo el 11.9% de las secuencias ST3 eran únicas. Dos unidades taxonómicas operacionales únicas (OTUs) de ST3 dominaron en esta población, representando el 80.2% de todas las observaciones de ST3. Este nivel bajo de variabilidad intra-subtipo 3 se ha detectado tanto en humanos como en ganado (Beghini et al., 2017). La homogeneidad de ST3 en esta población puede indicar que ST3 es altamente endémico en esta comunidad y puede adquirirse de una fuente común o puede transmitirse más fácilmente entre humanos que los otros subtipos observados en este estudio.

A nivel mundial, *Blastocystis* ST3 es el subtipo más frecuente en humanos (Alfellani et al., 2013; Stensvold y Clark, 2016). En nuestro estudio, la frecuencia de ST3 fue del 75%, alta en comparación con otras poblaciones de México, como los estados de Michoacán, donde la frecuencia de este subtipo fue del 21%, y en la Ciudad de México del 42% (Vargas-Sanchez et al., 2015; Villalobos et al., 2014).

En América Latina, la frecuencia de ST3 reportada es la siguiente: 14% en Colombia, 36% en Brasil, 84% en Ecuador, 30% en Bolivia, 92% en Perú y 63% en Argentina (Ramirez et al., 2016). Si bien ST3 está ampliamente distribuido en todo el mundo, es más frecuente en América Latina (Clark et al, 2013; Stensvold y Clark, 2016), lo que abre la posibilidad de que este subtipo se haya generado en esta área geográfica y se haya extendido al resto de los continentes.

Para contribuir a mejorar nuestro conocimiento sobre la magnitud de la diversidad genética de *Blastocystis* ST3 en el planeta, analizamos secuencias de la Ciudad de

México, América del Sur, Francia, Suiza, Italia, Nepal, Irak y las secuencias obtenidas en el presente estudio (169 secuencias en total, 54 de Morelos y 115 de la base de datos de NCBI). Los parámetros genéticos calculados para estas secuencias sugieren un aumento reciente en la población o una selección direccional / purificadora. Estos resultados están respaldados por la red de haplotipos, que muestra una distribución radial de topología en estrella que respalda las inferencias de una reciente expansión geográfica en *Blastocystis* ST3. Este comportamiento es similar al reportado en otros parásitos humanos, como *Plasmodium falciparum* (Joy, 2003).

En el presente estudio se encontró un total de 20 haplotipos considerando todas las secuencias analizadas. El haplotipo 1 fue el más abundante y ampliamente distribuido, siendo detectado en la mayoría de los países, principalmente en América Latina. Estos resultados sugieren que el haplotipo 1 es un tipo ancestral, a partir del cual se han generado recientemente los demás haplotipos, y que este haplotipo probablemente se originó en las regiones de América Latina, de donde recientemente ha colonizado otros lugares del mundo, probablemente asociado al fenómeno de la migración humana (El-Badry et al., 2017; Yomb et al., 2013; Oliveira-Arbex et al., 2018). Debido a la apertura de las fronteras políticas entre los países desarrollados, las personas infectadas con el haplotipo 1 de *Blastocystis* ST3 pueden propagar este parásito actuando como vectores de este microorganism (Yomb et al., 2013; Hagmann et al., 2014).

Es posible que los otros haplotipos de *Blastocystis* ST3 puedan estar colonizando diferentes áreas debido a esta migración, lo cual explicaría la alta representación de ciertos haplotipos en todo el continente americano y otros en países europeos. Por ejemplo, siete secuencias del haplotipo 9 corresponden a América del Sur y solo una a Europa (Suiza).

Sin embargo, para explorar estas hipótesis serán necesarias más secuencias y genomas completos de todo el mundo junto con buena información de diferentes parámetros del huésped, como su nacionalidad y/o antecedentes de viajes anteriores a áreas endémicas (Bühler et al., 2014), ya que su propagación podría relacionarse con la falta de síntomas que ocurre en muchos casos, que eso evita que haya tratamientos contra la infección por *Blastocystis* y, por lo tanto, exista una gran cantidad de portadores (Yomb et al., 2013; Cheong et al., 2009).

La infección por *Blastocystis* se ha asociado con factores como la edad, el contacto con animales y las prácticas de saneamiento (Lee et al., 2012; Leelayoova et al., 2008; Pandey et al., 2015). Para comprender mejor qué factores podrían influir en el riesgo de infección, en esta tesis se usaron datos sociodemográficos, socioeconómicos y un análisis de regresión logística para determinar si existían asociaciones entre la presencia de *Blastocystis*, infecciones mixtas o únicas de subtipos (ST1, ST2 o ST3) y estos factores sociodemográficos y socioeconómicos. En la población estudiada el único factor asociado con tener *Blastocystis,* fue la edad adulta, con mayores probabilidades de ser positivo en la categoría de adultos (> 15 años) (OR: 1.72, p= <0.0001). La edad se ha indicado como un factor de riesgo para *Blastocystis* en otros estudios, y los bebés humanos y animales jóvenes tienden a tener menores tasas de infección que los adultos (Maloney et al., 2019a; Pandey et al., 2015; Abdulsalam et al,. 2013; Mohammad et al., 2017). La edad adulta también se asoció con mayores probabilidades de tener una infección por ST2 o ST3 (OR: 1.6, p= 0.02; OR: 1.36, p= 0.03), pero esta asociación no se observó en infecciones mixtas o ST1. Estos resultados podrían indicar que las diferencias conductuales o fisiológicas entre adultos y niños pueden ser importantes para determinar el riesgo de infección para subtipos específicos de *Blastocystis*.

El material del piso se asoció significativamente con infecciones mixtas por ST1 y ST2, y las probabilidades de tener uno de estos tipos de infecciones disminuyeron en hogares con pisos de cemento (OR: -1.48, *p*= 0.02; OR: -1.61, *p*=0.005; OR: -1.14, *p*= 0.03). Este resultado puede capturar algunos efectos socioeconómicos, como la mejora de la higiene, un aspecto que no fue medido directamente en este estudio, ya que el estado socioeconómico se ha asociado con el riesgo de infección (Abdulsalam et al., 2013). Tener animales de granja aumentó significativamente las probabilidades de tener ST1 (OR: 1.51, *p*= 0.03). Ningún otro subtipo se asoció con animales en este estudio. Estos resultados pueden indicar que la transmisión zoonótica de ST1 se produce en esta población, aunque serían necesarios datos sobre los subtipos que circulan en los animales que poseen o manejan individuos con ST1 positivo para confirmar este hallazgo. En particular, los artiodáctilos, como los cerdos y el ganado, son el segundo huésped más común reportado para ST1 después de los humanos, lo que respalda el potencial de transmisión zoonótica de este subtipo (Stensvold, 2015).

Ser sintomático, definido por responder sí a uno o más criterios del cuestionario ROMA III, se asoció significativamente con la infección por ST3, aunque la asociación fue negativa (OR: -0.88, *p*= 0.04). Ser sintomático disminuyó las probabilidades de tener ST3. Esta asociación, aunque significativa, no fue fuerte y se debe tener precaución en la interpretación excesiva de este resultado ya que las asociaciones entre subtipos y sintomatología aún no están claras. Algunos estudios han indicado que no hay asociación entre la diarrea y ST1, ST2 o ST3, pero ST4 se asocia con diarrea y síndrome del intestino irritable (Domínguez-Márquez et al., 2009; Mattiucci et al., 2016; Stensvold et al., 2011). Sin embargo, ST1 y ST3 se han asociado con síntomas intestinales en otros estudios (Jones et al., 2009; Moosavi et al., 2012).

Se ha encontrado al subtipo 1 en el agua potable en Tailandia y en el agua de los ríos en Nepal y en estos estudios se sospechó la transmisión de *Blastocystis* por el agua a los humanos (Leelayoova et al., 2008). En el presente estudio no se encontró una asociación estadísticamente significativa entre la infección y la fuente de agua, lo que indica que la transmisión en esta comunidad puede ocurrir a través de otras rutas. No se encontraron asociaciones estadísticamente significativas entre los resultados de infección y la eliminación de aguas residuales, la presencia de animales domésticos, la presencia de pollos o la presencia de plagas domésticas.

Finalmente, *Blastocystis* es un huésped intestinal común que se encuentra en humanos y muchos otros vertebrados. La prevalencia y distribución de subtipos de *Blastocystis* se han descrito en poblaciones humanas de todo el mundo, sin embargo, aún faltan estudios moleculares de *Blastocystis* en América del Norte, por lo cual, los resultados obtenidos en este trabajo en relación con el estudio de *Blastocystis* han contribuido al estudio de este microorganismo.

CONCLUSIONES

1) Este estudio proporciona información importante sobre la epidemiología de *Blastocystis* y represento la primera aplicación de un protocolo NGS específico para *Blastocystis* en humanos.

2) Aunque la población de estudio descrita tenía una comunidad de subtipos de *Blastocystis* relativamente homogénea, el estado de infección y los subtipos individuales podrían estar vinculados a factores de riesgo específicos.

3) Se necesitan más estudios que tengan como objetivo caracterizar las infecciones de subtipos mixtos y la variación intra-subtipo, para comprender la dinámica de transmisión, la epidemiología y la patogenicidad de *Blastocystis* en humanos y animales.

4) Este es uno de los primeros estudios que analiza la diversidad y distribución de haplotipos de subtipos de *Blastocystis* ST3 en diferentes poblaciones humanas.

5) Este trabajo facilita la visión de una distribución global en *Blastocystis* ST3, proporcionando evidencia de una expansión reciente de este subtipo, que el haplotipo 1 es quizás un tipo ancestral a partir del cual se han generado, todos los otros haplotipos y puede estar relacionada con el fenómeno de migración.

6) Es necesario continuar estudiando este microorganismo, para generar un conocimiento más completo que nos permita conocer el curso de la infección por *Blastocystis*, su epidemiología y los factores causales que contribuyen a su distribución y dinámica de dispersión.

PERSPECTIVAS

La reciente investigación de *Blastocystis* deja un gran camino aún que recorrer en el conocimiento de este microorganismo. Existen muchas aréas de estudio que necesitan ser dilucidadas sobre la presencia de *Blastocystis* en el intestino humano, sin embargo, de manera particular tres líneas de investigación son de mi interés, que incluyen establecer un modelo experimental *in vitro* que permita evaluar si *Blastocystis* tiene capacidad patogénica. Asímismo, es necesario conocer cuál es el verdadero papel ecológico de éste microorganismo en su hospedero (ya sea patógeno, commensal o mutualista) y, finalmente, conocer el papel funcional de este protista en el intestino humano.

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Entamoeba histolytica and *Entamoeba dispar* infection in Mexican school children: genotyping and phylogenetic relationship

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Abstract

Background: This study aimed to determine the frequency of *Entamoeba histolytica* and *Entamoeba dispar* infection in school children in the community of Tlaltizapan, in order to understand the dynamics of infection within the school and family spheres of this population. Amoebiasis is an unsolved public health problem and an endemic disease in Mexico. The incidence rate varies depending on the state; the most affected states show the highest numbers of new cases of amoebiasis per year. Previously, we reported the molecular frequency of infection with *E. histolytica* and/or *E. dispar* in other rural communities of the state of Morelos.

Methods: Children from 3 schools were studied to estimate the frequency of intestinal parasites through microscopic examination of fresh stool samples. The number of studied individuals were 309 school children. The molecular characterization of *E. histolytica* or *E. dispar* was carried out by Polymerase Chain Reaction (PCR) using species-specific primers to amplify short tandem repeats (STR) in non-coding sequences associated with the tRNA gene; the amplified fragments were sequenced and analyzed.

Results: Eight different genotypes were obtained from *E. dispar* isolates with the molecular marker NKD3-D5. None of the cases in which the species *E. histolytica* was detected developed symptoms attributable to an invasive process of disease. Moreover, the parasitized condition appeared to have no significant impact on the development or nutritional status of affected children. Genotype 1, which corresponds to the reference strain *E. dispar* SAW760, considered a non-pathogenic amoeba, was the most prevalent.

Conclusions: The comparison of the genotypes of *Entamoeba* species did not show a correlation between children and their relatives. In this community, the species *Entamoeba dispar* genotype 1 was the most widespread. Based on the indicators of growth, development and nutrition status, the studied community seems to be reasonably adapted to constant exposure to intestinal parasites, since there were no evidences of a serious impact of the parasitized condition on the children's health.

Keywords: Frequency, E. histolytica, E. dispar, Genotype, Phylogenetic relationship, Patterns of transmission

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Background

Mexico has a highly diverse geography, and its sociodemographic conditions are even more diverse. This diversity is undoubtedly reflected on the morbidity rates of amoebiasis observed in different geographic areas of our country. The incidence rates of amoebiasis are different between the northern, northwestern, southeastern and highland regions of the country. However, in the last two decades, the age range of the population at risk has not changed; it still corresponds to children aged less than 18 years old. The highest rates are found in children aged 1–4 years (1034.04 cases/100,000 inhabitants in 2012) (the official incidence rate was 796.39 cases/ 100,000 inhabitants) [1].

Amoebiasis is one of the 20 main causes of disease in Mexico; however, the current frequency of infection due to both *E. histolytica* and *E. dispar* in the Mexican population is unknown. Some isolated epidemiological studies have been made using molecular tools to characterize *E. histolytica* and *E. dispar* [2–4].

Recently, we performed a trial study in an open population in the state of Morelos, and found a total prevalence of 21 % for infection with both species of *Entamoeba*, *E. histolytica* has a higher (1 3.8 %) compared with *E. dispar* (9.6 %) and mixed infections were 2.4 % [5]. Determining the real numbers of amoebic infection has been the aim of several studies in other endemic areas and in specific groups of individuals sharing close environments or with risky sexual practices; these studies have produced highly variable data [5–9]. Therefore, a worldwide estimation of the burden of disease due to each of the two species of amoebas [10] cannot currently be done.

Nevertheless, epidemiological studies of amoebiasis using molecular strategies have unveiled the extraordinary complexity of both species of *Entamoeba* and the genetic intra-specific variability in coding and in noncoding regions of DNA [5, 11–14]. Both species of *Entamoeba* are highly polymorphic; however, *E. histolytica* is clearly less polymorphic than *E. dispar* [14]. Now we know that both species have a peculiar geographic distribution [14], indicating that some genotypes have higher or lower geographic mobility; this characteristic can help us understand the patterns of transmission in specific communities or groups of individuals exposed to infection. Migrating infected individuals can become sources of new infections and potential outbreaks of amoebiasis, making the molecular tracking of *Entamoeba* isolates a valuable tool for global epidemiological and genealogical studies of *E. histolytica* and *E. dispar* [15]. The main objectives of the present work were to determine the molecular frequency and the dynamic of infection of *E. histolytica* and/or *E. dispar* in a school children population in Tlaltizapan, Morelos. For this purpose, we performed the genotyping of *Entamoeba* isolates and the phylogenetic reconstruction of parasite DNA sequences in order to detect the possible source of infection and the patterns of transmission. To estimate the impact of infection with parasitized condition on the studied children, we applied an index of anemia to both parasitized and non-parasitized children, and measured their body mass index according to the gender of the children.

Methods

Study area and sampling. A cross-sectional study was conducted from January to May 2011 among 309 children from 3 different schools randomly selected in the village of Tlaltizapan, in the state of Morelos.

The sample size was calculated taking into account the total number of school children in the town (5,921) within the same range of age (5–14). The required size of the sample was calculated to be 190 children; the expected frequency of intestinal parasitic infection was 15 %; the worst acceptable level was 10 %; the confidence level was 95 %, and the results were considered statistical significant when $p < 0.05$ % (Epi Info version 6) [16]. Even though the estimated sample size was less than the 309 individuals actually studied, the larger sample resulted in a strong statistical power, particularly for the frequency of the least prevalent parasites species.

The type of housing varies significantly in Tlaltizapan; in the center of the town there are well-constructed houses characteristic of urban settlements, with running water and drainage, electricity, and cement floors. However, the typical rural houses have no running water and no sewage facilities. In some cases, there is no latrine and the floors consist of compacted soil. The average number of inhabitants per house is seven individuals.

Ethical considerations

The study protocol was submitted for evaluation and was approved by the Health Ministry of the state of Morelos; it was also approved by the ethical committee of the Faculty of Medicine of the National Autonomous University of Mexico (UNAM). In both cases, the ethical committees applied the Mexican Official Norm NOM-012-SSA3-2007, which deals with human and animal research, to support their decision to approve this research.

Studied population

Three different schools were included in the study, all with similar characteristics and facilities (running water, toilet facilities, and 30–40 children per classroom). The age range studied was between 5 and 14 years, and there were no gender restrictions. The parents or legal guardians of each child, as well as the directors and teaching staff of the schools were informed about the details of

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the project, the advantages of voluntary participation, the sampling procedures and the potential risk of sampling. After the parents expressed their willingness to include their children in the study, they were asked to sign a letter of informed consent.

Each parent or guardian was interviewed by a member of our fieldwork team to assess the conditions of housing, access to potable water, waste disposal, and hygienic habits of the family. Pathological antecedents were also investigated, particularly recent episodes of diarrhea (in the previous 6 months). Information was collected through a previously validated questionnaire [5] and uploaded to a database for statistical analysis. Afterwards, wide mouth screw-capped containers, previously labeled with the complete name of the child and the respective code, were distributed to the parents or guardians with instructions for collecting and preserving the stool samples at home until they were collected by our fieldwork team.

With the purpose of determining the dynamics of transmission between each child and their near relatives, we also collected stool samples from the relatives of children who were microscopically positive for fecal parasites. A total of 167 stool samples were obtained from the relatives. The only condition to be included in the study was that the parasitized children and their relatives shared the same home.

Microscopic detection of intestinal parasites
The stool samples were kept at 4 °C and transported to the Health Center in Tlaltizapan, and then to the laboratory in Mexico City. They were analyzed as previously reported [5]. Briefly, the fecal samples were suspended in 4 % Lugol's iodine solution, and microscopic observation was performed at 10× and 40× magnification. To determine the concentration of ova and cysts, we used the Faust-Ferreira technique in the presence of a zinc-sulfate gradient solution ($d = 1.192$); a sample was collected from the flotation disk using a Pasteur pipette, suspended in 4 % Lugol's solution and microscopically observed as described above [17].

DNA extraction from stool samples
DNA was extracted from cysts found in stool samples subjected to the zinc-sulfate gradient flotation technique. Cysts were transferred to a 2 mL Eppendorf tube, washed 4 times with 0.15 M NaCl, and resuspended in 300 µl of lysis buffer (100 mM EDTA, pH 8, 0.25 % SDS) [18]. The tubes were subjected to five freezing cycles in ethanol-dry ice and thawed in a 37 °C water bath. Afterwards, 3 µl of 20 mg/mL of proteinase K were added. The samples were incubated for 1 h at 55 °C. After digestion with proteinase K, the lysates were brought to 0.7 M NaCl and 1 % CTAB (Sigma Chemical Co., St. Louis, MO). The mixture was

incubated at 65 °C for 30 min and the samples were then extracted with chloroform, phenol/chloroform and chloroform, followed by precipitation of DNA with ethanol. DNA was suspended in 50 µl of water and passed over a Sephadex G-25 spin column (Pharmacia Biotech, Uppsala, Sweden).

Molecular characterization of *E. histolytica* and *E. dispar*
The DNA obtained was used for amplification by polymerase chain reaction (PCR) in a 20 µl reaction mixture. Transfer RNA (tRNA) gene-linked short tandem repeats (STR) were amplified from the DNA using species-specific primers for *E. histolytica* and *E. dispar*. The primers used for the molecular characterization were DA-H5/H3 (Hsp 1–2), NK-H5/H3, S^{tga}D-H5/H3, SQ5/SQ-H3 for *E. histolytica* and DA-D5/D3 (Dsp 1–2), NK-D5/D3, S^{tga}D-D5/D3, SQ-D5/SQ-D3 for *E. dispar* [19].

These molecular markers amplify highly polymorphic intergenic sequences repeated in tandem (STR) associated to tRNA genes in both species of *Entamoeba*. In all cases, the PCR conditions were as follows: The reaction mixture consisted of 1 µL of clean extracted DNA added to Tris–HCl 10 mM, pH 8.3, KCl 50 mM, gelatin 0.001 %, MgCl$_2$ 2 mM, 0.2 mM of each nucleotide, 0.0025 U of polymerase (AmpliTaq platinum Polymerase, Invitrogen) and 20 µM of each primer; 5 min at 95 °C for the initial incubation, followed by 35 cycles of 30 s at 95 °C, 30 s at 72 °C and a final extension step of 10 min at 72 °C. Regarding the primers, the annealing temperature conditions were those reported by Ali et al., 2005. The PCR products were subjected to electrophoresis in 1.5 % agarose gels stained with ethidium bromide and visualized in an UV transilluminator. Sequencing of PCR products was carried out in a reaction mixture (15 µL) consisting of 2 µL of the Big Dye Terminator Sequencing Kit (Applied Biosystems, San Francisco, CA), 1.6 µM of the appropriate primer, and 5 µL of the purified PCR product. The amplification conditions were as follows: 1 cycle of 5 min at 95 °C, 45 cycles of 10 s at 95 °C, 10 s at 50 °C, and 4 min at 60 °C. Sequencing was performed in a capillary sequencer (ABI-Avant 100, University of Washington). The sequences were manually verified using the BioEdit program [20]. Taxonomic identity was established by comparing the obtained sequences with those available in GenBank (NCBI). The sequences were aligned using the program CLUSTAL X [21]. Phylogenetic reconstruction based on the molecular marker NKD3-D5 was carried out through the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) using the Statistical Package for the Social Sciences (SPSS), version 17.

Genetic diversity
With NKD3-D5 sequences obtained from the school and family isolates, and those reported in GenBank, we

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conducted a genetic diversity analysis using the program DnaSP version 5.0 [22]. The number of both segregate sites and haplotypes was estimated, as well as the average of nucleotide diversity per site (π) and the expected variation, by assuming a neutral site evolution (θ).

Estimation of the anemia index

The concentration of Hemoglobin (Hb) was determined from peripheral blood samples using a HemoCue analyzer (HemoCue AB, portable Hb Analyzer, Angelhom, Sweden). The values were stored in a database for analysis, contrasting the Hb g/dl with the standard curves of Hb concentration by age-group [23].

Body Mass Index

Body weight is expressed as the mean of two independent measurements using electronic scales (Medical Scales and Measuring Systems, Seca, Hamburg, Germany). Height was measured with a portable stadiometer (Medical Scales and Measuring Systems, Seca), taking the mean of two measurements. The Body Mass Index (BMI) was calculated using the following formula: weight (kg)/[height (m)] ^{2}. The BMI was used as an index of relative weight, with BMI-for-age z scores, and the percentiles were calculated according to age and sex using the Center for Disease Control and Prevention growth charts [24].

Statistical analysis

The frequency of intestinal parasitic infections was estimated as the number of positive cases/total school population studied; to calculate the relative frequency of single or multiple parasitic infections, we used the same denominator.

The search for an association between sociodemographic variables, health and anthropometric values was done using Pearson's chi-square and chi square for trend

assessment. In all cases, the statistical significance was set at p less than 0.05.

Results

General characteristics of the studied cohort

The distribution of children by age and gender in each of the three studied schools was equal; the total number of participants was 309 children, 156 boys and 153 girls. Table 1 shows the sociodemographic variables in the family environment investigated as potential risk factors for parasitic diseases; these were: presence of harmful fauna (cockroaches, fleas, rats, mice, flies); piped water inside the household, type of latrine (water latrine or septic tank), floor material of household dwelling (cement or soil) and the quality of water used for human consumption. The differences in these variables between infected and non-infected children were not statistically significant ($p > 0.05$). Table 2 shows that the frequency of intestinal parasite infection was not associated with age or gender, with a similar distribution for males and females. With regard to the risk factors in the schools, a hygiene index was established based on the conditions of the sanitary facilities, the cleanliness of bathrooms, the availability of trash cans, the presence of clean common areas, overcrowding conditions and access to health authorities. Using this index, the schools were ranked as good, regular or bad, similar to Webb et al. description [25]. The results of the hygiene index and the frequency of parasitization among children (by school) were subjected to a chi-square test for linear trend analysis, which showed that the differences between the schools were not statistically significant ($p = 0.74$) (Table 3).

Pathological antecedents specifically related to diarrhea episodes in the previous 12 months and the presence of mucus in the feces or blood were investigated in both parasitized and non-parasitized children; it was found that parasitized children had suffered 40 diarrhea episodes compared with 107 episodes among non-

Table 1 Analysis of the association between sociodemographic variables at home and the parasitized or non-parasitized condition of school children

Variables		Parasitized children				
		Positive	Negative	$^a p$	OR	IC 95 %
Harmful fauna	Yes	65	218	0.73	1.63	(0.35–7.58)
	No	2	11			
Availability of water inside the house	Yes	65	224	0.27	0.53	(0.20–1.40)
	No	7	13			
Type of latrine	Latrine	67	214	0.26	2.66	(0.59–11.8)
	septic tank	2	17			
Floor material	Cement	58	210	0.12	0.52	(0.24–1.16)
	Soil	11	21			

^{a} Fisher's exact test

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Table 2 Children distribution by age, gender and parasitized or non-parasitized condition

| Age range (years) | | Parasitized children | | | | |
		Positive	Negative	[a]p	OR	IC 95 %
5–9	Female	17	62	0.80	0.91	(0.44–1.89)
	Male	21	70			
10–14	Female	16	58	0.40	0.72	(0.33–1.56)
	Male	18	47			

[a] Chi^2 test

parasitized individuals; however, the differences between groups were not statistically significant ($p = 0.11$). With regard to bloody and mucous diarrhea, there were only eight such episodes in parasitized children.

Microscopy frequency of intestinal parasitic infection

The frequency of intestinal parasitic infection in the studied population ($n = 309$ children) was 23.3 % (72/309). The frequency of single infections was (48/72) 66.6 %, and 24 individuals (33.3 %) showed multiple infections (more than one parasite species); some of these were intestinal pathogens. It is worth noting the high frequency of *Blastocystis hominis* (25 %), *Giardia lamblia* (19.4 %), and *E. histolytica/E. dispar* (9.7 %).

Molecular frequency of *E. histolytica* and *E. dispar*

After microscopic screening for intestinal parasites, 72 stool samples were treated for DNA extraction and thereafter for PCR amplification; these were tested for *E. histolytica* and *E. dispar* using species specific primers; 30 out of 72 parasitized samples (41 %) were positive for *E. histolytica* and/or *E. dispar*; 8/72 samples corresponded to individuals infected with *E. histolytica* (11.1 %); 19/72 samples corresponded to *E. dispar* infected children (23.88 %); and 3 out of 72 samples belonged to children infected with both *E. histolytica* and *E. dispar* (4.16 %). With the purpose of establishing the dynamics of transmission between each child and their near relatives, we tested the relatives of the 72 parasitized stool samples and PCR tested for the presence of *E. histolytica* and *E. dispar*. There was a low

frequency of infection in this group of studied individuals; only 14 out of 48 parasitized samples (29.2 %) were positive for *E. dispar*, and we did not detect infection with *E. histolytica* or mixed infections in this group (Tables 4, 5).

Sequence analysis based on the number of motifs

Although the PCR was performed using all primers mentioned above, the NKD3-D5 marker showed the best performance, and thus we decided to work with the PCR products obtained with this specific marker for *E. dispar*. The sequence profiles (GenBank accession number KX461938-KX461956) were analyzed, manually aligned and compared with those reported in GenBank.

In order to establish the dynamics of infection of *E. dispar*, we analyzed sequences of school children and their families, including previous NKD3-D5 sequences reported in GenBank. The analysis of isolates using this molecular marker was based on the diversity and the number of motifs found in the sequences. Twenty-four different motifs were defined, each one represented with a specific color. In some cases, the motifs consisted of two or four sequences and we decided to include all of them into a single motif, since the differences between the sequences was only one nucleotide. Afterwards, we defined genetic patterns based on the number of repeated motifs; these patterns were compared between classmates and between the respective family members. The correlation study showed no clear route of transmission, since the different genetic patterns were shared between classmates and family members (Fig. 1).

Genotypes

Eight genotypes were found in parasitized children and their families; each genotype is represented by a color (Fig. 2). It appears that most of school children in Lazaro Cardenas school share genotype 1, the same genotype found in their closest relatives, except child LC020, whose genotype was genotype 8, entirely different to that of his classmates. Cases of persistent infection were also detected; for example, the child LC009, who also attended Lazaro Cardenas school, was infected by

Table 3 Number of parasitized and non-parasitized children per school

| School | Microscopic examination of stool samples | | | OR | IC 95 % |
	Positive (%)	Negative (%)	Total		
Sofía Vázquez[a]	29 (23.2)	96 (76.8)	125	1	–
Lázaro Cárdenas[b]	13 (28.9)	32 (71.1)	45	1.34	(0.58–3.09)
Emiliano Zapata[c]	30 (21.6)	109 (78.4)	139	0.91	(0.49–1.69)
Total	72	237	309		

[a]Good hygiene index
[b]Regular hygiene index
[c]Bad hygiene index
Chi^2-with lineal trend = 0.11 $p = 0.74$

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Table 4 *Entamoeba histolytica* and *Entamoeba dispar* frequency: microscopic and PCR analysis of stool samples of the school children

| Microscopy | PCR characterization | | | | |
	E. histolytica	*E. dispar*	*E. histolytica + E. dispar*	Negative	Total
Eh/Ed	1	6	0	4	11
Non Eh/Ed	7	13	3	38	61
Negative	–	–	–	237	237
TOTAL	8	19	3	287	309

Values are the frequency of *E. histolytica* and/or *E. dispar* species microscopically and/or PCR detected. *E. histolytica* frequency [*E. histolytica* + (*E. histolytica* + *E. dispar*)] was 3.55 %; *E. dispar* frequency [*E. dispar* + (*E. dispar* + *E. histolytica*)] was 7.12 %, and frequency of *Entamoeba* infection [*E. histolytica* + (*E. histolytica* + *E. dispar*) + *E. dispar*] was 9.70 %. PCR, polymerase chain reaction. (–) PCR was not performed

genotype 1 and his father was infected by *E. dispar* genotype 7; both remained infected throughout the entire follow-up. This phenomenon was not found in the Emiliano Zapata school, where there was more diversity of genotypes in the infected children, who did not share the genotypes with other classmates or with members of their respective families. The most frequent genotype was genotype 1, which, as we mentioned before, is the same showed by the reference strain SAW760 of *E. dispar* SAW760.

Genetic diversity
Table 6 shows the genetic diversity of *E. dispar* detected in this community using the NKD3-D5 marker. In the group of 19 sequences obtained from children and their relatives, we found 6 haplotypes, considering insertions, deletions and substitutions of nucleotides; 8 segregative sites, and π and θ values of 0.00337 and 0.00622 respectively. When the analysis included all corresponding sequences available in GenBank, we found 8 haplotypes from 22 sequences, also considering insertions, deletions and substitutions, with 9 segregative sites, and π and θ values of 0.00147 and 0.00354 respectively.

Phylogenetic reconstruction
For the phylogenetic reconstruction of isolate sequences, we created a matrix where the number of motifs detected was encoded as present (1) or absent (0) with a numeric value. Thereafter, we used the Social Sciences (spss) statistical package version 17 to carry out the phylogenetic reconstruction using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA). This analysis showed two major divergent groups, A and B. Fig. 3 shows that the majority of sequences from children and from their relatives belong to group A. As can be seen, this group is formed by subgroups; for example, subgroup I corresponds to sequences from Lazaro Cardenas school children and some of their relatives. These sequences are identical to the sequence of *E. dispar* SAW760 strain, which also belongs to group A, subgroup I. The rest of the sequences are dispersed among the other clades.

Anemia index
We estimated the concentration of hemoglobin in peripheral blood samples (Hb g/dl) from 299 out of 309 children and compared it with reference data by age range for diagnostic criteria for anemia [23]; we found that 21 out of 299 children had Hb g/dl values that, for their age, were indicative of anemia (6.9 %). However, the differences in Hb g/dl levels between the groups of parasitized and non-parasitized children were not statistically significant ($p = 0.53$) (Table 7).
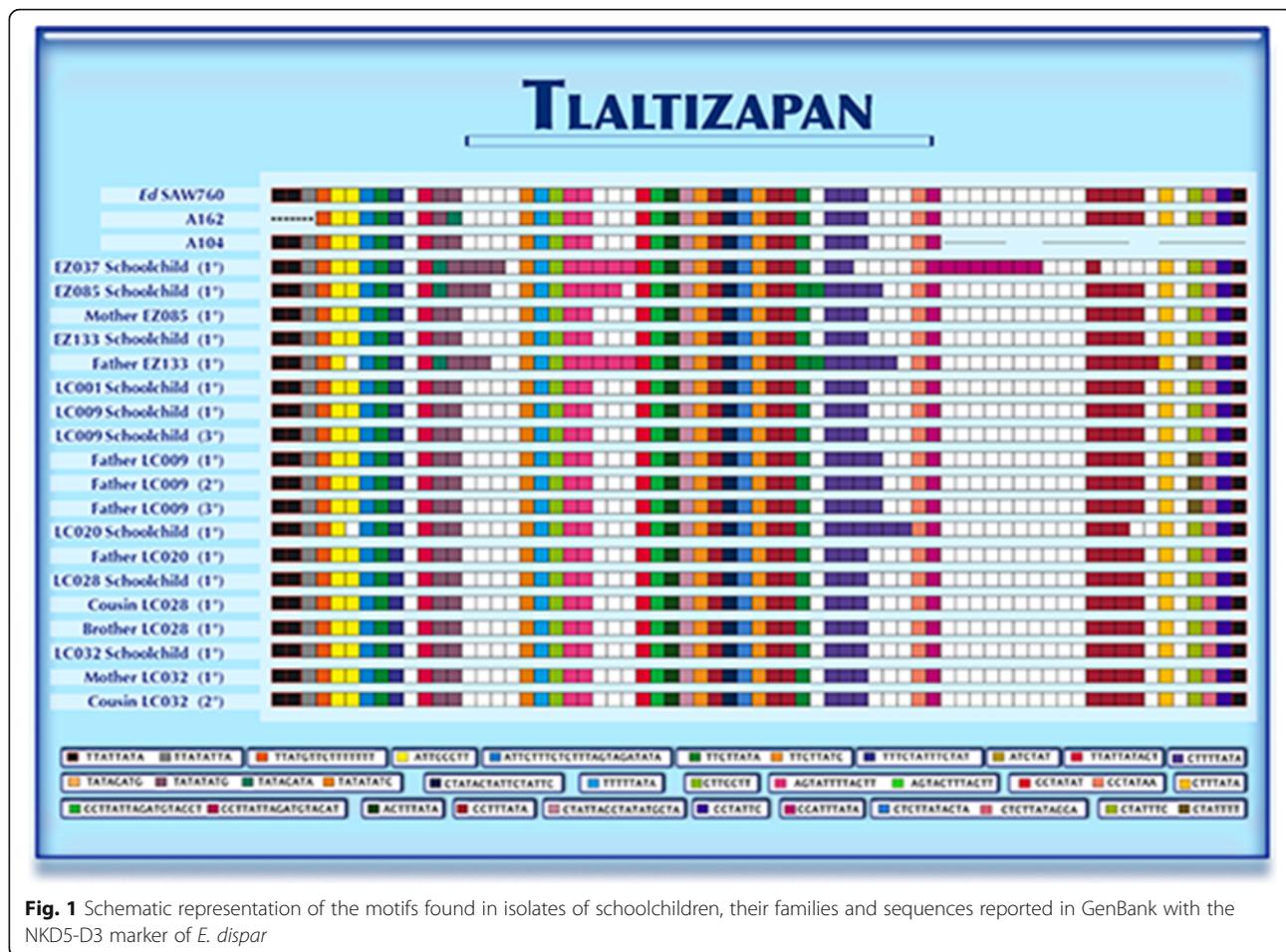
Anthropometric characteristics of the study population
The body mass index (BMI) calculations by sex and age of the 309 studied children showed differences in the population according to sex and age (data not shown). Over 50 % (192) of the children were between the 5th and 85th percentile, which corresponds to normal BMI.

Table 5 *Entamoeba histolytica* and *Entamoeba dispar* frequency: microscopic and PCR analysis of stool of the relatives of the parasitized school children

| Microscopy | PCR characterization | | | | |
	E. histolytica	*E. dispar*	*E. histolytica + E. dispar*	Negative	Total
Eh/Ed	0	6	0	0	6
Non Eh/Ed	0	8	0	34	42
Negative	–	–	–	119	119
TOTAL	0	14	0	153	167

Values are the frequency of *E. histolytica* and/or *E. dispar* species microscopically and/or PCR detected. *E. histolytica* frequency [*E. histolytica* + (*E. histolytica* + *E. dispar*)] was 0′%; *E. dispar* frequency [*E. dispar* + (*E. dispar* + *E. histolytica*)] was 8.38, and frequency of *Entamoeba* infection [*E. histolytica* + (*E. histolytica* + *E. dispar*) + *E. dispar*] was 0 %. PCR, polymerase chain reaction. (–) PCR was not performed

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Fig. 1 Schematic representation of the motifs found in isolates of schoolchildren, their families and sequences reported in GenBank with the NKD5-D3 marker of *E. dispar*

However, 13 children (4.2 %) were under the 5th percentile, which is considered underweight; 57 children (18.6 %) had a BMI between the 85th to 95th percentile, which defined overweight; moreover, 44 (14.3 %) children were classified as obese (over the 95th percentile). When the BMI was correlated with intestinal parasitic infection, the differences detected were not statistically significant ($p = 0.41$) (Table 8).

Discussion

Intestinal infections due to intestinal pathogens are endemic in developing countries all over the world [26]. Intestinal parasitic infections are closely associated with specific sociodemographic risk factors in the community environment, and particularly at home (Table 1). In addition, the highest rates of parasite infection were found in children aged 1–4 years (Table 2); however, no differences were found in the sociodemographic variables studied between infected and non-infected children. Furthermore, differences in age or gender between parasitized and non-parasitized children were not statistically significant ($p > 0.05$. This suggests that the main sources of infection for the children may be outside the

family environment. The other place where children spend most of their time is the school. The three schools showed differences in the hygiene index, and we expected to find some important differences in the parasitized condition of children; however, the differences between the hygiene index of schools were not statistically significant (chi-square for linear trend = 0.11; $p = 0.74$). In summary, the demographic variables of family, age, gender and school environment of the studied children were not directly responsible for the intestinal parasitic infection (Tables 1, 2 and 3); this circumstance points out to the existence of wide spread sources of intestinal parasites in the community environment.

Actually, the frequency of intestinal parasitic infection in the state of Morelos (23.3 %) is not the highest in our country compared with those observed in rural communities in the neighboring states of Guerrero or Chiapas, where the recently reported prevalence of intestinal parasitic infection exceeds that observed in Tlaltizapan [26, 27].

Our results on the frequency of pathogenic parasites in 2011 in Tlaltizapan can be contrasted with those obtained in 2005 in a rural community located 34 km southeast of Tlaltizapan [5]. In both cases, we detected
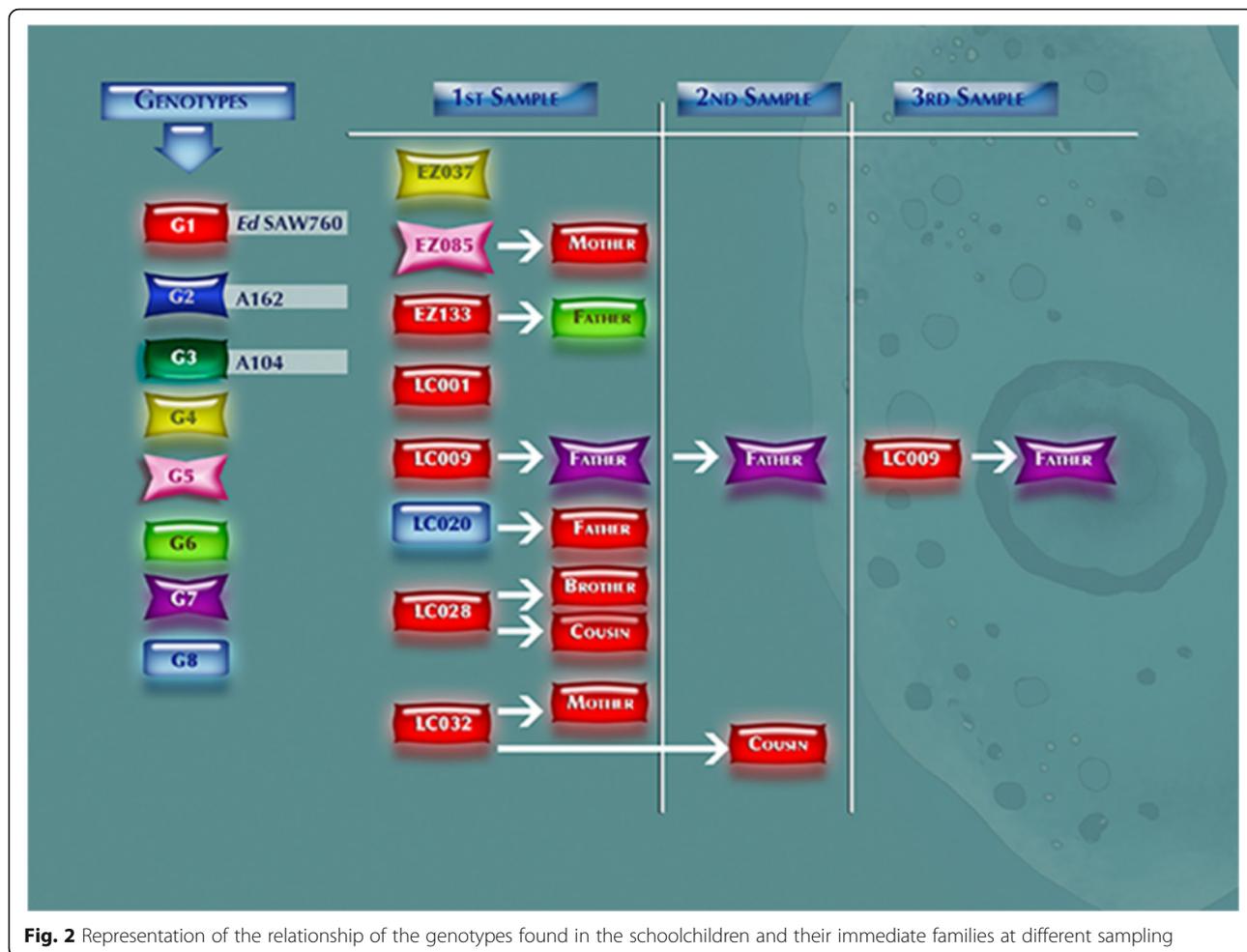
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Fig. 2 Representation of the relationship of the genotypes found in the schoolchildren and their immediate families at different sampling

protozoan pathogens such as *E. histolytica*, *E. dispar* and *G. lamblia*, in addition to a number of protozoa indicators of fecalism. However, the changes in the frequency of intestinal protozoan infection in children are worth noting; the frequency of *Blastocystis sp* in our children was remarkably high (25 %), in contrast to the complete absence of *Blastocystis sp.* observed in our previous study [5], although this figure is similar to that observed in other countries such as Thailand, Philippines or Brazil, where the prevalence are 20–40 % in 5–10 years old children [28, 29].

Intestinal parasites are etiological agents of diarrhea in both adults and children; however, children under 15 years old are the main group at risk. Children under 5 years old develop serious adverse effects related to cognitive skill, nutritional status and short stature, as consequence of repeated diarrhea events [30]. In our cohorts, we documented cases of diarrhea in both parasitized (55.5 %) and non-parasitized (44.9 %) children; however, the differences between the groups were not statistically significant. Clinically, none of the cases of diarrhea was attributed to an amoebic disease; in all cases the outcome was treated with oral rehydration solution. Some diarrheal events were self-limiting and resolved spontaneously within 48–72 h; in some other cases, the physician in charge indicated oral antibiotic administration.

Table 6 Parameters related to the genetic diversity of the sequences in the specie *Entamoeba dispar* using the molecular marker NKD3-D5 (603pb)

Groups[a]	No. of sequences	No. of haplotypes	Ss[b]	π	θ
School and Fam. *E. dispar*	19	6	8	0.00337	0.00622
E. dispar total	22	8	9	0.00114	0.00185

[a]School and Fam *E. dispar*: Number of samples of school children and their relatives obtained in this study; total *E. dispar* total: number of analyzed sequences available in the GenBank data
[b]Number of segregated sites

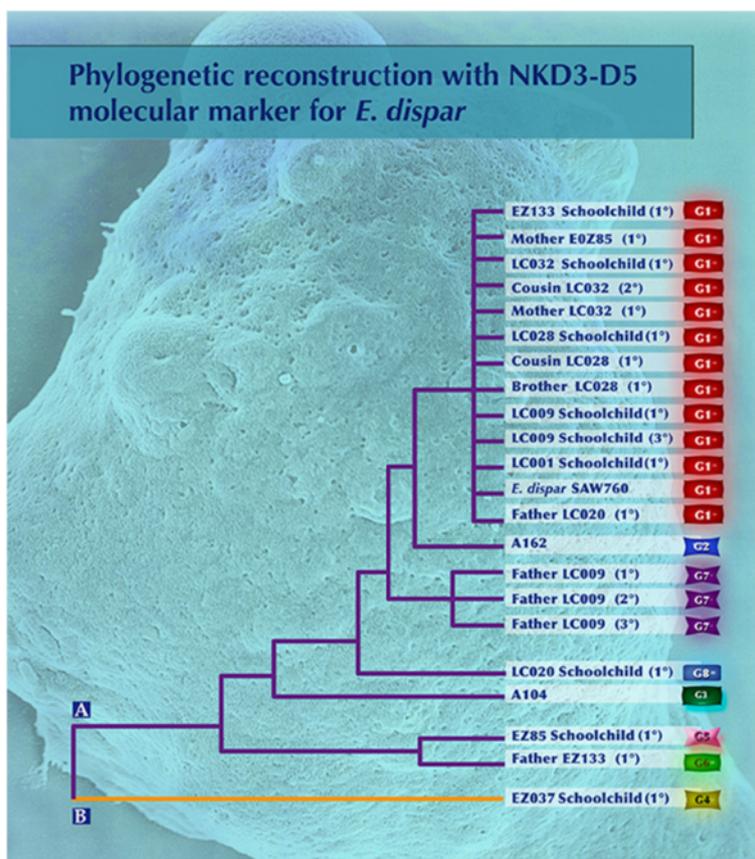Rojas *et al. BMC Infectious Diseases* (2016) 16:485

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Fig. 3 Phylogenetic representation of isolates obtained in schoolchildren, their families and sequences reported in GenBank

It is worth noting the evident tolerance of the school children to intestinal parasitic infection, not only to those parasites considered as commensal but also to pathogenic parasites. Furthermore, it appears that parasitism has no impact on the anemia index (Table 7); actually, the overall frequency anemia was 6.9 %, but the differences between infected and non-infected groups were not statistically significant. This contrasts with previous studies that reported the presence of iron deficiency anemia in children with repetitive events of diarrhea [31, 32].

Furthermore, intestinal parasitic infection did not substantially affect the growth and development of the children, as indicated by the analysis of differences in the BMI between infected and non-infected groups ($p < 0.05$) (Table 8); nevertheless, there are studies in which the presence of parasitism had detrimental effects on the growth and development of children with repeated episodes of diarrhea [31, 32].

We should look for cryptosporidium and coccidian infection in the feces samples of the children studied here; however, we decided not to use the kinyoun staining method due to the irrelevant frequency of these parasites in the state of Morelos compared with the frequency of these infections in other states of Mexico [33].

The availability of molecular tools to study the molecular epidemiology of *E. histolytica* and *E. dispar* infection in endemic regions allows us to determine the distribution of genetic variants of the two species in different geographic regions; hypothetically, knowing the geographic distribution of virulent genotypes of *E. histolytica* may help predict possible morbidity surges in at risk communities. Unfortunately, hitherto there is no convincing evidence of the relationship between genotypes and the type of amoebic outcome of the infected host. Efforts in this direction have been made using a number of polymorphic molecular targets [15, 34, 35].

Table 7 Analysis of the association between hemoglobin and intestinal parasitic infection

Hemoglobin Levels	Microscopic examination of stool samples			
	Positive (n = 67)	%	Negative (n = 232)	%
Hb[a]	5	7.46	16	6.89
Hb[b]	62	92.5	216	93.1

[a] children with Hb levels associated to anemia (Lanzkowsky [23])
[b] children with normal levels of Hb
Exact Fisher's test $p = 0.79$

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Table 8 Anthropometric characteristics: association between body mass index (BMI) and parasitized status

Microscopic examination (n = 306)	<5th percentile (under-weight)	5th to 85th percentile (normal weight)	85th to 95th percentile (over-weight)	≥95th percentile (obese)	Total (%)
Parasitized	3	51	10	8	72 (23.5)
Non-parasitized	10	141	47	36	234 (76.4)

Chi^2 test = 2.85, p = 0.41

The most studied molecular targets used to analyze the association of genotypes with the different outcomes of disease (amebic colitis, amebic liver abscess or asymptomatic intestinal infection) are the STRs of the intergenic region related to tRNA genes [19, 36]. These were the targets selected for our study; the NKD3-D5 marker was the one that produced the best PCR products and sequences in a large number of samples. The NKD3-D5 marker allowed us to indentify 8 different genotypes in the studied children and their relatives; the most prevalent was genotype 1 (Fig. 2).

It is worth noting that the Lazaro Cardenas school, which has particular characteristics such as overcrowded classrooms, was the school attended by most of the school children belonging to a close community in Tlaltizapan; the people of this community live in extreme poverty. This school was the one with the lowest value in the hygiene index (Table 3); it was also the school with the highest frequency of intestinal parasitic infection (28.9 %). Given these facts, one would expect a higher frequency of *E. histolytica*, and greater genetic diversity of *E. histolytica*. However, it seems to be a common phenomenon, which has been observed in other endemic communities, that the same genotypes circulate from one individual to another within the community; in our study, the transmission patterns of the parasites went from the homes to the school and viceversa [5, 12, 13, 37, 38].

The analysis of the genetic diversity of *E. dispar* isolates obtained in our group of children and their relatives using the NKD3-D5 marker showed what can be considered a low diversity (Table 6), since we obtained only 6 genotypes from 19 sequences with 8 segregative sites (Ss), π value of 0.00337 and θ of 0.00622. Considering the total number of sequences (our sequences and those available in the databases), the values were: 8 genotypes from 22 sequences, 9 Ss, π and θ values of 0.00114 and 0.00185 respectively; these values correspond to a low diversity.

Previously, we reported the genetic diversity of *E. dispar* isolate from amoebic liver abscess material in which, using the D-A marker, we obtained 15 genotypes and π and θ values of 0.081 and 0.064 respectively. Even though there is not enough information about the genetic diversity estimated through the π and θ rates, the previous values are higher than those obtained in the present work [14]. Escueta-de Cadiz et al. (2010) [39] reported the genotyping of *E. histolytica* using the marker

NK2H3-H5; they identified 5 motifs with 8 nucleotides each, which indicates that *E. histolytica* is less polymorphic than *E. dispar*; the use of the D-A genetic marker [13] demonstrated that *E. histolytica* is indeed less polymorphic than *E. dispar*. The low genetic diversity of our isolates suggests that most of the segregative sites are found in only a few sequences.

Conclusions

The comparison of the genotypes of *Entamoeba* species did not show correlation between children and their relatives; this was also observed in the most frequent genotypes in the schools, suggesting that in this community the species of *E. dispar* genotype 1 is the most widespread.

Finally, based on the indicators of growth, development and nutrition status, we can conclude that the studied community seems to be reasonably adapted to constant exposition to intestinal parasites, not only to those considered commensal but also to pathogens, since there were no evidences of a serious impact of the parasitized condition on the children's health.

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alignment. EH, OP, MN, EG, AV contributed reagents/material/analysis tools. EH, OP, MN, MG, UM, PM, AV, LR, EG collected samples in the community of Tlaltizapan and acquisition of data related to the studied population. All the authors read and approved the final version of the manuscript.

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CORRECTION

Correction: Differential expression of pathogenic genes of *Entamoeba histolytica* vs *E. dispar* in a model of infection using human liver tissue explants

Cecilia Ximénez, Enrique González, Miriam Nieves, Ulises Magaña, Patricia Morán, Marco Gudiño-Zayas, Oswaldo Partida, Eric Hernández, Liliana Rojas-Velázquez, Ma. Carmen García de León, Héctor Maldonado

Table 4 is incorrect. Please see the correct Table 4 here.

Table 4. Expression levels of genes linked to mechanisms of pathogenesis in the ex vivo infection model, selected by bibliographic revision.

CLAVE	Time (h)	RQ ± SD		p value
		E. histolytica	E. dispar	
Ehoxired	1	6049 ± 1527	3822 ± 1231	0.072
	3	2320 ± 127.3	820.5 ± 269	0.045
	24	592.2 ± 125	75 ± 25	0.033
	48	21 ± 12.5	0.8 ± 0.23	0.025
Ehaig-1	1	80.3 ± 32.3	16.3 ± 2.6	0.04
	3	361.1 ± 72.4	0.64 ± 0.4	0.03
	24	4154. 4 ± 201	0.21 ± 0.1	0.02
	48	3.4 ± 1.2	0.96 ± 0.4	0.017
Eh hsp-20kDa	1	957.2 ± 191	27.4 ± 6.6	0.036
	3	1236.8 ± 327	39.2 ± 6.1	0.023
	24	29.3 ± 5.6	9.5 ± 6.5	0.03
	48	35.8 ± 9.1	0.05 ± 0.0006	0.04
Ehpeptidase	1	2.1 ± 0.6	0.044 ± 0.014	0.015
	3	8.25 ± 1.7	0.66 ± 0.25	0.026
	24	2.7± 1.9	0.43 ± 0.12	0.045
	48	2.23 ± 0.23	0.05 ± 0.03	0.04
Eh20kDa	1	0.37 ± 0.1	0.66 ± 0.4	0.93
	3	70.7 ± 28	0.3 ± 0.2	0.042
	24	4.1 ± 0.33	0.71 ± 0.4	0.015
	48	2.3 ± 0.3	0.55 ± 0.4	0.14
Eh dovasa	1	0.41 ± 0.03	0.9 ± 05	0.18
	3	2.1 ± 0.65	0.51 ± 0.2	0.032
	24	0.9 ± 0.3	0.15 ± 0.01	0.046
	48	0.9 ± 0.14	0.12 ± 0.043	0.042
Ehthiored	1	0.31 ± 0.023	0.31 ± 0.023	0.083
	3	0.22 ± 0.1	0.13 ± 0.05	0.067
	24	0.5 ± 0.03	0.2 ± 0.1	0.042
	48	0.7 ± 0.14	0.2 ± 0.1	0.03
Ehhypo1	1	69 ± 32	0.23 ± 0.1	0.032
	3	44.4 ± 1.3	0.9 ± 0.2	0.03
	24	61.4 ± 25	0.53 ± 0.14	0.02
	48	40 ± 15.4	0.5 ± 0.1	0.02
Ehhypo3	1	1.02 ± 0.4	1.7 ± 0.001	0.125
	3	0.21 ± 0.1	13.2 ± 4.7	0.031
	24	0.98 ± 0.7	7.4 ± 0.01	0.046
	48	0.22 ± 0.01	0.76 ± 0.5	0.062
Ehhypo4	1	34.82± 0.4	5.4 ± 2.3	0.017
	3	8.73 ± 2.5	6.2 ± 1	0.82
	24	18.3 ± 6.1	17.1 ± 5	0.062
	48	15.1 ± 6	0.53 ± 0.3	0.032
Ehhypo5	1	191.6 ±71.5	914.9 ± 95.5	0.15
	3	1053 ± 33.6	3.6 ± 2.2	0.017
	24	3.9 ±1.3	0.27 ± 0.1	0.033
	48	0.75 ± 0.31	0 .63 ± 0.4	0.072

(Continued)

Table 4. (Continued)

CLAVE	Time (h)	RQ ± SD		p value
		E. histolytica	*E. dispar*	
Ehhypo6	1	73.01 ± 32	0.01 ± 0.001	0.034
	3	145.1 ± 63	0.034 ± 0.014	0.021
	24	2.6 ± 0.7	0.02 ± 0.005	0.045
	48	1.71 ± 0.7	0.01 ± 0.01	0.043
Ehhypo7	1	0.00011 ± 0.00006	0.0083 ± 0.0006	0.45
	3	3.7 ± 0.85	0.48 ± 0.05	0.29
	24	286.1 ± 57.1	0.87 ± 0.45	0.017
	48	2.1 ± 0.45	0.15 ± 0.05	0.124
Ehypo8	1	6.4 ± 2.6	3.2 ± 0.5	0.083
	3	13.6 ± 0.43	4.8 ± 1.5	0.02
	24	2.83 ± 0.5	1.3 ± 0.23	0.31
	48	0.95 ± 0.3	0.31 ± 0.02	0.073
Ehhypo9	1	3.1 ± 1.2	8.13 ± 0.95	0.02
	3	0.56 ± 0.29	0.56 ± 0.4	0.03
	24	129.4 ± 27.6	0.18 ± 0.1	0.025
	48	2.2 ± 1.1	0.44 ± 0.04	0.04
Ehhypo10	1	0.74 ± 0.3	0.05 ± 0.013	0.097
	3	0.012 ± 0.006	0.045 ± 0.004	0.94
	24	0.041 ± 0.003	0.01 ± 0.004	0.31
	48	0.05 ± 0.01	0.00023 ± 0.0001	0.88

After human PCLS were ex vivo infected with *E. histolytica* or *E. dispar* trophozoites for different time spans (1, 3, 24 and 48 h), the relative quantification (RQ) was determined (by qPCR) for the mRNA of some genes obtained by a bibliographic analysis. (RQ) relative quantification, (SD) standard deviation, data expressed as the mean of 5 separate assays. Shown the RQ of some genes which codified to hypothetical proteins, the cardinal number added is only for personal identification. p value calculated by Student t-test.

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Reference

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REVIEW ARTICLE

Human Intestinal Microbiota: Interaction Between Parasites and the Host Immune Response

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The human gut is a highly complex ecosystem with an extensive microbial community, and the influence of the intestinal microbiota reaches the entire host organism. For example, the microbiome regulates fat storage, stimulates or renews epithelial cells, and influences the development and maturation of the brain and the immune system. Intestinal microbes can protect against infection by pathogenic bacteria, viruses, fungi and parasites. Hence, the maintenance of homeostasis between the gut microbiota and the rest of the body is crucial for health, with dysbiosis affecting disease. This review focuses on intestinal protozoa, especially those still representing a public health problem in Mexico, and their interactions with the microbiome and the host. The decrease in prevalence of intestinal helminthes in humans left a vacant ecological niche that was quickly occupied by protozoa. Although the mechanisms governing the interaction between intestinal microbiota and protozoa are poorly understood, it is known that the composition of the intestinal bacterial populations modulates the progression of protozoan infection and the outcome of parasitic disease. Most reports on the complex interactions between intestinal bacteria, protozoa and the immune system emphasize the protective role of the microbiota against protozoan infection. Insights into such protection may facilitate the manipulation of microbiota components to prevent and treat intestinal protozoan infections. Here we discuss recent findings about the immunoregulatory effect of intestinal microbiota with regards to intestinal colonization by protozoa, focusing on infections by *Entamoeba histolytica*, *Blastocystis* spp, *Giardia duodenalis*, *Toxoplasma gondii* and *Cryptosporidium parvum*. The possible consequences of the microbiota on parasitic, allergic and autoimmune disorders are also considered. © 2017 IMSS. Published by Elsevier Inc.

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Epidemiological Considerations of Parasites

During the 1990's, great efforts were taken to control and reduce infections caused by soil-transmitted helminths in schoolchildren. The most severe consequences of worm infections are seen in young children who can die of acute roundworm obstruction of the gut and severe malnutrition. During the second half of the 20th Century, World Health Organization (WHO) developed effective community-based treatment programs in various developing countries around the world (Sub-Saharan Africa, Asia, Latin America and Caribbean countries). These programs had different results depending on resources available for its implementation. However, reports clearly show the reduction of

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morbidity rates of soil transmitted-helminthes, intestinal nematodes and roundworm parasites worldwide.

This program was also implemented in Mexico. After 1995, when this program began, the incidence rate of nematode infections decreased considerably, and in some geographic areas of Mexico had almost disappeared (http://www.dgepi.gob.mx). The empty ecological niche left by this change has been occupied by other commensal or pathogenic intestinal protozoa (1).

Globally, the most prevalent pathogenic protozoa are (in order of frequency of infection): *Blastocystis* spp (2−70%), *Giardia duodenalis* (5−20%), the *Entamoeba histolytica/E. dispar* complex (2−21%), and *Cryptosporidium* spp (1−17%) (2−4). The latter is a neglected parasite previously considered as opportunistic, affecting only immunocompromised patients. It is now one of the most frequent parasites detected in children under five who suffer from diarrhea. Whereas the prevalence of infection in Mexico of *Cryptosporidium* is around 3%, recent studies in developed countries have found that there might be slight differences in the frequency of intestinal parasitic infections, depending on sociodemographic and cultural characteristics of particular communities (5−8).

In general, intestinal parasitic infections due to protozoa represent a key component of the burden of infectious disease worldwide, constituting an important public health problem in most underdeveloped countries. However, these diseases remain a neglected issue in extended geographic areas. Since the composition of the intestinal bacterial population modulates the progression of protozoan, the regulation of different components of the microbiota could be used to prevent or attenuate intestinal protozoan infection and ultimate outcome of parasitic disease. Parasites and microbes have co-evolved together throughout evolution. For that reason, bacteria and parasites display complex interactions in the human mucosa. Since the underlying mechanisms remain poorly understood, the aim of the present review is to summarize recent findings in this area (9,10).

General Characteristics of Intestinal Microbiota

Fecal and metagenomic studies have revealed that the healthy intestinal microbiota is a complex ecological community of trillions of microorganisms, containing viruses, bacteria, protozoa and fungi. These microorganisms interact with the intestinal mucosa and carry out critical physiological functions for the host (11). The intestinal microbiota has coevolved with the intestinal immune system of the human host, maintaining a mutualistic host-microbial relationship. It has a significant influence on the maturation, development and modulation of the intestinal immune response, regulating the expression of immune mediators as well as the development, recruitment and differentiation of local immune cell populations (12,13).

The intestinal microbiota is highly diverse and varies over time as well as between individuals and regions of the intestine. Despite the large number of distinct bacterial taxa, they belong to a comparatively small number of phyla. Bacteroidetes and Firmicutes are the most abundant taxa in the intestinal microbiota (14). As with other elements of the microbiota, the relative levels of their respective populations are variable between individuals. The structure of the microbial community is a vital factor for host immunity under certain environmental contexts. Disruption of the microbiota from the normal balance and its interaction with the immune system can upset host homeostasis and susceptibility to disease, and thus determine the outcome of infections by intestinal pathogens. An imbalance in the population structure of the intestinal microbiota is called dysbiosis (15−17).

The application of high-throughput approaches, including next generation sequencing of the small subunit ribosomal RNA (16s rRNA), has led to a revolution in the identification of intestinal microbiota components. It is now known that approximately 500−1000 bacterial species inhabit the human adult intestine, the predominant genera being *Bacteroides*, *Bifidobacterium*, *Eubacterium*, *Clostridium*, *Peptococcus*, *Peptostreptococcus*, *Lactobacillus* and *Ruminococcus* (18).

Parasites (particularly intestinal parasites) are ancient in evolution. Although parasites have co-evolved with the human host, there is a relatively small number to which we are exposed. Helminths, for example, show a strong immunoregulatory activity, as do some bacteria of the intestinal microbiota. Thus, these parasites can modify the structure of intestinal microbiota, colonize this organ, and persist among the distinct populations of microorganisms (19−22). On the other hand, certain compositions of the bacterial community of microbiota are able to impede gut colonization by helminths, or prevent their persistence in case of colonization (23).

The interaction of intestinal parasites with the bacterial community of the gut microbiota forms a complex interacting system. Any modification of the microbiota has an effect on the host immune response, in part because these parasites and microbiota metabolize substrates in an interactive fashion and generate products that affect one another. The products of the microbiota may interfere with the survival and physiology of different parasites and consequently with the outcome of many parasitic infections (24). Likewise, intestinal parasites (both helminths and protozoa) continually secrete molecules that may change the environment and therefore cause an alteration in the structure of the gut microbiota (25). It is important to conceptualize the intestinal environment as an ecosystem in which biological and biochemical interactions occur at various organizational levels between the parasites, the microbial communities, and the host immune response (26,27).

Human Intestinal Protozoa

There is a rather wide range of protozoa that colonize the human gastrointestinal tract. Protozoans are not a homogeneous group and their physiology and biochemistry are largely geared to the parasitic habitat. Distinct mechanisms of host invasion are displayed by intracellular parasites (e.g., *Cryptosporidium parvum* and *Toxoplasma gondii*) and extracellular, host-specialized protozoa (e.g., *Entamoeba histolytica* and *E. dispar*). A number of parasites, including *Giardia duodenalis*, are adapted to more than one host. Since they do no real damage, a few protozoa can be regarded as commensal (e.g., *Entamoeba coli* or *E. hartmanni*). Other intestinal protozoans, such as *Blastocystis* spp, occasionally give rise to symptoms related to intestinal damage (e.g., diarrhea). The prevalence of *Blastocystis* has increased worldwide, especially some subtypes that infect human hosts. In Mexico, the prevalence of this parasite has gradual raised to its current level of about 60% (28−31).

The increased prevalence of *Blastocystis* today is linked to clear changes in the composition of the microbiota in the human host (32−35) and coincides with the decrease in the prevalence of helminth infection and morbidity in Mexico, a change that began in 1995 when the massive deworming program for schoolchildren was launched. *Blastocystis* infection was previously considered as an opportunistic disease associated with human immunodeficiency, or occasionally found in patients suffering from irritable bowel syndrome (IBS) or chronic inflammatory bowel disease (IBD) (36−40). The question of whether these protozoa are able to regulate the human intestinal inflammatory immune response remains controversial.

The intestinal parasites most prevalent in Mexico are apicomplexans (e.g., *Toxoplasma gondii* and *Cryptosporidium parvum*), *E. histolytica*, *Blastocystis* spp and *Giardia duodenalis*. The prevalence of these parasites might be comparable between Mexico and other countries or regions with very similar sociodemographic and socioeconomic characteristics. The remainder of the present review describes the unique properties of such microorganisms.

Apicomplexan Parasites

Intestinal infections caused by Apicomplexa have been detected worldwide, both in developed and developing countries. One third of humans have been exposed to at least one of these parasites and is seropositive. Recent observations in Mexico suggest that two apicomplexan parasites, *Toxoplasma gondii* and *Cryptosporidium parvum*, are increasingly present in infant populations under five years of age (41).

Infection by *T. gondii* leads to mild-moderate self-limited diarrhea in patients without immunological compromise. However, in immunocompromised patients it is often a lethal opportunistic organism (42−44). On the other hand, a *C. parvum* infection in immunocompetent individuals provokes acute diarrhea as the major symptom, which can be severe in infants under five (the most frequent population affected by a symptomatic infection). Whereas *C. parvum* is localized in the small intestine, *T. gondii* is able to invade extra-intestinal organs (the CNS) and other tissues (muscles) (45,46).

After parasites transmigrate into the intestinal lumen, neutrophils recruited to the site of infection transport them to other villi (44). Following *T. gondii* dissemination in the intestine and its infection of neutrophils and other phagocytic cells, such as dendritic cells, it can invade the lamina propria and eventually reach the nervous system and muscular tissue. Both apicomplexan parasites induce a MyD88-dependent Th1-driven immune response characterized by the production of IFN-γ and IL-12 (47,48). This response is essential for parasite control, but may be harmful for the host. In the case of *T. gondii* infection in the mouse model, the overzealous Th1 response can result in high mortality due to severe ileitis. Although *T. gondii* promotes inflammatory disease in various species, it does not lead to ileitis in the human host. Indeed, humans lack Toll-like receptor (TLR)-11, important in the intestinal pathology of mice (44,49).

As the main cytokine for controlling an infection by either *C. parvum* or *T. gondii*, IFNγ is expressed during the innate and adaptive immune response and has several functions. Apart from triggering Th1 cytokine production, it stimulates the production of nitric oxide, reactive oxygen species, antimicrobial peptides (AMPs) and the immunity-related GTPases, which all inhibit intracellular parasite growth (50,51). Other Th1 pro-inflammatory cytokines contribute to the immune response against *T. gondii* and *C. parvum*, including TNF, IL-1β and IL18. These cytokines attract essential cell types to the site of infection (52,53).

Both *T. gondii* and *C. parvum* also elicit a Th2 response, which is more pronounced with the latter. *Cryptosporidium parvum* produces and/or elicits an up regulation of some Th2 cytokines (e.g., IL4, IL5, IL6, IL10 and TGF-β) that may be involved in parasite clearance. TGF-β possibly dampens the inflammatory response following the resolution of the infection (54−56). Since a complete lack of Th1 cytokines in mice guarantees a lethal outcome during a *T. gondii* infection and frequently leads to the same effect during a *C. parvum* infection, the main role of Th2 cytokines is probably to modulate the strong Th1 cytokine response (57).

What is known about the effect of intestinal microbiota on the capacity or incapacity of these two intracellular intestinal parasites to survive and persist? Intestinal bacteria compete very efficiently for space and nutrients in the gut, which apparently is the first defense mechanism against pathogen colonization. Nevertheless, an inappropriate host immune response against commensals represents the onset of an inflammatory response that gives rise to IBD and its corresponding severe pathology.

Except for *T. gondii*, there is very limited information regarding the impact that a particular composition of the microbiota may have on protozoan infection. Commensals are key to various aspects of *T. gondii*-mediated disease. Most knowledge about the role of microbiota in *T. gondii* and *C. parvum* infections has been obtained from studies on genetically manipulated mice.

For both these apicomplexan parasites, TLR11 recognition is essential for the induction of the Th1 cell response. This is particularly important when *T. gondii* is administered intraperitoneally to infect mice. Contrarily, TLR2, TLR4 and TLR9 seem to be indispensable for an efficient Th1 response in orally infected mice. The Th1 protective response is dependent on the intestinal microbiota, suggesting that TLR2, 4 and 9, play a role in the development of the Th1 response against the parasite by sensing bacterial products (58,59). For a *C. parvum* infection, the Th1 response is enhanced when mice are fed a TLR9 agonist. Hence, intestinal bacteria perhaps carry out a similar function during infection with this parasite as that observed in the process of a *T. gondii* infection (60).

Interestingly, the absence of intestinal commensal bacteria impeded the development of ileitis after a *T. gondii* infection in wild type mice (58,61), supporting the idea that intestinal microbiota contributes to the development of immunopathology in the gut. Indeed, *Enterobacteriaceae per se* might induce an inflammatory pathology that is characteristic of *T. gondii* infection in mouse models (61,62). In the absence of TLR11 in genetically manipulated mice, oral infection with this parasite does not lead to ileitis, apparently because the Th1 response elicited by the intestinal bacteria population is more efficient. These findings have recently been reported in a large number of excellent studies in the literature. Hence, the elimination of some factors that contribute to the development of the Th1 response diminishes the negative effects of the immunopathology during a *T. gondii* intestinal infection (62–65).

Significant changes take place in the composition of the microbiota during a *T. gondii* infection. For example, there is a decrease or elimination of Bacteroidetes and Firmicutes followed by an important but temporary predominance of Enterobacteriaceae, particularly *Escherichia coli* (62,66). This microbiota profile has been described in various mouse models of ileitis and in human patients with IBD (67,68). Although the increase in the intestinal population of *E. coli* in mice orally infected with *T. gondii* can provoke lethal gut inflammation, a *T. gondii* infection in the presence of elevated levels of other populations of commensals is not lethal. Expansion of the *E. coli* intestinal population during a *T. gondii* infection triggers an intense Th1 response, causing high levels of IFNγ. This in turn leads to severe tissue damage as well as the loss of Paneth cells and AMPs. The same pattern of immunopathology is observed in patients with IBD (58,69).

In a *C. parvum* infection, on the other hand, INFγ and CD4$^+$ lymphocytes are protective and the role of *E. coli*

or other groups of commensal bacteria in the immunopathology is unknown. Due mainly to the lack of reliable animal models, the immune response during a *C. parvum* infection is poorly understood. This represents an open opportunity for research that is a priority for the protection of public health, because the number of individuals suffering from some type of immunodeficiency has been steadily rising over the last few decades.

Entamoeba histolytica Infection

Amebiasis is acquired by the ingestion of food and/or water contaminated with amebic cysts, and these are highly resistant to dry environments and the acid barrier in the stomach that filters transit to the small intestine. The complete excystation of cysts and colonization by trophozoites occur in the terminal ileum.

In asymptomatic individuals, the colonization of the intestinal mucosa by *E. histolytica* manifests a commensal relationship with the human host in which the excretion of cysts of non-pathogenic trophozoites takes place during the entire lifecycle of the parasite. Epidemiological studies of individuals in endemic areas suggest that most infected individuals are asymptomatic cyst passers, and only a minority is susceptible to developing amoebic disease (mainly amoebic colitis or self-limited dysentery). A small but significant number of patients with intestinal amoebiasis develop severe extraintestinal amoebiasis (most commonly, amoebic liver abscesses).

This ancient protozoan has co-evolved with the human host for thousands of years. The recent unveiling of its genome revealed the genetic diversity of the two species of *Entamoeba*: *E. histolytica* and *E. dispar* (the latter being the more diverse) (70–72). The existence of distinct genotypes of *Entamoeba* strains may explain the different outcomes of human *Entamoeba* infection (73,74). Human host factors have also been examined in light of the various effects of an amoebic infection, testing the possible genetic basis of susceptibility and resistance of humans to invasive disease (75–77).

It is becoming clear that the interaction between *Entamoeba* species and intestinal bacteria is an important factor in the modulation of amebic virulence (72,78–80). The close relationship between *E. histolytica* and intestinal bacteria is evidenced in several ways. The trophozoite stage of *Entamoeba* feeds on intestinal commensal bacteria, and the phagocytosis of bacteria in the colon is deemed a triggering mechanism of the invasiveness of *E. histolytica*. Moreover, certain *E. dispar* strains display a pathogenic behavior in individuals with particular microbiota structures, generating non-dysenteric colitis (81,82).

Genital skin ulcers infected with pyogenic bacteria are found to be co-infected with one or both *Entamoeba* species. We recently detected an *E. dispar* infection in pyogenic liver abscess and an *E. histolytica* and

E. dispar co-infection in patients of amoebic liver abscess (79). The *E. dispar* finding in pyogenic material from hepatic abscesses points to the modulation of virulent expression of parasites by some bacterial groups (72).

On the other hand, there is evidence that the presence of *E. histolytica* induces considerable changes in the structure of intestinal microbiota populations in the human host (83). In Northern India, Verma AK, et al. (2012) carried out an absolute quantification of 16S rRNA using quantitative PCR on positive patients to *E. histolytica* amoebic colitis. They identified a significant decrease in *Bacteroides*, the *Clostridium coccoides* subgroup, the *Clostridium leptum* subgroup, *Lactobacillus*, *Campylobacter* and *Eubacterium*, and a significant increase in *Bifidobacterium* compared to healthy control individuals (84).

In a longitudinal study of the first two years of life of a cohort of Bangladeshi children infected with *E. histolytica*, an association was observed between the prevalence of diarrhea and the expansion of the *Prevotella copri* population. Since this bacterium has been linked to inflammatory disorders, the authors suggest that the enhanced *P. copri* population possibly causes excessive immune activation. Interestingly, the abundance of *Bacteroides thetaiotaomicron* was not related to more frequent diarrheal episodes (83).

In an evaluation of the microbiota composition in rural populations of Cameroon, Morton ER, et al. (2015) described a strong correlation between the presence of *E. histolytica* and certain bacterial groups of the intestinal microbiota. Seven phyla were significantly different when samples from *Entamoeba* positive and negative individuals were compared. In *E. histolytica*-positive samples, a greater frequency of *Firmicutes* and a lesser frequency of *Bacteroidetes* were present (85).

In mice susceptible to *E. histolytica*, Burgess SL, et al. (2014) established an association between acquired protection and an increase in the level of IL-17A, antimicrobial peptide (AMP) and serum amyloid A (SAA). The authors also transferred SFB-mono-associated feces into the susceptible mice and found resistance against *E. histolytica* colonization together with higher levels of IL-17A and IL-23 before and after infection. Following amebic infection, they detected an elevated intestinal concentration of neutrophils and dendritic cells and a rise in the serum level of SAA in SFB-colonized mice. In addition, the authors carried out adoptive transfer of bone marrow dendritic cells (BMDCs) from SFB-colonized mice into the susceptible mice and observed that these BMDCs were capable of migrating to the intestine and conferred protection against *E. histolytica* colonization in an IL-17-dependent manner (86).

The Blastocystis Controversy

Blastocystis is a genus of parasitic unicellular organisms that inhabit the digestive tract of various metazoans, including fish, amphibians, birds, reptiles, rodents and humans. From the early 1900s until 1996, *Blastocystis* was considered a saprophyte yeast of the digestive tract (87,88). Through phylogenetic studies, *Blastocystis* was placed in the group of Stramenopiles (88,89).

Blastocystis is genetically diverse, with 17 subtypes defined by the small subunit rRNA gene (SSU rRNA), each having distinct distribution in the world and types of hosts. Among the nine subtypes of *Blastocystis* that infect humans, the most frequent are ST1, ST2, ST3 and ST4 (90−93). The possible relationship between parasite virulence and the genetic subtype of the protozoa is still controversial (90,91). For example, ST3 was the most prevalent subtype identified in both asymptomatic individuals and symptomatic patients in Iran (91).

Blastocystis is a common parasite with worldwide distribution and extremely variable prevalence. Infection is linked to poor hygiene, animal contact, and contamination of food and water sources (92,94). Recent studies have found a significant increase in the prevalence of *Blastocystis*. Sohail and Fisher (2005) reported that the prevalence of *Blastocystis* in asymptomatic adults is 30−50% in developing countries and 1.5−10% in developed countries (93). Bart A, et al. (2013) found that 38% of 107 symptomatic patients in the Netherlands were infected with *Blastocystis* (95). In Mexico, there are studies were measured the co-infection of *Blastocystis* and other intestinal parasites (96,97). For example, in the district of Xochimilco in Mexico City, the rate of infection of *Blastocystis* was 41.7% among 115 food vendors, with a co-infection of *Entamoeba coli* and *Endolimax nana* in 14.8 and 8.7% of the cases, respectively (30).

Some studies report that *Blastocystis* is a member of the normal mammalian eukaryotic microbiota (98). Scanlan PD, et al. (2014) evaluated different subtypes (species) of *Blastocystis* in individuals sampled over a period of 6−10 years, finding that this parasite is a prevalent and diverse member of the healthy gut microbiota, and that it is capable of long-term host colonization without prompting disease (99). In addition, Parfrey LW, et al. (2014) examined bacteria and parasites in healthy individuals from two populations: one with a traditional, agrarian lifestyle and another with a modern, Westernized lifestyle. After characterizing the human eukaryotic microbiota in the gut via high-throughput sequencing, they determined that it is less diverse and more patchily distributed than bacteria (100). Regarding the protists in the gut (typically considered as parasites), many are commensal and some are beneficial.

Nevertheless, human intestinal infection with *Blastocystis* can cause a variety of gastrointestinal signs and symptoms. In most cases, the infection is self-limited and the most frequent intestinal symptoms are diarrhea and abdominal pain. Evidence exists that a *Blastocystis* infection might exert effects on the inflammatory status of the intestine (101). This and other intestinal parasites can increase

oxidative damage and the production of pro-inflammatory cytokines in animal models (102,103). A solubilized antigen obtained from *Blastocystis* down-regulated the response of peripheral blood mononuclear cells (PBMCs) and exacerbated the proliferation of colorectal cancer cells *in vitro* (102). On the other hand, there seems to be more prevalent *Blastocystis* infection and a higher pathogenicity in people with stressed lifestyles in developing countries (94). All these reports suggest the pathogenic potential of *Blastocystis*.

IBS is the disease most frequently correlated with *Blastocystis* infection (101,104). This parasite could participate in the development of IBS (105,106). The symptoms corresponding to a *Blastocystis* infection are apparently a consequence of the innate immune response triggered by the disruption of the intestinal barrier. The infiltration of the intestinal epithelial barrier involves a host of immune receptors and cells, including TLRs and IgM/IgG/IgA antibodies as well as CD8+ T cells, macrophages and neutrophils (107). However, the role of *Blastocystis* colonization in patients with gastrointestinal symptoms is still unclear.

Some studies have detected a greater prevalence of *Blastocystis* in asymptomatic individuals, meaning that infection does not always induce gastrointestinal pathology (108). Our group has observed an association between *Blastocystis* colonization and an anti-inflammatory state of the intestine in asymptomatic individuals (manuscript in preparation). In IBS patients, sigmoidoscopic biopsies have demonstrated that *Blastocystis* can generate an imbalance in the intestinal microbiota, thus provoking chronic inflammation (109). Furthermore, patients with IBS and a *Blastocystis* intestinal infection show a significant decrease in the *Bifidobacterium* genus. In individuals presenting with a *Blastocystis* infection without IBS, there was a significant decline in *Faecalibacterium prausnitzii,* a bacterium known for its anti-inflammatory properties, supporting the hypothesis that links *Blastocystis* to the pathophysiology of IBS and an intestinal microbiota imbalance (34).

According to a recent study performed by our group in a rural cohort in Mexico (in the state of Morelos), asymptomatic individuals infected with *Blastocystis* show more diversity in the bacteria of their intestinal microbiota, which contains elevated levels of the *Alistipes, Oscillospira* and *Ruminococcus* genera and reduced levels of *Bacteroides, Bifidobacterium* and *Prevotella*. The presence of *Blastocystis* and its associated microbiota did not seem to induce an inflammatory state. Indeed, it appeared to generate an anti-inflammatory scenario, characterized by a lower concentration of fecal inflammatory markers (manuscript in preparation).

Whether *Blastocystis* is pathogenic or not may depend on the effects of infection on the bacterial microbiota. Future studies are needed that focus on the possible influence of intestinal *Blastocystis* colonization on the bacterial composition of the microbiota in healthy asymptomatic individuals and patients with IBS. Particular attention should be paid to the link between a *Blastocystis* infection and the presence of biomarkers related to free radical production, oxidative stress and inflammation.

Giardia duodenalis Infection

Giardia duodenalis (*G. lamblia*/*G. intestinalis*) is a protozoan that infects both humans and animals around the world. Among the parasites that cause intestinal infection, it is one of the most common (110). The infection rate for *Giardia duodenalis* is generally lower for the people of developed countries, usually under 10% of the population and can cause a self-limited illness (i.e., giardiasis) characterized by diarrhea, abdominal cramps, bloating, weight loss and malabsorption (111−113).

G. duodenalis is a useful model for demonstrating the importance of the intestinal barrier (the commensal microbiota, mucosal layer and intestinal epithelium) in the context of many gastrointestinal diseases. Researchers have found that *Giardia* attempts to avoid this barrier through direct or indirect strategies, which include inducing pronounced changes in the biofilm architecture of the host microbiota, disrupting the mucosal layer, and damaging the physiology and survival of the intestinal epithelium (114−116).

Accordingly, Beatty JK, et al. (2017) observed that *G. duodenalis* could induce abnormalities in the biofilm architecture of the host microbiota, mediated by *Giardia* cysteine secretory/excretory proteases. Such biofilm disruption allowed for bacterial invasion, which resulted in epithelial apoptosis and the translocation of bacteria across the intestinal epithelial barrier. Moreover, these dysbiotic microbial communities in the host stimulated the activation of TLR signaling pathway 4 and the overproduction of proinflammatory cytokine IL-1ß. The authors suggest that the interaction between *G. duodenalis* and the intestinal microbiota of the host may cause persistent dysbiosis, possibly predisposing infected individuals to gastrointestinal disorders (117).

Likewise, Bartelt JK, et al. (2017) found that *Giardia* gives rise to signs of lesion in the intestinal mucosa, although *Giardia*-infected mice did not generate the proinflammatory bowel responses identified in pediatric patients with endemic *G. duodenalis* infection. Nevertheless, the presence of this parasite had a profound effect on the metabolism of the intestinal microbiota of the mice. The authors also tested co-infection with enteroaggregative *Escherichia coli*-*Giardia duodenalis* in mice, finding an increase in the signs of intestinal lesion as well as the levels of IL1α and CCL11 in a synergistic manner (118).

Because *G. duodenalis* does not invade the surface of cells in the small intestine, some researchers pose that it constantly interacts with the intestinal microbiota, thus fostering a close protozoan/microbiota relationship (119). This interaction has been examined in various studies employing probiotics as an experimental model. For example,

Lactobacillus species (*L. johnsonii*, *L. casei* and *L. rhamnosus* GG) promote *Giardia* clearance, while bacteriocins produced by *L. acidophilus* (P106) and *L. plantarum* (P164) reduce parasite adhesion (120−122).

Barash NR, et al. (2017) evaluated the impact of a *Giardia* infection on the intestinal microbiota of mice, finding that colonization of the small intestine by *G. duodenalis* engenders systemic dysbiosis of aerobic and anaerobic commensal bacteria. *Giardia* colonization was characterized by higher levels of aerobic microorganisms (*Proteobacteria*), and lower levels of anaerobic microorganisms (*Firmicutes* and *Melainabacteria*). They proposed that the resulting dysbiosis may be directly mediated by anaerobic *Giardia* metabolism and/or indirectly by the stimulation of intestinal inflammation. Hence, the Giardia-related alteration of commensal diversity could be the cause or consequence of inflammatory and metabolic changes throughout the murine intestine (110).

Beatty JK, et al. (2017) determined that these microbiota abnormalities are mediated in part by secretory-excretory *Giardia* cysteine proteases. With *in vitro* cell culture and germ-free murine infection models, *Giardia*-induced disruptions of the microbiota enabled bacterial invasion, which in turn provoked epithelial apoptosis, tight junction disruption, and bacterial translocation across the intestinal epithelial barrier. Furthermore, these dysbiotic microbiota communities prompted the activation of the TLR4 signaling pathway and elicited the overproduction of pro-inflammatory cytokine IL-1 β in humanized germ-free mice (117).

Many studies have been conducted on the correlation between *Giardia* and other pathogens to examine the role of their combination in infectious diarrhea (112,123).

Future Perspectives: Therapeutic Potential of Regulating the Relationship Between Parasites, Microbiota and the Host Immune Response

Bacteria, nematodes and protozoan parasites are components of the gastrointestinal tract microbiota that are recognized as important regulators and/or modulators of the host immune response (23). Indeed, several intestinal immunoregulatory species have co-evolved with their host for thousands of years. The regulation of the host immune response by an immunoregulatory element of the gut microbiota can, under certain environmental circumstances, undermine host antimicrobial immune defenses mechanisms, allowing parasites to adapt to the new ecological niche. This in turn leads to colonization and persistence of infection (124−126).

The metabolism and composition of bacteria in the microbiota is affected not only by infection with bacteria, nematodes and protozoa, but also by aspects of our modern
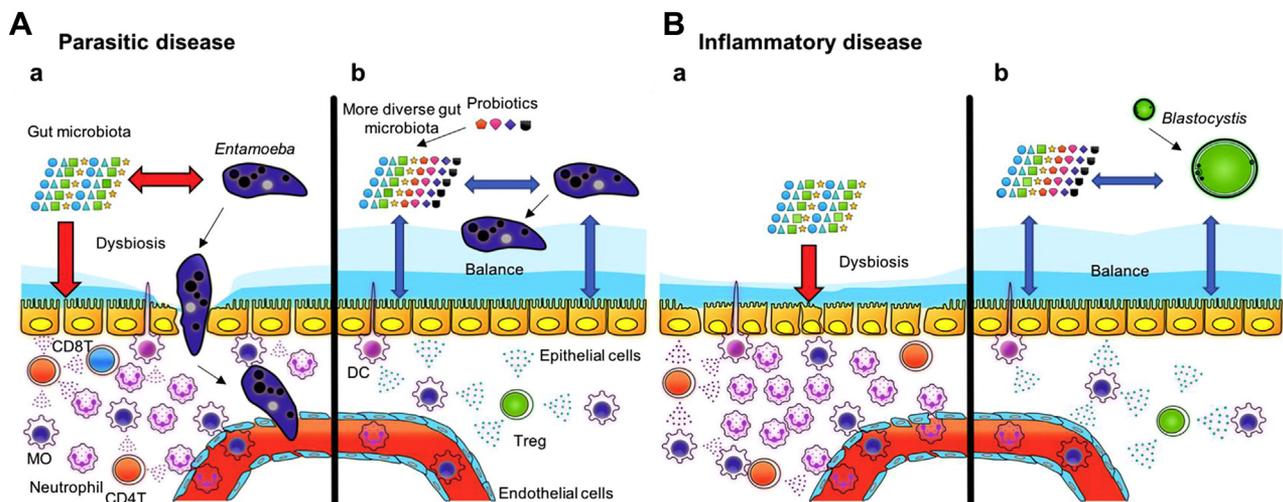

Figure 1. Potential therapeutic benefits of the Protozoa-Microbiota-Immunity relationship. (A) In parasitic disease, such as in *Entamoeba* infection (A), the gut microbiota associated with the pathogen can establish a pro-inflammatory environment by its interaction with epithelial and dendritic cells (DC). The production of inflammatory cytokines, such as IFNγ, TNF, IL-4, IL-6, IL-8 and IL-17, attracts and activates neutrophils, monocytes, and CD4T and CD8T lymphocytes. This scenario of dysbiosis makes the inflamed epithelium and endothelium more permeable, thus facilitating parasite invasion. The administration of probiotics or prebiotics (B) could change the microbiota composition by increasing its diversity, consequently disrupting the relationship between the parasite and the elements of the microbiota associated with it. The metabolites produced by the intestinal microbiota can stimulate the host cells to produce an anti-inflammatory cytokine response, which induces Treg cell production, creates a better structure of the epithelium, and establishes a balanced state between the host, the intestinal microbiota and the parasite. (B) Chronic inflammatory diseases, such as IBD, are characterized by a constant pro-inflammatory state perpetuated by an altered intestinal microbiota composition having reduced diversity (A). Although there is controversy about the possible role of *Blastocystis* in intestinal inflammatory diseases, recent research on this protozoan has exposed its ability to increase the diversity of the intestinal microbiota and promote an anti-inflammatory state in the intestinal mucosa (B). More research is needed to determine the metabolites produced by *Blastocystis* to communicate with bacteria, as well as their mechanisms of inducing an anti-inflammatory effect. Nevertheless, this bacterium could possibly have potential as a therapy for chronic inflammatory diseases. (A color figure can be found in the online version of this article.)

lifestyle (e.g., type of diet, psychological and/or physical stress). The influence of the latter factor might also facilitate the colonization and persistence of potentially pathogenic microorganisms, which generate toxic products that are part of the pathophysiology of numerous chronic and degenerative disorders in the human host (127). These are two possible causes of dysbiosis, a state in which the host microbiota brings about harmful effects through one or more of three mechanisms: inducing qualitative and/or quantitative changes in the intestinal flora itself, altering its metabolic activity, and/or modifying its composition.

Since distinct compositions of the microbiota may be favorable or unfavorable for parasite colonization of the host, modulation of the microbiota can potentially be utilized for therapeutic purposes. For instance, such modulation could possibly impede or suppress immunological disorders by avoiding the inadequate immune response directed against harmless antigens, which occurs with allergic reactions and autoimmune diseases (127). A variety of studies emphasize the role of certain components of the microbiota in protecting the host against allergic and hypersensitivity responses as well as other immunological disorders (128−132). Hence, examining the characteristics of different interactions between protozoa and bacteria might be useful for the design of strategies to treat dysbiosis due to protozoan pathogens and/or an overzealous immune response.

Probiotics, prebiotics or fecal transplantation can prompt changes in the composition of the microbiota, thus altering the metagenomic, transcriptomic, proteomic, and metabolomic structure of the gut (131,132). This type of intervention might be beneficial under certain circumstances during colonization by a pathogenic protozoan Figure 1A, such as *E. histolytica*, *G. duodenalis*, *Cryptosporidium* or *Toxoplasma* (127). Thus, the introduction of a particular protozoan could possibly modify the interaction of bacteria with another protozoan and affect pathogenic behavior and the outcome of disease in the host. Further research is needed to search for the mechanisms that govern these relationships, for which purpose new animal models must be used, such as germ-free and gnotobiotic organisms. These models have been extraordinary research tools for studying the population structure of intestinal microbiota, the corresponding effector molecules, and the possible mechanisms for lowering the virulence of pathogenic protozoa.

When inflammatory mechanisms are involved, the interaction between dysbiosis in the intestinal microbiota and the human immune response tends to manifest itself as a predisposition to disease (127). Therefore, in microbiota co-infected with some protozoa (e.g., *Blastocystis* spp), the anti-inflammatory potential and the greater bacterial diversity could possibly exert a therapeutic effect in treating chronic inflammatory intestinal disease or other autoimmune diseases Figure 1B. Accordingly, *Clostridium difficile* infection, primary IBD, Crohn's disease (CD) and ulcerative colitis (UC) are associated with a decreased diversity

of the gut microbiota that causes a shift to dysbiosis. These are potential targets for personalized therapeutics using *Blastocystis* as a probiotic to increase diversity and restore balance in the composition of the bacterial microbiota, which might be able to revert inflammatory intestinal disease (133).

There is increasing evidence about the anti-inflammatory activity resulting from intestinal colonization by *Blastocystis* protozoa, apparently by boosting levels of *Firmicutes* and the diversity of bacteria in the intestinal microbiota. Before exploiting this possibility, however, it is necessary to determine the role of *Blastocystis* in the disease process and explore the conditions under which it or other probiotics may foster an anti-inflammatory immune response. Based on infectious inflammatory models of intestinal disease in the human host, the study of the relationship between protozoa, bacteria and the immune response has produced many advances during the last few decades. Perhaps sufficient advances have been made for the development of therapeutic alternatives (e.g., probiotics, prebiotics and fecal microbiota transplantation) to control infectious and inflammatory processes.

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Entamoeba histolytica Calreticulin Induces the Expression of Cytokines in Peripheral Blood Mononuclear Cells Isolated From Patients With Amebic Liver Abscess

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Calreticulin (CRT) is a highly conserved protein in the endoplasmic reticulum that plays important roles in the regulation of key cellular functions. Little is known about the participation of *E. histolytica* CRT (*Eh*CRT) in the processes of pathogenicity or in the modulation of the host immune response. The aim of this study was to evaluate the role of CRT in the proliferation and the cytokine profile in peripheral blood mononuclear cells (PBMCs) from patients with amebic liver abscess (ALA) during the acute phase (AP-ALA) of the disease compared to patients during the resolution phase (R-ALA). The PBMCs from each participant were cocultured with *Eh*CRT and tested by the colorimetric method to evaluate their proliferation index (PI). The supernatants were subjected to an enzyme-linked immunosorbent assay (ELISA) to evaluate the concentration of cytokines. The mean values of all groups were compared using the independent *t*-test. When the PIs of individuals without diagnosis of liver abscess (NEG) were compared, there were no statistically significant differences in the proliferation of PBMCs between patients with AP-ALA and R-ALA when stimulated with *Eh*CRT or concanavalin A (ConA). However, the levels of interleukins [IL-6, IL-10, granulocyte colony stimulating factor (GCSF), and transforming growth factor β1 (TGFβ1)] were higher in patients with AP-ALA, whereas in patients with R-ALA, higher levels of interferon gamma (IFNγ) were detected. These results suggest that *Eh*CRT acts as a mitogen very similar to the activity of ConA. In addition, *Eh*CRT is an excellent immunogen for the specific activation of PBMCs, inducing the differential expression of ILs depending on the outcome of disease, determining the type of immune response: a Th2 cytokine profile during the acute phase and a Th1 profile during the resolution phase.

Keywords: amebic liver abscess, calreticulin, *Entamoeba histolytica*, mitogen, proliferation index, interleukins

INTRODUCTION

Infection with the enteric protozoan *E. histolytica* is one of the leading causes of death worldwide. The disease is a consequence of the parasite's abilities to invade the colon, causing amebic colitis. *E. histolytica* can disseminate to the liver via the portal venous system, resulting in amebic liver abscess (ALA). However, approximately 90% of infected people are asymptomatic cyst carriers (Haque et al., 2003). The molecular mechanisms by which this parasite causes invasive amebiasis are not fully understood. *E. histolytica* has adherence and cytotoxicity factors that are essential for its survival, but they are not directly responsible for ALA formation. It is known that the limitation and prevention of recurrent invasive amebiasis requires the development of an effective immune response. Thus, it is likely that the acute inflammatory response associated with *E. histolytica* infection is a key factor for the development of ALA (Chadee et al., 1985).

Parasite-specific immune responses are regulated by cytokines and chemokines that lead to the development of immunity, but these responses also contribute to infection, inducing pathogenesis and parasite persistence (Talvani et al., 2004). Little is known regarding the amebic signals that initiate an acute inflammatory response.

It has been reported that in mice infected with *E. histolytica,* host tissue damage is attributed primarily to the lectin activity of the galactose/*N*-acetyl-D-galactosamine (Gal/GalNAc) from *E. histolytica,* which promotes the accumulation of mononuclear cells, including neutrophils, inflammatory monocytes, and macrophages, at the site of infection (Blazquez et al., 2007). Intestinal epithelial cells infected with *E. histolytica in vitro* produce elevated levels of the cytokines, interleukin-8 (IL-8), growth-regulated protein alpha (GRO-α), granulocyte macrophage colony-stimulating factor (GMCSF), and IL-1 (Eckmann et al., 1995). Treatment of cultured human intestinal cells with the lectin Gal/GalNAc from pathogenic and nonpathogenic entamebas (*E. histolytica* and *E. dispar*) results in the secretion of chemoattractant and proinflammatory cytokines (Sharma et al., 2005), suggesting that these cells and cytokines also contribute to tissue damage, participating in the mechanisms of initiation, amplification, or limitation of the inflammatory processes during invasive amebiasis.

The identification of the mediators involved in leukocyte activation during infection by *E. histolytica* is of fundamental importance for understanding host responses in amebiasis. Cellular interactions and cytokines have been reported during amebic infections, and cytokines have been shown to be able to regulate monocyte function and increase the amebicidal activity of monocytes (Seydel et al., 2000; Lotter et al., 2013).

It is still not clear what other elements in the dynamics of host–parasite relationship define the outcome of infection, especially regarding the regulation of the immune response against *E. histolytica.*

To obtain further evidence about the relationships of immune cells with *E. histolytica,* other proteins have been studied to investigate the intracellular signals that promote the host immune response. Some examples include the cysteine proteinases 1 and 5 (CP1 and CP5) that breakdown IgA1 and IgA2 antibodies. These proteins cleave the Fc region that interacts with parasite surface receptors and mediates effector functions that can mask immunogenic surface molecules with inert Fab fragments, thus helping to prevent the parasite expulsion from the intestinal lumen (Garcia-Nieto et al., 2008).

The role of calreticulin (CRT) in host–parasite interactions has recently become a major area of research. The CRT genes from many parasites (*Trypanosoma, Leishmania, Entamoeba, Onchocerca, Schistosoma,* and *Haemonchus*) have been cloned and sequenced (Rokeach et al., 1994; Joshi et al., 1996; El-Gengehi et al., 2000; Marcelain et al., 2000; González et al., 2002; Suchitra and Joshi, 2005).

Although the functions of CRT are conserved in vertebrates, some CRT functions differ among parasites (Nakashi et al., 1998; Ferreira et al., 2004); parasite CRTs bind host C1q and inhibit C1q-dependent complement activation. The CRT of *Haemonchus contortus* binds host C-reactive protein and C1q (Naresha et al., 2009). The ecto-parasite *Amblyomma americanum* secretes CRT during feeding, suggesting that the anticoagulant ability of CRT may prevent blood clotting and allows the parasite to feed on the host and induce host antiparasite responses (Jaworski et al., 1995). The presence of CRT in the penetration gland cells of *Schistosoma* suggests that this molecule may be important for the host skin penetration (Khalife et al., 1994).

Previously, we reported the presence of CRT in *E. histolytica* (*Eh*CRT) and that this protein induces an important immunogenic response in the human host. More than 90% of patients with ALA develop high levels of serum antibodies against *Eh*CRT (González et al., 2002). We also reported the cloning of the CRT gene from *E. histolytica,* and the preparation of mono-specific antibodies against recombinant CRT. The immunohistochemical assays on trophozoites show that *Eh*CRT is in cytoplasmic vesicles and in vesicles that are in close contact with the inner cytoplasmic membrane (González et al., 2011). In addition, it was demonstrated that the CRT from both pathogenic *E. histolytica* and nonpathogenic *E. dispar* species specifically interact with human C1q molecules and inhibit the activation of the classical complement pathway (Ximénez et al., 2014). This activity is consistent with that reported by Vaithilingam et al. (2012). The trophozoites activated by the presence of jurkat cells clearly show the binding of C1q to CRT on the surface of the phagocytic mouths during the process of erythrophagocytosis.

However, the activation of the host immune response and the cytokine profile induced by *Eh*CRT have not yet been investigated.

In this study, we analyzed the proliferation and cytokine production of peripheral blood mononuclear cells (PBMCs) cultures isolated from ALA patients during the acute phase of the disease and from individuals who resolved ALA in order to characterize the cytokines profiles produced in response to *Eh*CRT.

METHODS

Ethics Statement

The present work was designed according to the guidelines for the management of human samples for experimental purposes as indicated in the Official Regulation NOM-12SSA3-2007 included in the General Health Law of Mexican Health Ministry. In addition, the project was approved by the Scientific and Ethics Committee of the Faculty of Medicine from the National Autonomous University of Mexico. Patients were informed about the purposes of the project, the sampling, and the potential risks during procedure, and the patients were invited to voluntarily participate by signing an informed consent letter.

Study Groups and Biological Samples

Patients with diagnosis of ALA admitted to the Internal Medicine, Gastroenterology, and Infectiology Services of the General Hospital of Mexico Dr. Eduaro Liciaga were recruited. These patients formed the group with acute phase amebic liver abscess (AP-ALA). The patients in the resolution phase of ALA (R-ALA) were recruited from a search of the archives of discharged patients from the General Hospital of Mexico, and they formed the ALA resolution group (R-ALA). Three fecal samples were collected from each patient from both groups at 1-week intervals after their hospitalization for microscopic examination for parasites. At the time of collection of the third sample, 10 ml of blood was drawn to evaluate serum antibodies against *E. histolytica* by enzyme-linked immunosorbent assay (ELISA), using an $OD_{490nm} \geq 0.5$ as an indicator of a positive result (Morán et al., 2007). The remaining sample was used to isolate PBMCs.

A third group, named the negative control (NEG), was formed with clinically healthy individuals from the Blood Bank of the General Hospital of Mexico, with ELISA serum levels of antiamebic antibodies below the OD threshold (OD<0.5). Using the same protocol for ALA groups, three fecal samples and 10-ml blood samples were taken for microscopic examination and PBMC isolation, respectively. Ten individuals for the group were included.

Isolation and Culture of PBMC

The PBMCs were isolated from 10 ml peripheral blood samples collected from each participant in tubes with K2 ethylenediaminetetraacetic acid (EDTA) as anticoagulant (BD Vacutainer, Ref 368171). Cells were separated on a Ficoll-Hypaque gradient (Gibco, Life Technologies, Grand Island, NY, USA), and the PBMC pellet was separated and washed three times with phosphate-buffered saline PBS. After separation, PBMCs were centrifuged and resuspended in Roswell Park Memorial Institute medium RPMI culture medium supplemented with 10% fetal bovine serum. The cells (1×10^6 cells/ml) were incubated with r*Eh*CRT (5 μg/ml) at 37°C with 5% CO_2; concanavalin A (ConA) (Sigma Chemical St. Louis, MO USA) was used as a stimulating factor at 10 μg/ml for different periods of time (24, 48, 72, and 96 h). Each experiment was performed in duplicate. At each time point, the cultures were centrifuged for 10 min at $1,000 \times g$, and the supernatant was reserved for cytokine analysis.

Recombinant *Eh*CRT Production

Full-length r*Eh*CRT protein was expressed and purified as previously described (González et al., 2011). Briefly, the plasmid pBluescript-KS+ (pbKS+) was used to clone and express the 1,178 bp *Eh*crt gene (GB-EAL649855.1) (Loftus et al., 2005), which produces the full-length protein; it was subcloned into the prokaryotic expression vector pProEX HT-b (Gibco Life Technologies) to express the CRT protein with a six-histidine tag at the N-terminal end. Competent *Escherichia coli* BL21 cells were transformed with the recombinant plasmid. The expression of recombinant protein r*Eh*CRT was induced with a final concentration of 1 mM isopropyl-β-D-thiogalactoside. The QIAexpressionist system (Qiagen, Valencia, CA, USA) was used to purify the recombinant protein, and, briefly, the cells were harvested by centrifugation at $3,000 \times g$ for 12 min, and the bacterial pellet was resuspended in 5 ml lysis buffer (8 M urea, 0.1 M NaH_2PO_4, and 0.1 M Tris-HCl, pH 8.0). The lysate was added to a 50% suspension of Ni-NTA agarose (Qiagen,). The mixture was passed through a filtration column, and the recombinant protein was eluted with 8 M-urea buffer pH 4.5. The selected fractions were dialyzed against 19 mM PBS. Protein concentration was determined by the Bradford method, and the quality was evaluated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE). The reactivity grade of the recombinant protein was tested against sera from patients with ALA and for anti-*E. coli* lipopolysaccharide antibody (LPS) antibody (ab211144, Abcam) by Western blot.

Proliferation Index (PI)

To obtain the PI at 24, 48, 72, and 96 h, 20 μl of 5 μg/ml [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT) (Sigma) was added to each well containing PBMCs incubated with r*Eh*CRT or ConA and incubated at 37°C for 1 h in a humidified incubator with 5% CO_2. After incubation, the plates were centrifuged at $1,000 \times g$ for 10 min and the supernatant was discarded. The cells were resuspended in 300 μl dimethyl sulfoxide (Sigma) and the optical density (OD) was measured at 570 nm in an ELISA plate reader (EL × 800 BioTek). The proliferation index was calculated by [OD of test sample—OD of negative control/OD of negative control]. (Verma et al., 2010)

Cytokine Detection by ELISA

The supernatants of the PBMC cultures, treated with r*Eh*CRT, ConA, or without stimulus (RPMI-10% SFB), were tested for detection of interleukins IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IL-13, IL-17A, interferon gamma (IFNγ), tumor necrosis factor alpha (TNFα), granulocyte colony stimulating factor (GCSF), and transforming growth factor beta1 (TGFβ1) using a multianalytic ELISA array kit (MEH-003A, Qiagen), according to manufacturer's instructions. The concentrations of the cytokines are given in pg/ml and were calculated using the standard curve provided in the kit.

Statistical Analysis

All values are expressed as the means ± S.D. The student's *t* test for unpaired results was used for the evaluation of differences

TABLE 1 | Demographic characteristics of individuals in the AP-ALA, R-ALA, and healthy control (NEG) groups.

Groups	Age (range in years)	Male	Female	Total	ELISA (OD)
AP-ALA	39.11	9	1	10	0.87 ± 0.043
R-ALA	39.72	6	4	10	1.0 ± 0.025
NEG	47.62	7	3	10	0.15 ± 0.024

The antibodies against E. histolytica antigens present in all belonging to different studied groups were evaluated by ELISA. The data are expressed as the average OD ± SD in each group.

between cytokine concentrations in each group. Differences were statistically significant when $P \leq 0.05$.

RESULTS

Study Participants and *E. histolytica* Antibody Concentration

The demographic features of each group are shown in **Table 1**. Among all the individuals, the mean age was 39 ± 7 years old, and 70% were male and 30% were female. The microscopic examinations were negative in all samples. Results of ELISA assay are shown as OD_{490nm} values, considering negative results when values were under the cut value of 0.520. The mean value for the negative group was 0.15, the AP-ALA group was 0.87, and the R-ALA group was 1.0.

Purification of r*Eh*CRT and PI

The purified r*Eh*CRT from *E. coli* appeared as a single band at 60 kDa on 12% SDS-PAGE after bromophenol blue staining (**Figure 1A**). The results indicate that r*Eh*CRT was a good immunogenic factor, and this was previously confirmed by the antibody production in animal models (rabbits and mice) (González et al., 2011). The r*Eh*CRT conserved its reactivity when it was tested as an antigen using serum from ALA patients in ELISA assays (**Table 1**) and in Western blots, and no reactivity was observed with anti-*E. coli* LPS antibody (**Figure 1B**).

The role of r*Eh*CRT as a costimulatory factor in the proliferation of PBMC was verified when cellular proliferation was measured by 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assays, comparing the stimulatory capacity of r*Eh*CRT vs. the mitogen activity of ConA. The differences in the PI values were not statistically significant for any time point when comparing between the NEG and ALA groups (AP-ALA, R-ALA) (**Figure 2**). However, at 72 h, we can see the highest PI (**Figure 3**) and the PI ≥ 1 is considered as positive proliferation (Verma et al., 2010).

Detection of Cytokines in the Supernatants of PBMC Cultures

The concentrations of cytokines in PBMC samples obtained for sera from the ALA and NEG groups and treated with EhCRT were measured through ELISA only at 72 h (**Figure 4**). The
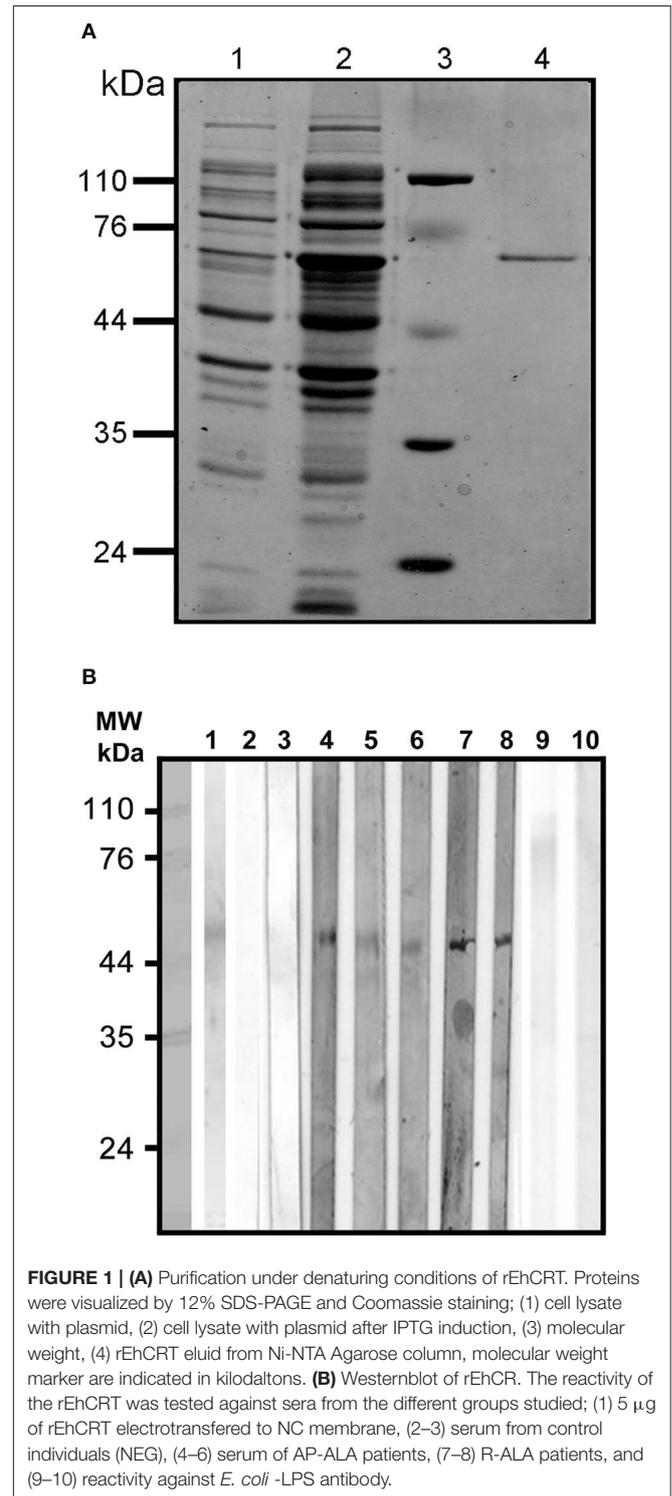
FIGURE 1 | (A) Purification under denaturing conditions of rEhCRT. Proteins were visualized by 12% SDS-PAGE and Coomassie staining; (1) cell lysate with plasmid, (2) cell lysate with plasmid after IPTG induction, (3) molecular weight, (4) rEhCRT eluid from Ni-NTA Agarose column, molecular weight marker are indicated in kilodaltons. (B) Westernblot of rEhCR. The reactivity of the rEhCRT was tested against sera from the different groups studied; (1) 5 μg of rEhCRT electrotransfered to NC membrane, (2–3) serum from control individuals (NEG), (4–6) serum of AP-ALA patients, (7–8) R-ALA patients, and (9–10) reactivity against *E. coli* -LPS antibody.
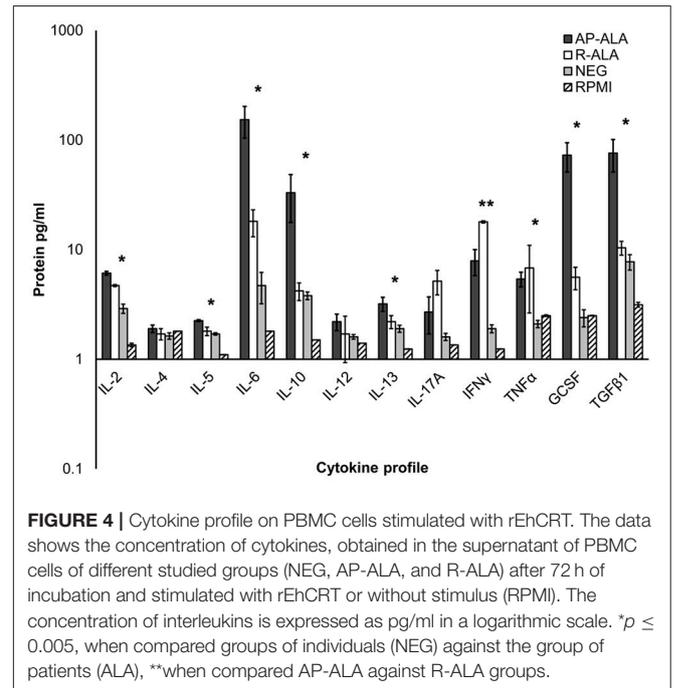
pattern of pro-inflammatory cytokines from the AP-ALA and R-ALA groups were compared to the NEG group, who did not produce the mentioned cytokines in the absenceof EhCRT.

The trend of the data agrees with an overexpression of all interleukins analyzed in all groups.

FIGURE 2 | Proliferation Index at different times, of PBMC cells obtained from control subjects (NEG) and ALA patients stimulated with rEhCRT or Con A.

FIGURE 3 | Proliferation Index at 72 h post incubation of PBMC cells obtained from control subjects (NEG), AP-ALA or R-ALA patients with rEhCRT or Con A. PI ≥1 is considered as positive stimulus.

FIGURE 4 | Cytokine profile on PBMC cells stimulated with rEhCRT. The data shows the concentration of cytokines, obtained in the supernatant of PBMC cells of different studied groups (NEG, AP-ALA, and R-ALA) after 72 h of incubation and stimulated with rEhCRT or without stimulus (RPMI). The concentration of interleukins is expressed as pg/ml in a logarithmic scale. *$p \leq$ 0.005, when compared groups of individuals (NEG) against the group of patients (ALA), **when compared AP-ALA against R-ALA groups.

DISCUSSION

The aim of this study was to examine the proliferation of PBMCs obtained from the blood of patients with AP-ALA and R-ALA stimulated *in vitro* with r*Eh*CRT or ConA and determine the cytokine profiles induced in the different groups. The responses of the PBMC show that *Eh*CRT is one of the many immunogenic proteins in *E. histolytica* that can induce activation and proliferation of PBMCs similarly to ConA. These results reinforce our previous observations that the *Eh*CRT is highly immunogenic in humans, mice, and rabbits (González et al., 2002, 2011).

When comparing the cytokine profiles between the AP-ALA and R-ALA groups in contrast with negative or PBMC without stimulus, it is clear that *Eh*CRT functions as a specific antigenic costimulator, inducing a different pattern of cytokines between different groups. This stimulatory action was specific because no reactivity with *E. coli* -LPS antibody was detected for the recombinant protein EhCRT (**Figure 1A**).

The expression levels of IL-2, IL-5, IL-6, IL-10, GCSF, and TGFβ1 were increased in patients with AP-ALA, while the expression levels of IL-17, INFγ, and TNFα were mainly upregulated in the R-ALA group. Nevertheless, there were statistically significant differences only for INFγ.

E. histolytica has different proteins that modulate the host immune response. The Gal/GalNAc-lectin induces the T cells to production of IL-2 and INFγ (Schain et al., 1992), whereas in macrophages, the amebicide activity of Gal/GalNAc-lectin induces the production of TNFα (Seguin et al., 1997). In dendritic cells, Gal/GalNAc-lectin favors a Th1 response in addition to inducing the production of major histocompatibility complex

However, in the ALA groups, the overexpression was, in general, larger than in the negative group, and significant differences were measured in the expression of the following interleukins; IL-2, IL-5, IL-6, IL-10, IL-17, IFNγ, TNFα, GCSF, and TGFβ1 (p = 0.0035, 0.0023, 0.019, 0.006, 0.0076, 0.0049, 0.0039, 0.0035, 0.0059, respectively).

The comparisons of differential expressions of the interleukins between groups of AP-ALA and R-ALA are shown in **Figure 4**. We found differences in the overexpression of interleukins IL-2, IL-5, IL-6, IL-10, GCSF, and TGFβ1 (p = 0.0067, 0.026, 0.0045, 0.0016, 0.0051, 0.0047, respectively), which were higher in the AP-ALA group. In the R-ALA group, we observed higher overexpression of the interleukins IL-17, INFγ, and TNFα; however, statistically significant differences were detected only for INFγ (p = 0.73, 0.0014, 0.096).

(MHC) class II molecules, and the costimulatory molecules CD80, CD86, and CD40 (Ivory and Chadde, 2007).

Another protein called monocyte locomotion inhibitory factor (MLIF) is produced by *E. histolytica* in axenic cultures that induces the production of pro-inflammatory cytokines (IL-1β, IL-2, IL-5, IL-6, IFN-γ) and anti-inflammatory cytokines such as IL-10, as well as the low expression of chemokines CCL1, CCL4, and the receptor CCR1 in human monocytes (Rico et al., 2003; Utrera-Barillas et al., 2003).

In the group of patients with AP-ALA, the cytokines IL2, IL5, Il6, IL17A, IFNγ, and TNFα displayed an increase in their concentration and demonstrated that the immune response had a pro-inflammatory profile. This response has been observed in stimulation assays using Gal/GalNAc-lectin in intestinal cell cultures (Sharma et al., 2005) and in other parasitic diseases such as malaria (Vazquez et al., 2015). It is important to highlight the effect on IL2, IL6, IL-17, IFNγ, and TNFα, whose levels were the highest and overexpressed in comparison with the profile observed in the negative group.

Guo et al. (2011), demonstrated in an *Entamoeba histolytica* vaccination model that IL-17 provides protection to mice vaccinated with the recombinant LecA fragment of the Gal/GalNAc-lectin. Interestingly, the major source of IL-17 in these mice was the CD8 T cells, whereas CD4 T cells express elevated levels of IFN-γ. The authors suggest that IL-17 may enhance the protective functions of Th1 immune response.

These results lead us to propose that treatment of PBMC in culture with *Eh*CRT favors the production of IFNγ and increases the production of IL-17A, thus directing the cellular immune response to a Th1 Profile, in PMBCs obtained from R-ALA.

Our results agree with those published by Ghadirian and Denis (1992), who showed that IFNγ could activate mouse peritoneal macrophages, which, in turn, were able to eradicate the *E. histolytica* trophozoites from colon tissue *in vitro*. Studies in animal models (Seydel et al., 2000) and human infections (Haque et al., 2007) have established that amoeba-specific IFN-γ production is critically involved in the clearance of infection and in host protection. In addition, Meza et al. (2014) demonstrated that virgin T-cell differentiation into Th17 cells producing IL-17 occurred after the direct stimulation with other cytokines such as TGFβ, IL-6, and IL-1 in a murine model of infection with *Trypanosoma cruzi*. Moraes et al. (2015) reported that mononuclear cells collected from healthy individuals incubated with *E. histolytica* in culture induced the production of *IFNγ* and TGFβ, and that both had a beneficial effect on the modulation of the activity of these cells. Our results agree with data of Moraes regarding the effects on IFNγ. These cytokines are important in the control *E. histolytica* infection.

The R-ALA group showed an increase in the concentration of the cytokines IL-10 and TGFβ, which agrees with results of Bansal et al. (2005). These authors mention that, in addition to the production of these cytokines and the increased production of IL-4, a suppressive immune response was also induced in patients infected with *E. histolytica*, which, in turn, favored a symptomatic infection. The symptomatic group in Bansal et al. differs with our R-ALA group because the latter had no symptoms and they were all ALA with negative microscopic examinations and a resolved

E. histolytica infection. In our opinion, this immunosuppressive effect is due to the direct stimulation of the PBMC by *Eh*CRT, and through autocrine, paracrine, and pleiotropic effects on cytokine production, which favored the increase in other types of cytokines such as IL-5, IL-6, and IL-13 capable of generating a Th2 immune response.

In addition, we found that the increased cytokine IL-10 in the AP-ALA group in our study was in contrast with the results reported by Bansal. These results suggest that IL-10 is an immunomodulator resulting in proinflammatory cytokine profiles that could promote immunosuppression in the R-ALA individuals. The effect attributed to IL-10 differs in other parasitic diseases such as *Leishmania donovani* (Andreani et al., 2015), *Trypanosoma cruzi* (Longhi et al., 2014), and *Giardia duodenalis* (Babaei et al., 2016). In these reports, a decrease in IL-10 was observed that favored the spread of parasites into the hepatic tissue.

On the contrary, the IL-10 level was increased in dysenteric and ALA patients (Utrera-Barillas et al., 2003; Bansal et al., 2005). These studies indicate that invasion of the colon and liver by *E. histolytica* elicits an anti-inflammatory immune response and may successfully suppress immune reaction to the amebae.

In summary, the ameba needs to balance IL-10 and the pro-inflammatory cytokine to allow establishment of infection. In contrast, peritoneal monocytes and macrophages exposed to lipopeptidophosphoglycan (LPPG) secreted TNF-α, IL-6, IL-8, IL-12, and IL-10 via TLR2 (Maldonaldo-Bernal et al., 2005). Thus, LPPG-driven signaling may activate a negative feedback loop that attenuates inflammatory responses.

Host protective immunity involves participation of both humoral and cellular responses; however, the mechanism involved in the immune evasion of *E. histolytica* is not clear. One of these mechanisms could be associated with the ability of parasites to modulate cytokine expression in the inflammatory process, which is initiated by expression of proinflammatory cytokines. *E. histolytica* infections induce a state of transient suppression of cell-mediated immunity in early stages of inflammation in amebic hepatic abscess, and a complex signaling system of cytokines is triggered by pathogen invasion (Eckmann et al., 1993; Romagnani, 2000).

CONCLUSIONS

The data obtained in this study confirmed that the *Eh*CRT behaves like an amebic immunogenic protein for humans and suggest that the *Eh*CRT participates in the specific stimulation of immune cells.

Our results suggest that the r*Eh*CRT can stimulate human PBMC proliferation independently of the presence of *E. histolytica* trophozoites, acting as a specific costimulator of the immune response like that induced by ConA. In addition, these results underline *Eh*CRT as a parasitic factor that can modulate the immune response, from the stimulation of proinflammatory cytokines to the immunosuppressive effects, depending on the progression of the ALA, thus inducing the development of a Th2 cytokine profile in the acute phase of

disease and a Th1 profile once the individuals had resolved the ALA.

The functions of *Eh*CRT and its role in the pathogenesis of ALA need further research, particularly on the interaction with the cells of immune system and the induction of chemokines and cytokines regulators that hopefully will allow a better understanding of the pathogenesis of ALA.

AUTHOR CONTRIBUTIONS

CX conceptualized the manuscript. EG, MN-R, UM. PM, OP-R, EH, LR, and AS curated the data. CX, EG, MN-R, PM, OP-R, EH, LR, and AS performed the formal analysis. CX acquired the funding. EG, MN-R, and PM performed the investigation. MN-R, PM, AS, and UM provided the resources. EG, OP-R, and EH performed the software analysis. CX, EG, MN-R, and PM provided the supervision. CX, EG, MN-R, and PM executed the validation. CX, EG, MN-R, and PM wrote the original draft of the manuscript. CX, EG, MN-R, PM, OP-R, EH, UM, and AS wrote, reviewed and edited the manuscript.

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Asymptomatic Intestinal Colonization with Protist *Blastocystis* Is Strongly Associated with Distinct Microbiome Ecological Patterns

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ABSTRACT *Blastocystis* is the most prevalent protist of the human intestine, colonizing approximately 20% of the North American population and up to 100% in some nonindustrialized settings. *Blastocystis* is associated with gastrointestinal and systemic disease but can also be an asymptomatic colonizer in large populations. While recent findings in humans have shown bacterial microbiota changes associated with this protist, it is unknown whether these occur due to the presence of *Blastocystis* or as a result of inflammation. To explore this, we evaluated the fecal bacterial and eukaryotic microbiota in 156 asymptomatic adult subjects from a rural population in Xoxocotla, Mexico. Colonization with *Blastocystis* was strongly associated with an increase in bacterial alpha diversity and broad changes in beta diversity and with more discrete changes to the microbial eukaryome. More than 230 operational taxonomic units (OTUs), including those of dominant species *Prevotella copri* and *Ruminococcus bromii*, were differentially abundant in *Blastocystis*-colonized individuals. Large functional changes accompanied these observations, with differential abundances of 202 (out of 266) predicted metabolic pathways (PICRUSt), as well as lower fecal concentrations of acetate, butyrate, and propionate in colonized individuals. Fecal calprotectin was markedly decreased in association with *Blastocystis* colonization, suggesting that this ecological shift induces subclinical immune consequences to the asymptomatic host. This work is the first to show a direct association between the presence of *Blastocystis* and shifts in the gut bacterial and eukaryotic microbiome in the absence of gastrointestinal disease or inflammation. These results prompt further investigation of the role *Blastocystis* and other eukaryotes play within the human microbiome.

IMPORTANCE Given the results of our study and other reports of the effects of the most common human gut protist on the diversity and composition of the bacterial microbiome, *Blastocystis* and, possibly, other gut protists should be studied as ecosystem engineers that drive community diversity and composition.

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Blastocystis colonization influences gut #microbiome ecology

Blastocystis is the most prevalent protozoan resident of the human intestine, colonizing approximately 0.5 to 30% of humans in industrialized countries and 30 to 100% in nonindustrialized societies (1–7). *Blastocystis* is transmitted via the oral-fecal route, and colonization has been linked to poor hygiene, animal contact, and contamination of food and water sources (4, 8). And yet, *Blastocystis* is also common in industrialized populations, with 20 to 50% prevalence among healthy cohorts in Europe (9, 10), and interestingly, is associated with high income in Denmark (11). Similarly, its role in human health and disease is controversial. *Blastocystis* has been associated with a variety of gastrointestinal symptoms (12–14) and inflammation, both within the gut (15) and systemically (16, 17). Several studies have suggested that *Blastocystis* may play a role in the development of irritable bowel syndrome (IBS) (11, 18, 19). However, *Blastocystis* has often been described as an asymptomatic member of the normal intestinal microbiota (7, 9, 10, 20) and has even been inversely associated with body mass index and Crohn's disease (7). It remains unclear how *Blastocystis* is capable of acting as an opportunistic pathogen or a commensal or a beneficial microbe and to what extent this depends on the specific circumstances of its host.

The plethora of human-associated microbiome studies documenting the role of the microbiome in health and disease has directed considerable attention to the factors that influence microbiome diversity and composition. To date, diet (21, 22), antibiotics (23), age (24), inflammation (25), and to a lesser extent, host genetics (26–28) have been shown to impact the ecology of the gut bacterial community, yet most studies are limited to profiling bacteria and seldom consider interdomain, multitrophic interactions as one of these factors. The relationship between human-associated gut protists and the resident gut bacterial community has only recently begun to be explored. Pathogenic protists, such as *Giardia* and *Entamoeba histolytica*, are associated with an increase in gut bacterial diversity or with compositional differences (29–32), likely due to the mucosal damage and inflammation they cause in the intestinal mucosa. Interestingly, nonpathogenic protists may also cause major shifts in the bacterial microbiota: Morton et al. document an association between the presence of *Entamoeba* and increased diversity and compositional shifts in the bacterial microbiota in asymptomatic individuals, a portion of whom likely harbor nonpathogenic *Entamoeba dispar* (33). Recent reports in humans also suggest that *Blastocystis* is associated with compositional and diversity differences of the bacterial gut microbiota (2, 7, 34). However, given that this protist is known to cause disease in certain individuals and that the reported results are confounded by disease status, it remains unknown whether the changes in bacterial microbiomes are due to the presence of *Blastocystis* or to ongoing inflammation. Differentiating whether *Blastocystis* causes direct ecological effects on the bacterial microbiome or if microbiota shifts are mainly mediated through immune and physiologic changes associated with *Blastocystis*-associated inflammation will help determine the ecological consequences of decreased *Blastocystis* incidence in the westernized gut human microbiome.

In light of this, we aimed to study the gut microbiome in relation to *Blastocystis* colonization in a large sample of healthy individuals residing in a semi-industrialized setting in rural Mexico. Through this investigation, we detected marked taxonomic and functional bacterial differences associated with asymptomatic colonization of *Blastocystis* that support its role as a common eukaryotic gut commensal that drastically influences the bacterial microbiota through currently unknown mechanisms.

RESULTS

Prevalence of *Blastocystis*. We studied 156 individuals from the town of Xoxocotla, in the state of Morelos, Mexico. This population was deemed free of gastrointestinal symptoms associated with disease, according to physical examination by a clinician and

data obtained from the Rome III questionnaire (see Materials and Methods for criteria in this questionnaire). Besides the Rome III questionnaire, a complete medical history was carried out for each individual, emphasizing inflammatory symptoms and antibiotic use in the 6 months preceding the clinical assessment and sampling. None of the selected samples came from individuals that reported antibiotic use or previous inflammatory conditions. The gender distribution was 84 women (53.8%) and 72 men (46.2%), with a median age of 27 years (10 to 53 years). The majority of individuals in the study had some degree of education (84.6%). The most frequent level was secondary school (33.0%), followed by primary school (32%), preschool (26%), technical education (8.4%), and professional studies (0.49%) (see Table S1 in the supplemental material for baseline characteristics of study participants). The status of colonization by *Blastocystis* and other protozoa (*Cryptosporidium parvum*, *Entamoeba histolytica/E. dispar*, and *Giardia intestinalis*) was determined by microscopy and quantitative PCR (qPCR) assays targeting regions of the small subunit (SSU) rRNA gene to detect and quantify levels directly from human stool specimens. *Blastocystis* colonization was detected in 65% of individuals (102/156), 52% of whom (53/102) were women. There was no difference in the mean ages of *Blastocystis*-positive versus -negative individuals (27.8 ± 8.4 years [mean ± standard deviation] versus 28.1 ± 7.9 years, respectively; Student's *t* test, $P = 0.83$). Targeted 18S rRNA gene sequencing results allowed further resolution of *Blastocystis* taxonomy, showing that *Blastocystis* subtype 3 (ST3) is the prevalent subtype colonizing this human population. We did not identify any individuals infected by *Crysptosporidium parvum* or *Giardia intestinalis* but did observe coinfection with *Entamoeba histolytica/E. dispar* in five individuals, who were excluded from the bioinformatics analysis in order to assess the effect of *Blastocystis* only.

Intestinal microbiota composition and *Blastocystis* infection. (i) Prokaryotes. We determined the bacterial community by amplification and sequencing of the 16S rRNA gene (V3 region). *Blastocystis* colonization was associated with profound changes in bacterial alpha and beta diversity (Fig. 1) (for analysis of alpha diversity, Chao1 and Shannon indices and Mann-Whitney test were used [$P < 0.001$], and for analysis of beta diversity, principal-component analysis [PCoA] and permutational multivariate analysis of variance [PERMANOVA] were used [$P < 0.001$]). The marked change in beta diversity can be explained by large differences in the abundances of predominant bacterial taxa, including *Prevotella copri*, *Prevotella stercorea*, *Ruminoccoccus bromii*, *Alistipes putredinis*, *Bacteroides* species, *Bifidobacterium longum*, and *Oscillospira* species (DESeq2, Wald test, and false discovery rate [FDR], $P < 0.05$) (Table 1; the full list of differential operational taxonomic units [OTUs] is in Table S2). The presence of *Blastocystis* was associated with significant differences in 7 of the 10 most abundant OTUs, including *Prevotella copri* and *Ruminococcus bromii*, the two most abundant taxa in the data set (Fig. 1D; Table 1). Similarly, at the genus level, 7 of the 10 most abundant genera, comprising ~65% of all bacterial sequences, were significantly different in relation to their abundances in the bacterial community associated with *Blastocystis* colonization (Fig. S1).

Given the metadata collected in this cohort (age, education level, and family relationships), we captured the effects of the *Blastocystis* colonization on the gut bacterial microbiome while controlling for potential confounding variables using MaAsLin (35). MaAsLin is a multivariate linear modeling tool with boosting that tests for associations between specific microbial taxa and continuous and/or Boolean metadata. This method reduces the total amount of correlations to be tested, therefore allowing for improvements in the speed and the robustness of the additive general linear models. With MaAsLin, we found a significant association between most of the differential taxa (previously obtained through the DESeq2 analysis) and *Blastocystis* status, whereas no other variable explained the taxonomic differences observed in colonized individuals (Table S4).

We also controlled for the effect of sequencing depth influencing our results, as previously described by Weiss et al. (36), and found that while sequencing depth did impact the ordination of our data, the influence of *Blastocystis* species described in our
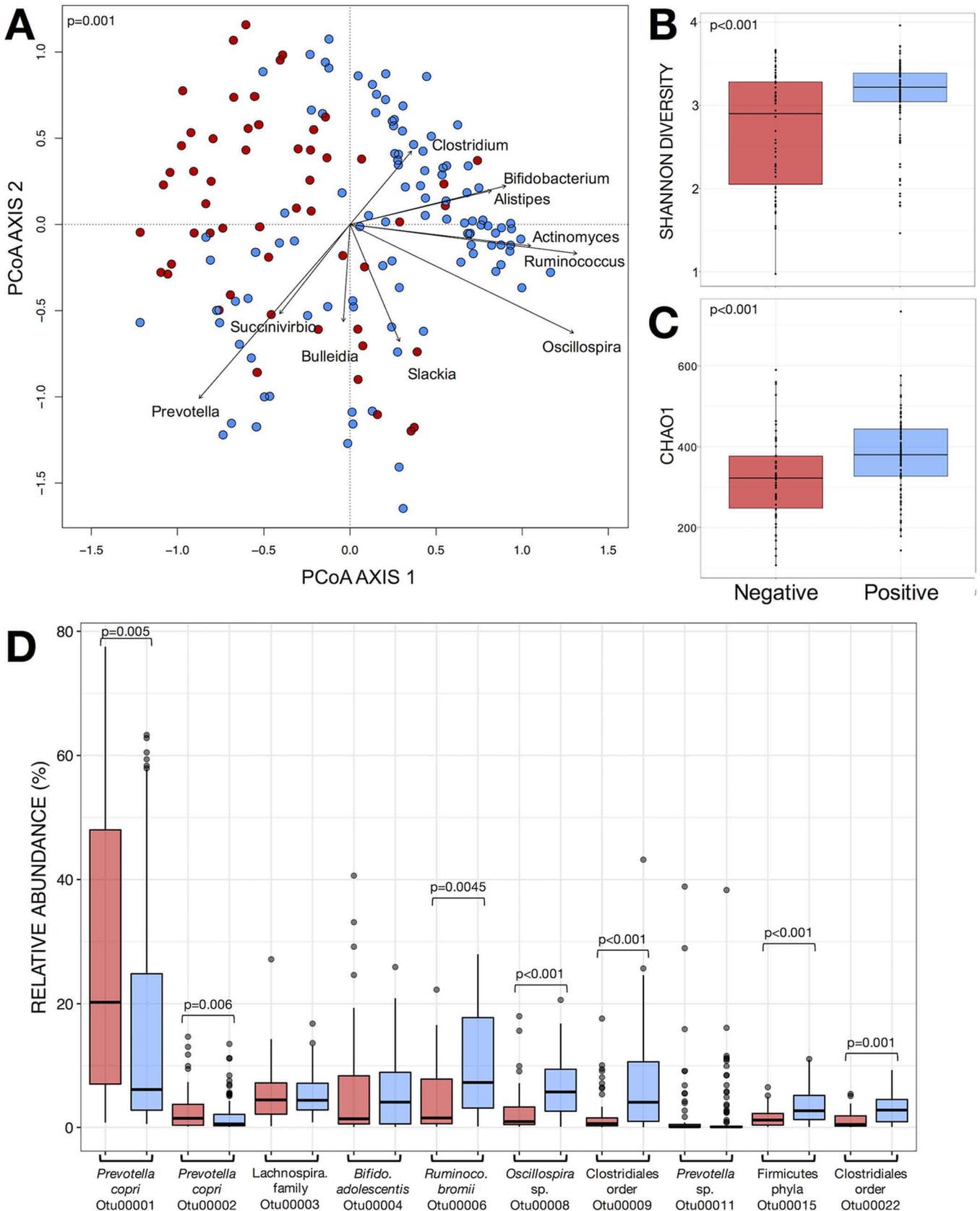
FIG 1 Variations in beta and alpha diversity of gut microbiome bacterial communities in relation to presence of *Blastocystis*. (A) Principal-component analysis (PCoA) ordination of variation in beta diversity of human gut bacterial communities based on Bray-Curtis dissimilarities among fecal samples. Colors represent the presence of *Blastocystis* in gut microbial communities (red for negative and blue for positive), and arrows represent the significant ($P < 0.001$) correlations

(Continued on next page)

TABLE 1 Differential abundances of taxa in relation to *Blastocystis* colonization calculated by DESeq2[a]

OTU_taxon	DESeq2 value for:					
	baseMean	log$_2$ fold change	lfcSE	stat	*P* value	padj
Bacteria						
Otu00001_*Prevotella_copri*	58,635.572	−1.063	0.275	−3.863	1.12E−04	5.39E−03
Otu00002_*Prevotella_copri*	6,577.601	−1.179	0.309	−3.818	1.34E−04	6.41E−03
Otu00006_*Ruminococcus_bromii*	13,423.877	1.768	0.358	8.463	3.42E−04	4.50E−03
Otu00008_*Oscillospira*_sp.	2,952.294	0.983	0.138	7.133	9.79E−13	2.41E−10
Otu00009_*Bacteria*	3,050.404	1.348	0.217	6.211	5.26E−10	7.28E−08
Otu00013_*Prevotella_stercorea*	3,848.363	−1.614	0.358	−4.508	6.53E−06	4.30E−04
Otu00014_*Clostridiales*	1,022.155	0.707	0.142	4.987	6.12E−07	4.91E−05
Otu00015_*Firmicutes*	2,174.964	2.464	0.365	−6.741	1.57E−11	3.00E−09
Otu00018_*Alistipes_putredinis*	927.977	0.970	0.173	5.617	1.95E−08	2.18E−06
Otu00019_*Bifidobacterium_longum*	3,202.069	−2.947	0.242	−12.156	5.35E−34	5.92E−30
Otu00022_*Clostridiales*	1,449.441	0.890	0.153	5.802	6.55E−09	7.71E−07
Otu00025_*Oscillospira*_NA	1,524.800	−2.683	0.254	−10.575	3.91E−26	8.74E−23
Otu00030_*Bacteria*	2,201.012	−1.727	0.268	−6.446	1.15E−10	1.79E−08
Otu00032_*Barnesiellaceae*	499.952	−1.970	0.247	−7.964	1.67E−15	5.77E−13
Otu00041_*Rikenellaceae*	382.230	1.081	0.228	4.745	2.09E−06	1.51E−04
Otu00043_*Clostridiales*	482.388	1.111	0.209	5.313	1.08E−07	1.04E−05
Eukaryota						
OTU_8514_*Blastocystis*_sp._subtype_3	40.584	5.362	0.503	10.656	1.64E−26	5.80E−24
OTU_8487_*Blastocystis*_sp._subtype_3	36.126	5.202	0.498	10.444	1.56E−25	2.75E−23
OTU_5808_*Hymenolepis_nana*	42.245	−3.377	0.557	−6.066	1.31E−09	3.56E−08
OTU_5817_*Hymenolepis_nana*	37.953	−3.581	0.545	−6.574	4.91E−11	1.73E−09

[a]Results from the 30 most abundant taxa in the data sets are included. For a complete list, see Table S2. baseMean, mean of normalized counts; lfcSE, standard error of log fold change; stat, Wald statistic; padj, adjusted *P* value.

study is not driven by the variation in sequencing depth across samples (Fig. S3A) (PERMANOVA, *P* = 0.5).

(ii) Eukaryotes. We determined the eukaryotic microbiota by amplification and sequencing of the 18S rRNA gene (V4 region). In the 18S rRNA gene data set, 63 samples had fewer than 1,000 sequences per sample after applying filtering steps (see Materials and Methods), which prompted their removal from the data set. Thus, 93 samples (N_{pos} = 60 and N_{neg} = 33, from colonized and noncolonized individuals, respectively) were retained for ecological analysis of this data set. There were no significant differences with respect to baseline characteristics between the reduced number of samples and the original data set used for 16S rRNA gene analysis (Table S5).

Blastocystis colonization was associated with more discrete differences in the eukaryotic microbiota (Fig. 2). As expected, our analysis detected *Blastocystis* subtype 3 as the protist most significantly different in abundance between *Blastocystis*-positive and -negative individuals (Table 1; see full list in Table S2). *Blastocystis* colonization was also associated with increases in yeast and fungal species (*Debaryomyces hansenii, Mucor mucedo, Aspergillus flavus, Mucor racemosus,* and *Issatchenkia terricola*) and a decrease of *Hymenolepis nana* (Table 1; Table S2). Since *H. nana* is a known cestode colonizer in the human gut, often with pathological consequences, we evaluated whether the presence of *H. nana* influenced bacterial community structure and confounded our results. However, we found that *H. nana* did not drive a significant effect in bacterial community structure (Fig. S2A). The detection of a significant difference in *H. nana* abundances between *Blastocystis*-positive and -negative individuals is likely due to a high variation in abundance in both groups and relatively few samples where they are represented. Likewise, we investigated the impact of the presence of *Debaryomyces hansenii* in the microbiota because this yeast species has been associated with human

FIG 1 Legend (Continued)

between PCoA axes versus the relative abundances of bacterial genera in communities. (B and C) Shannon diversity (B) and Chao1 estimated richness (C) display differences in alpha diversity. Significant differences are shown by *P* values of Mann-Whitney tests for comparison between 2 groups. (D) Relative abundances of the 10 most abundant bacterial OTUs relative to the presence of *Blastocystis*. Significant differences are shown, calculated by DESeq2 (Wald test plus FDR).
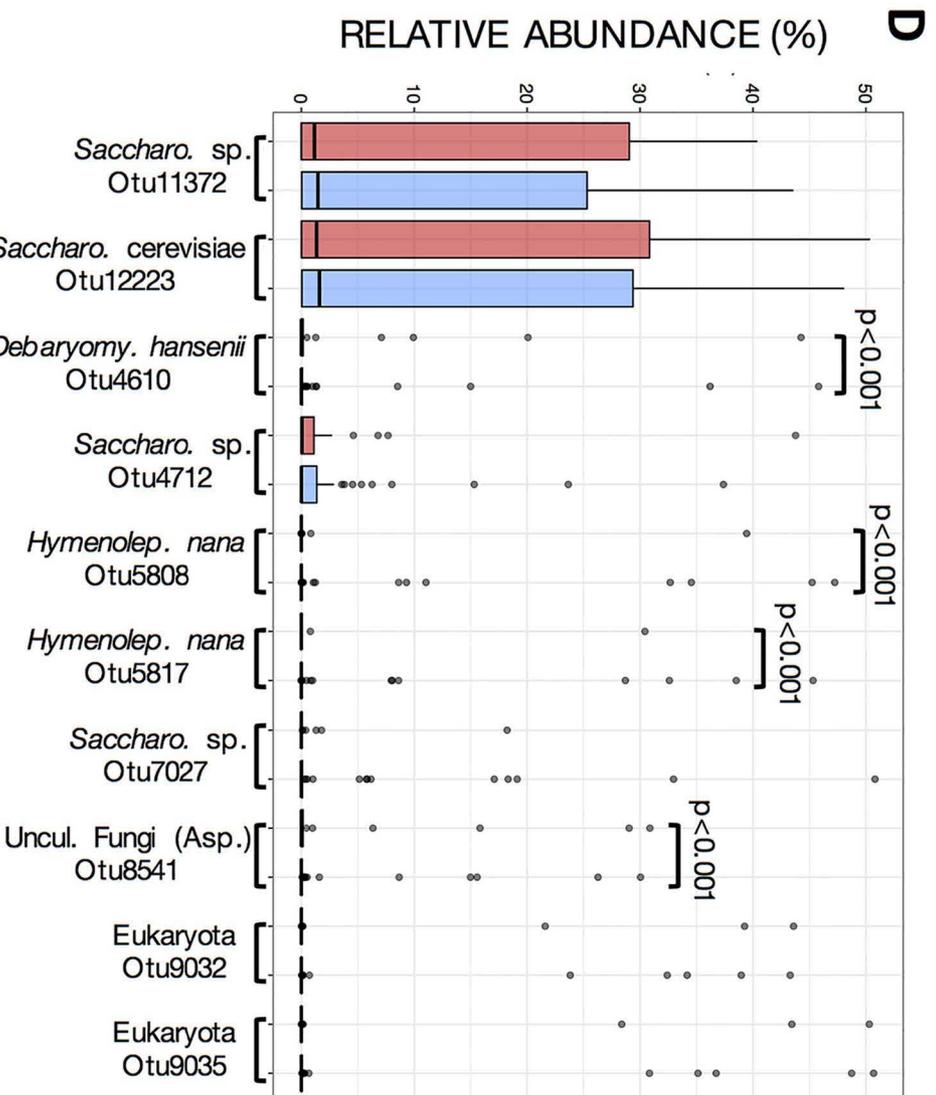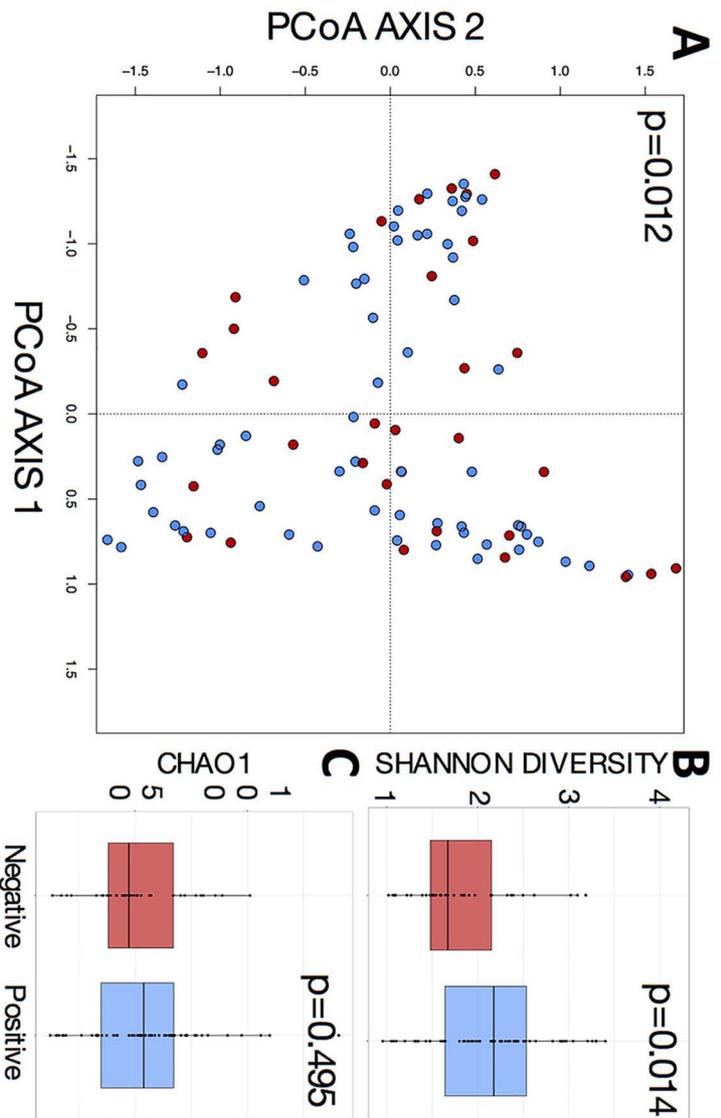
FIG 2 Variations in beta and alpha diversity of gut microbiome eukaryotic communities explained by presence of *Blastocystis*. (A) Principal-component analysis (PCoA) ordination of variation in beta diversity of human gut eukaryotic communities based on Bray-Curtis dissimilarities among samples. Colors represent the presence of *Blastocystis* in gut microbial communities (red for negative

(Continued on next page)
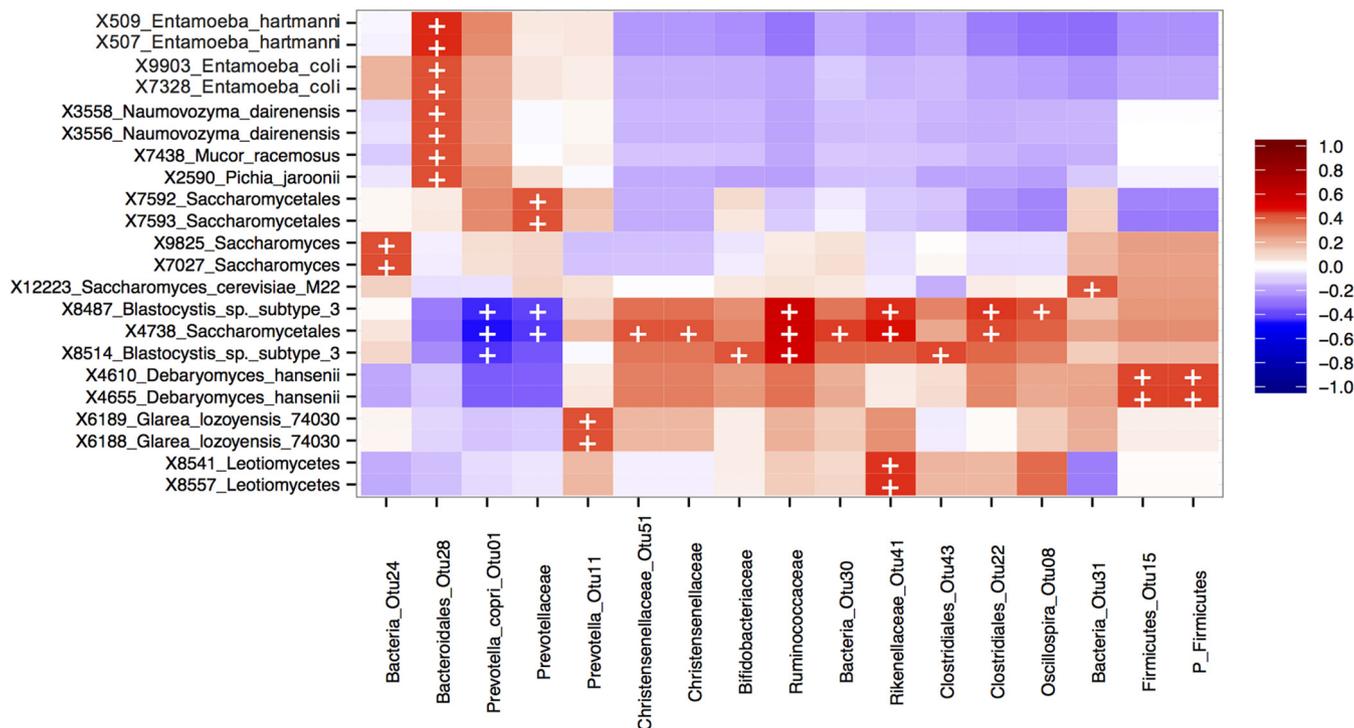
FIG 3 Heatmap of biweight correlations (bicor method) between top 100 bacterial (*x* axis) and top 100 taxon (*y* axis) OTUs in fecal samples from study participants. Colors denote positive (red) and negative (blue) correlation values. Significant correlations are denoted with a plus sign ($P < 0.05$; FDR).

infections (37). Interestingly, the presence of this yeast explained 3.3% of bacterial community variation (PERMANOVA, $P > 0.001$). However, the distribution of *D. hansenni* did not overlap the distribution of *Blastocystis* (Fig. S2B), and their effects on the bacterial community are independent from each other (PERMANOVA, $P = 0.68$).

While controlling for sequencing depth, we observed that it interacted significantly with the effect of *Blastocystis* species colonization on community structure (PERMANOVA, $P = 0.02$) (Fig. S3B). This result is biologically intuitive since the presence of *Blastocystis* species in itself is contributing to the number of sequences detected. However, because of this result, we carried out the DESeq2 analysis using variance-stabilizing-transformed data (38). This analysis yielded the same numbers and identities of differential OTUs between *Blastocystis*-colonized and -noncolonized individuals as we obtained with nontransformed data.

Altogether, the eukaryomes' compositional differences in relation to *Blastocystis* colonization resulted in only a marginal difference in eukaryome beta diversity (PCoA, PERMANOVA, $P = 0.01$) (Fig. 2A). *Blastocystis* colonization was associated with a smaller, yet significant increase in eukaryome alpha diversity (Shannon index, Mann-Whitney, $P = 0.014$) (Fig. 2B) compared to the changes in bacterial diversity. There were no changes in community richness (Chao1, Mann-Whitney, $P = 0.49$).

(iii) Interdomain associations. Correlative analysis of the abundances of the top 100 most abundant taxa of the 16S versus 18S rRNA gene data sets showed, as expected, that *Blastocystis* subtype 3 was positively correlated with members of the *Ruminoccaceae* family and inversely correlated with *Prevotella copri* (Fig. 3).

FIG 2 Legend (Continued)
and blue for positive). (B and C) Shannon diversity (B) and Chao1 estimated richness (C) display differences in alpha diversity. Significant differences are shown by *P* values of Mann-Whitney tests for comparison between 2 groups. (D) Relative abundances of the 10 most abundant eukaryotic OTUs relative to the presence of *Blastocystis*. Significant differences, calculated by DESeq2 (Wald test plus FDR), are shown.
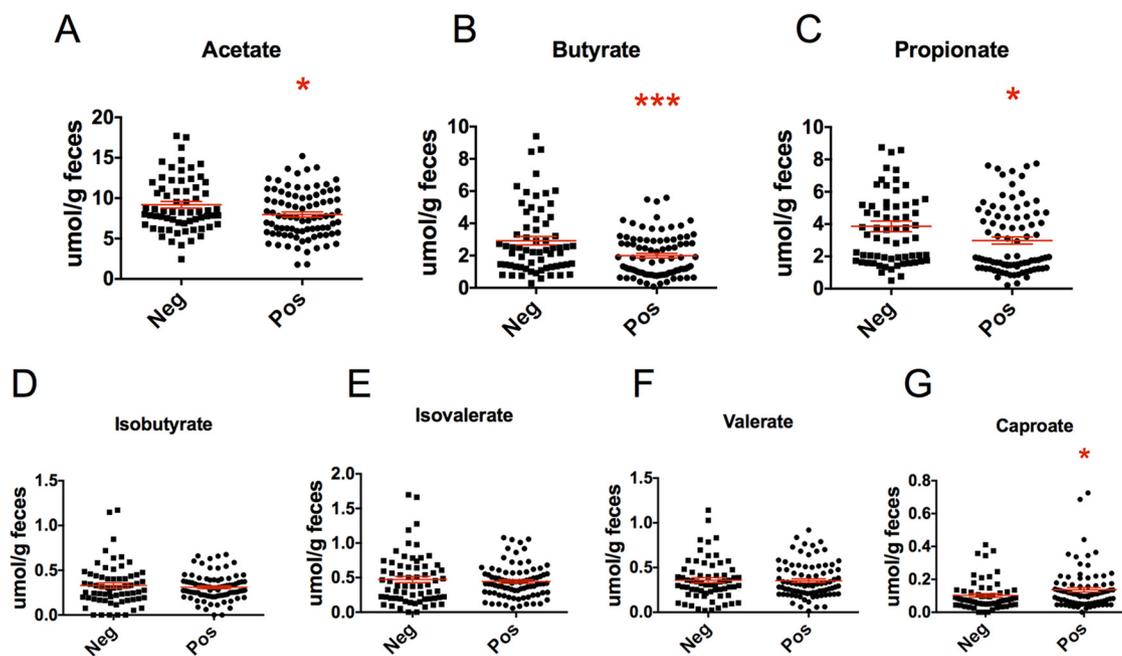
FIG 4 Short-chain fatty acid production in relation to *Blastocystis* colonization. Concentrations of fecal acetate, propionate, butyrate, isobutyrate, isovalerate, valerate, and caproate were measured by gas chromatography ($N_{pos} = 102$, $N_{neg} = 54$; *, $P < 0.05$; ***, $P < 0.001$ [Mann-Whitney]).

Bacterial-community-derived functional changes associated with *Blastocystis* colonization. To determine the functional differences associated with *Blastocystis* colonization, we inferred metagenomics potential using PICRUSt (39). Metagenomes predicted from the 16S rRNA gene data revealed 202 differential biochemical pathways out of 266 biochemical pathways associated with *Blastocystis* colonization (Welch's *t* test) (Table S3), strongly suggesting that the microbiomes in the two groups were functionally distinct. The functional differences involved diverse metabolic functions, including metabolism of secondary bile acids, lipids, tryptophan and other amino acids, and carbohydrates. To confirm the predicted changes in carbohydrate metabolism, we next directly measured the concentration of fecal short-chain fatty acids (SCFA) in all samples. As predicted by our PICRUSt analysis, colonization with *Blastocystis* was associated with vastly different bacterial colonic fermentation patterns, as measured by production of SCFA. The three main SCFA, acetate, butyrate, and propionate, were reduced in colonized individuals, whereas caproate was increased (Fig. 4).

Immunological differences in *Blastocystis*-colonized individuals. (i) Fecal cal-protectin. To determine whether *Blastocystis* colonization was not associated with intestinal inflammation (as inferred by the Rome III criteria questionnaires), we measured fecal calprotectin in a subset of samples ($N_{pos} = 26$ and $N_{neg} = 17$). Calprotectin is a neutrophil cytosolic protein commonly used as a marker of gut inflammation (40). Strikingly, lower concentrations of fecal calprotectin were observed in *Blastocystis*-colonized individuals than in noncolonized individuals (Mann-Whitney, $P = 0.0003$) (Fig. 5A). All calprotectin values were well under levels consistent with pathological gut inflammation (>200 μg/mg), in accordance with the lack of clinical symptoms noted in our study cohort.

(ii) Fecal IgA. Immunoglobulin A (IgA) is the most abundant antibody in mucosal surfaces, and it has a critical role in maintaining homeostasis with the microbiome (41). It acts by binding to and neutralizing invading pathogens, and it can specially target microbes close to the mucus layer (42). IgA secretion into the lumen is elicited by parasitic infections with helminths, and it is important in limiting parasite fecundity and providing immune protection against reinfection (43).
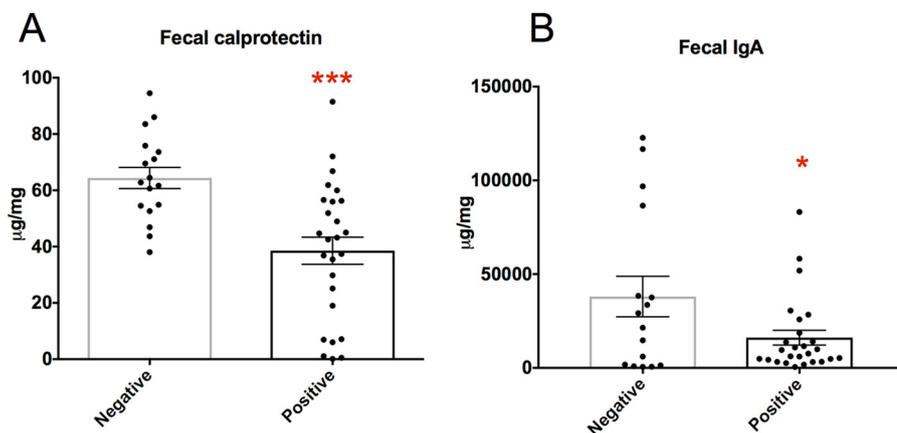
FIG 5 Gut mucosal immune changes associated with *Blastocystis* colonization. Concentrations of calprotectin (A) and total IgA (B) were determined by ELISA in feces in a subset of samples from this study ($N_{neg} = 17$, $N_{pos} = 26$; *, $P < 0.05$; ***, $P < 0.001$ [Mann-Whitney]).

We measured the total IgA concentration in feces to determine if asymptomatic *Blastocystis* colonization was associated with changes in this important humoral immune factor. In a subset of individuals ($N_{pos} = 26$ and $N_{neg} = 17$), we observed that, similar to the calprotectin finding, colonized individuals exhibited lower levels of fecal IgA (Mann-Whitney, $P = 0.03$) (Fig. 5B).

(iii) Oxidative stress markers in urine. It has been described that oxidative stress and the presence of free-radical activities in hosts colonized by parasites correlate with the production of specific metabolites, such as advanced oxidative protein product (AOPP) and malondialdehyde (MDA) (44–46). Previous studies have evaluated MDA levels among healthy individuals and people infected by *Ascaris lumbricoides* (45), *Entamoeba histolytica*, and *Plasmodium vivax* (44), and all revealed increased levels of MDA in the infected individuals, suggesting that oxidative stress acts as a mediator of tissue damage concurrent with different parasites. We measured these biomarkers in urine samples of most study participants ($N_{pos} = 102$ and $N_{neg} = 53$), as described previously (13, 47), and found no significant differences between colonized and non-colonized individuals (Table S6), suggesting that *Blastocystis* colonization is not accompanied by oxidative stress in this population.

(iv) Serum cytokines. *Blastocystis* ST7 has been shown to induce the expression of proinflammatory cytokines in mouse intestinal explants (15) and in serum of patients with IBS (16). However, in a reduced subset of our population, no differences in the serum concentrations of cytokines interleukin-2 (IL-2), IL-4, IL-6, IL-10, IL-17, tumor necrosis factor alpha (TNF-α), and gamma interferon (IFN-γ) were detected between *Blastocystis*-infected and noninfected individuals ($N_{pos} = 16$ and $N_{neg} = 9$, Mann-Whitney) (Table S7), further supporting the finding that *Blastocystis* colonization in our cohort is not associated with inflammation. This may reflect on the different immune effects exerted by different *Blastocystis* subtypes; previous work reported differential induction of IL-10 but not of IL-12 or IL-8 by subtype (17). However, *Blastocystis* pathogenicity does not directly correlate with subtype identity (4, 10), and pathogenicity is likely a better predictor of cytokine induction. These results reflect data from a very reduced number of blood samples available in this study and should be confirmed in a larger sample size.

DISCUSSION

The intestinal microbiota is highly variable among individual humans, and its diversity is affected by factors like diet, sociogeographic setting, antibiotic use, disease, age, and to a lesser degree, genetics (24, 27, 48). A direct association of intestinal parasites, such as helminths and protozoa, with human intestinal microbiota composition and diversity has been previously reported (33, 49). However, the influence of

gastrointestinal inflammation had not been previously accounted for, and thus, it remained unanswered whether the microbiome differences associated with parasite colonization were linked to ecological parameters and/or were a direct result of gut inflammation. Our study focused on residents from a rural Mexican community that were confirmed to be healthy and without clinical symptoms. This population provided an ideal cohort to study how the most common human protist altered the ecology of the bacterial and eukaryotic microbiome in the absence of inflammation. Similar to previous studies (2, 34), our results confirm that colonization with *Blastocystis* is strongly associated with broad shifts in the gut-resident bacterial community and an increase in bacterial alpha diversity, providing very strong evidence that the presence of this protist may influence human gut microbial ecology through mechanisms that remain unknown.

In this population, *Blastocystis* colonization was very strongly correlated with a decrease in *Prevotella copri* and an increase in *Ruminococcus bromii*, the first and second most abundant bacterial phylotypes in this data set, respectively. These taxa are so commonly observed within the human gut microbiome that individuals are often described as either *Prevotella* rich or *Ruminococcus* rich (50). *Prevotella* species are dominant colonizers of the human gut in nonwesternized populations that consume a plant-rich diet (24, 51), whereas *Ruminococcus bromii* has been proposed to be a keystone species of the human gut, due to its colonization capacity and exceptional ability to degrade resistant starches (52, 53). In this context, this study reveals a potential role of *Blastocystis* as a discriminant feature between two fundamental bacterial species of the human gut, as *Prevotella copri* is positively correlated with *Blastocystis* subtype 3 (ST3), while *Ruminococcus bromii* is negatively correlated with *Blastocystis* ST3. *Blastocystis* colonization is thus a potentially important factor in structuring the gut microbiota architecture.

While other variables could explain these striking differences in microbial communities, age, family relationships, and health status were controlled for in this study. Although a full dietary assessment was not carried out in this population, dietary patterns in this rural population have historically been observed to be highly homogenous, characterized by a high intake of both plant-based sources (corn, legumes, chilies, tomatoes, and other fruits and vegetables) and meat (mainly chicken and beef). Thus, we do not expect our results to be explained by dietary differences among individuals in our study.

Instead, we postulate that *Blastocystis* may exert a predatory effect on bacteria of highly abundant taxa, such as *Prevotella copri*, which accounts for ~50% of the bacterial abundance in *Blastocystis*-negative individuals. *Blastocystis* is known to graze on bacteria. Dunn et al. (54) reported that the ameboid form of the protist was capable of bacterial engulfment, a process that has been suggested to serve the nutritional needs of encystation (55). Additional evidence of the grazing of *Blastocystis* on bacteria includes (i) the low frequency of the ameboid form in axenic cultures (56) and (ii) the contact between *Blastocystis* pseudopodia and bacteria (57). Bacterial predation is also known to occur in the case of a group of free-living amoebas, which are the only group of organisms that can cause a decrease of a bacterial population from 10^8 down to 10^5 per gram of soil (58, 59). Bacterial engulfment has also been described during colonization by the intestinal protist *Entamoeba histolytica* (60). Interestingly, a previous study showed that the presence of *Entamoeba* (likely including *E. histolytica* and *E. dispar*) was associated with an effect on *Prevotellaceae* and *Ruminococcaceae* that was similar to what we found in this study (33).

In the absence of *Blastocystis*, a strong bacterial competitor like *Prevotella copri* may dominate the community, which limits species richness and community evenness, as was observed in this study (Fig. 1). When present, *Blastocystis* predation on the most abundant bacterial taxa (also known as density-dependent predation) may lower the competition for nutrients and space, which may lead to an increase in bacterial richness and community evenness, as has been shown in this and previous studies (2, 61). This is well supported by food web theory and examples from macro- and microecology (62,

63), where the increase in community diversity through grazing or predation occurs through a top-down control on the strongest competitors, which consequently allows for the colonization and persistence of weaker competitors in the community. Our results do not eliminate the possibility of a bottom-up control, where specific bacterial community structures may favor colonization of the intestine by *Blastocystis*. However, this alternative explanation, where a more diverse community could favor the entry of a predator into the ecosystem, is not well supported by ecology theory (64). Future studies considering both bottom-up and top-down mechanisms are necessary to test various scenarios, including a deterministic influence of competition and predation on the gut microbial communities, which are impossible to assess in an observational cross-sectional study like this one.

Other potential indirect mechanisms for *Blastocystis* to affect the abundance and diversity of the bacterial microbiome are via direct interaction with the intestinal epithelium and the underlying immune tissue. Indeed, our study revealed differences in calprotectin and IgA. IgA-mediated modulation of the bacterial microbiome has been reported in mice lacking IgA (65, 66) or when comparing low-IgA mice to high-IgA mice (67). However, the result observed for IgA in this study was subtle and unlikely to cause such drastic changes in microbiome composition and function. Likewise, the levels detected for fecal calprotectin (a granulocyte marker protein produced at high levels in the cytosol of neutrophils and a well-known marker of intestinal inflammation [40]) are well below levels consistent with clinical inflammation (68), and *Blastocystis* was associated with lower levels. Still, indirect mechanisms need to be ruled out experimentally before concluding that they are not at play in the results obtained in this study.

These subclinical immune differences may also be a result of (i) the large differences in bacterial community structures observed in relation to *Blastocystis* colonization and/or (ii) a direct anti-inflammatory effect of *Blastocystis* on the intestinal mucosa. Evidence of the former mechanism clearly exists. Changes in IgA production have been observed in cohousing and fecal transfer experiments carried out in mice (67), where high-IgA mice that adopted the microbiota from low-IgA mice experienced a decrease in IgA levels, demonstrating the IgA modulatory capacity of the bacterial microbiome (67). There is also evidence of direct effects of *Blastocystis* on the immune system. *Blastocystis* is associated with reduced neutrophil counts in blood (69) and is known to produce serine proteases that degrade secretory IgA (sIgA) (70). These mechanisms must be further studied to understand if *Blastocystis* creates an anti-inflammatory environment in the intestine and if this may explain the asymptomatic status in this population. Ideally, this could be studied in a longitudinal cohort study that includes participants that transition from a noncolonized to a colonized status.

The large taxonomic differences observed were, unsurprisingly, accompanied by broad functional differences, inferred computationally by PICRUSt and measured directly through the determination of fecal SCFA concentration. Interestingly, the difference in the bacterial microbiome associated with *Blastocystis* colonization resulted in an overall decrease in carbohydrate fermentative metabolism. We attribute this to (i) the decrease in abundance of *Prevotella copri*, an efficient fiber degrader and acetate producer (71), and (ii) the reduced cross-feeding by other bacterial taxa due to the decrease in *Prevotella copri*. Cross-feeding, whereby a species produces a metabolite that can be used by other species, is a common feature in many microbial communities (72). In the mammalian gut, fiber is degraded into hexoses and pentoses, which are subsequently used as substrates in pathways that lead to the production of acetate, propionate, and butyrate (71). Butyrate can also be produced from acetate (via acetyl coenzyme A [acetyl-CoA]) (71), which could explain the larger decrease in butyrate concentrations in *Blastocystis*-colonized individuals.

Our study found similarities with previous reports. For example, Audebert et al. also reported increases in bacterial alpha diversity and in the abundance of *Ruminococcus* species in association with *Blastocystis* colonization (2). However, our study found taxonomic discrepancies with previous reports. Previously, O'Brien Andersen et al. (34)

had reported an increase in *Prevotella* species associated with *Blastocystis* in a cohort of Danish patients referred to their study by doctors. Likewise, Audebert et al. (2) reported a positive association between *Prevotellaceae* and *Blastocystis* colonization in French individuals. These discrepancies may arise from comparing westernized populations, expected to have different microbiomes than rural Mexicans. Members of the *Prevotellaceae* family account for ~30% of the total gut bacterial taxa in this study, compared to ~5% of the French population studied previously (2). A study that compared the microbiota in relation to *Blastocystis* using shotgun metagenomics data from 11 studies across four continents found an association between *Blastocysis*, *Prevotella copri*, and *Ruminococcus gnavus* that was the opposite of what we observed in this study (7) and did not detect a difference in bacterial alpha diversity. These differences may be due to comparing our results to studies that have analyzed a combination of healthy and diseased individuals or because of differences in *Blastocystis* subtypes (2, 34). For example, while colonization was mostly explained by *Blastocystis* subtype 3 in this study, the samples analyzed by Audebert et al. included colonization with many more subtypes, including ST1, -2, -3, -4, -6, and -7 (2). Notably, an ongoing study in asymptomatic individuals from Cameroon has revealed findings similar to those of our study in relation to *Blastocystis* colonization (Laure Segurel, personal communication), suggesting that the effect of *Blastocystis* on the bacterial microbiome may be dependent on the sociogeographical setting studied or on similarities in subtype colonization. The important interstudy discrepancies reflect the need to study *Blastocystis* in a context-specific manner, accounting for the effects of subtypes, inflammation, geographical setting, and other covariates that may influence microbiome composition and diversity.

Altogether, our work is the first to show the important ecological association between the most common human-associated protist and the fundamental species of the bacterial microbiome in the absence of gastrointestinal disease or inflammation. While our findings are in agreement with the well-supported top-down effect of a predator on ecosystem diversity, this remains to be tested experimentally. If proven, the presence of *Blastocystis* or similar bacterial predators may be an important mechanism of microbial diversity maintenance that is not currently being considered in the human microbiome field. Including microbial eukaryotes in human gut microbiome surveys will provide a more integrated understanding of the ecological processes that shape the gut microbiome and the mechanisms by which they relate to health and disease.

MATERIALS AND METHODS

Study population and study design. This cross-sectional study was carried out in Xoxocotla, Morelos, Mexico, about 120 km south of Mexico City (longitude 99°19′W, latitude 18°3′N). This semirural area spans 29,917 km² and hosts a total population of 5,163 people. The region is characterized by tropical climate (warm subhumid), and agriculture is the main source of economic income. Sample collection was carried out between January and September 2014. A total of 156 urine and stool samples were collected from individuals at Xoxocotla Health Center as a part of a routine medical examination of people living in the town or within the vicinity. Urine and stool samples were collected in sterile plastic containers, immediately placed at 4°C for transport, and then stored at −20°C until analysis. Prior to sample and data collection, the nature of the study was explained to the participants and informed verbal and written consents were obtained. Demographic, socioeconomic, and environmental data and the history of gastrointestinal symptoms in the 3 months preceding sampling (abdominal pain or discomfort and defecation patterns) were obtained based on the standardized Rome III questionnaire in face-to-face interviews. The Rome classification system is based on the symptom clusters that remain consistent across clinical and population groups and is currently the gold standard to determine gut health (73). The protocol was approved by the Medical Ethics Committee of the National Autonomous University of Mexico.

Real-time qPCR analysis for detection of *Blastocystis* and other parasites. The presence of *Blastocystis*, *Cryptosporidium parvum*, *Entamoeba histolytica/E. dispar*, and *Giardia intestinalis* was assessed by quantitative PCR (qPCR) using QuantiTect SYBR green master mix (Qiagen). The *Blastocystis*-specific primer BhRDr (5′ GAG CTT TTT AAC TGC AAC AAC G 3′ [74]) and the broad-specificity eukaryote-specific primer RD5 (5′ ATC TGG TTG ATC CTG CCA GT 3′ [75]), the *Cryptosporidium*-specific primers CrF (forward [F], 5′ CGC TTC TCT AGC CTT TCA TGA 3′) and CrR (reverse [R], 5′ CTT CAC GTG TGT TTG CCA AT 3′), the *Entamoeba histolytica/E. dispar*-specific primers Ehd-239F (5′ ATT GTC GTG GCA TCC TAA CTC A 3′) and Ehd-88R (5′ GCG GAC GGC TCA TTA TAA CA 3′), and the *Giardia*-specific primers Giardia-80F (F, 5′ GAC GGC TCA GGA CAA CGG TT 3′) and *Giardia*-127R (R, 5′ TTG CCA GCG GTG TCC G 3′) (76) were used. Amplification reactions were performed in 10-μl reaction mixture volumes with the *Taq* PCR master mix

kit (Qiagen) and 6.25 pmol each of primers BhRDr-RD5, CrF-CrR, Ehd-239F–Ehd-88R, and *Giardia*-80F–*Giardia*-127R. The amplification conditions consisted of 35 cycles of 1 min each at 94, 59, and 72°C, with an additional step of 95°C for 15 s, 60°C for 1 min, 95°C for 30 s, and 60°C for 15 s (77). The qPCR plates included positive controls (samples known to be positive for each parasite), as well as standard curves using DNA from each parasite from ATCC's enteric protozoon DNA panel.

The 18S rRNA gene was amplified using primers 5′ GTA CAC ACC GCC CGT C 3′ (F) and 5′ TGA TCC TTC TGC AGG TTC ACC TAC 3′ (R). The amplification conditions consisted of 35 cycles of 1 min each at 94, 59, and 72°C, with an additional step consisting of 95°C for 15 s, 60°C for 1 min, 95°C for 30 s, and 60°C for 15 s. All qPCRs were performed on an Applied Biosystems 7500 machine. The parasitic loads in the samples were calculated as the difference between the average cycle threshold (C_T) value of each parasite and the average C_T value of the 18S rRNA gene of each sample.

Fecal microbial community analysis. (i) Extraction. DNA was extracted from ~50 mg of stool. Samples were mechanically lysed using Mo Bio dry bead tubes (Mo Bio Laboratories, Inc.) and the FastPrep homogenizer (FastPrep instrument; MP Biochemicals) before DNA extraction with the Qiagen DNA stool minikit.

(ii) Amplification. For 16S rRNA gene amplification, samples were amplified by PCR in triplicate using bar-coded primer pairs flanking the V3 region of the 16S rRNA gene as previously described (78). Each 50 ml of PCR mixture contained 22 ml of water, 25 ml of TopTaq master mix, 0.5 ml of each forward and reverse bar-coded primer, and 2 ml of template DNA. The PCR program consisted of an initial DNA denaturation step at 95°C (5 min), 25 cycles of DNA denaturation at 95°C (1 min), an annealing step at 50°C (1 min), an elongation step at 72°C (1 min), and a final elongation step at 72°C (7 min). Controls without template DNA were included to ensure that no contamination occurred. Amplicons were run on a 2% agarose gel to ensure adequate amplification. Amplicons displaying bands at ~160 bp were purified using the illustra GFX PCR DNA purification kit. Purified samples were diluted 1:50 and quantified using PicoGreen (Invitrogen) in the Tecan M200 plate reader (excitation at 480 nm and emission at 520 nm).

(iii) Sequencing. For 16S rRNA gene sequencing, each PCR pool was analyzed on the Agilent Bioanalyzer using the high-sensitivity double-stranded DNA (dsDNA) assay to determine approximate library fragment size and verify library integrity. Pooled-library concentrations were determined using the TruSeq DNA sample preparation kit, version 2 (Illumina). Library pools were diluted to 4 nM and denatured into single strands using fresh 0.2 N NaOH. The final library loading concentration was 8 pM, with an additional PhiX spike-in of 20%. Sequencing was carried out using a Hi-Seq 2000 bidirectional Illumina sequencing and cluster kit, version 4 (Macrogen, Inc.). The 18S rRNA gene was sent to the Integrated Microbiome Resource at Dalhousie University for amplification and sequencing. The 18S rRNA gene was amplified with the primers E572F (5′ CYGCGGTAATTCCAGCTC 3′) and E1009R (5′ AYGGTATC TRATCRTCTTYG 3′), and the reaction mixture included a PNA blocking primer (5′ TCTTAATCATGGCCTC AGTT 3′) to reduce amplification of mammalian sequences. Amplification was carried out in duplicate, with one reaction mixture using undiluted DNA and the other using DNA diluted 1:10 in PCR water. Amplification was conducted according to previously described protocols (79). PCR products were visualized on E-gels, quantified using Invitrogen Qubit with PicoGreen, and pooled at equal concentrations, according to a previous report (79). PhiX was spiked in at 5%, and the resulting library was sequenced at Dalhousie University on the Illumina MiSeq using the MiSeq 500-cycle reagent kit, version 2 (250 × 2).

Bioinformatics. Sequences were preprocessed, denoised, and quality filtered by size using the Mothur MiSeq SOP (standard operation protocol) (16S rRNA gene [80]) or QIIME (18S rRNA gene [81]).

(i) 16S rRNA gene sequences. All sequences were processed using Mothur according to the standard operating procedure as previously described (82). Quality sequences were obtained by removing sequences with ambiguous bases, a low-quality read length, and/or chimeras identified using chimera.uchime. Quality sequences were aligned to the SILVA bacterial reference alignment, and OTUs were generated using a dissimilarity cutoff of 0.03. Sequences were classified using the classify.seqs command.

(ii) 18S rRNA gene sequences. Demultiplexed reads were trimmed to a uniform length of 250 bp using the FastX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/) and clustered into operational taxonomic units (OTUs) using the minimum entropy decomposition (MED) method (83) as implemented in the oligotyping microbial analysis software package (84). MED performs *de novo* taxonomic clustering using Shannon entropy to separate biologically meaningful patterns of nucleotide diversity from sequencing noise; the processed data are partitioned into phylogenetically homogeneous units (MED nodes) for downstream bacterial diversity analyses. This analysis was carried out with the minimum substantive abundance parameter (−M) set at 250 reads. All other parameters were run with default settings; the maximum variation allowed per node (−V) was automatically set at 3 nucleotides.

Representative sequences were classified by clustering against the Greengenes Database at 97% similarity (16S rRNA gene [85]) or SILVA release 123 at 99% similarity (18S rRNA gene [86]). The 16S rRNA gene data set was filtered to remove mitochondrion and chloroplast sequences and OTUs present in fewer than three samples. The 18S rRNA gene data set was filtered to remove mammalian and plant sequences and all OTUs present in fewer than three samples. Both data sets were filtered to exclude singletons and doubletons. Following filtering, a cutoff of 1,000 reads per sample was applied. All 16S rRNA gene samples passed the cutoff, while 63 samples were excluded from the 18S rRNA gene data set.

PICRUSt. We used PICRUSt (39) to generate a profile of putative functions (via metagenome prediction) from the 16S rRNA OTU data. Predicted metagenomes from all the samples were categorized by function at KEGG Orthology level 3. To test for significant differences in functional category

abundances between colonized and noncolonized individuals, we used the Welch's t test implementation of STAMP (87).

SCFA analysis. Fecal samples were combined with 25% phosphoric acid, vortexed, and centrifuged until a clear supernatant was obtained. Supernatants were submitted for GC analysis to the Department of Agricultural, Food and Nutritional Science of the University of Alberta. Samples were analyzed as previously described (88), with modifications. Briefly, samples were combined with 4-methyl-valeric acid as an internal standard, and 0.2 ml was injected into the Bruker Scion 456 gas chromatograph, using a Stabilwax-DA 30-m by 0.53-mm by 0.5-μm column (Restek). A standard solution containing acetic acid, propionic acid, isobutyric acid, butyric acid, isovaleric acid, valeric acid, and caproic acid combined with internal standard was injected in every run.

The PTV (programmable temperature vaporization) injector and FID (flame ionization detector) detector temperatures were held at 250°C for the entire run. The oven was started at 80°C and immediately ramped to 210°C at 45°C/min, where it was held for 5.11 min. Total run time was 8.00 min. Helium was used at a constant flow of 20.00 ml/min. Sample concentrations were normalized to the wet weight of feces.

Calprotectin assay. Fecal calprotectin was determined using a human calprotectin enzyme-linked immunosorbent assay (ELISA) kit (Hycult Biotech, Inc., Uden, Netherlands). Stool extracts were prepared and analyzed according to the manufacturer's instructions. After normalizing fecal weight to 1-mg/μl solutions, samples were diluted 10× in phosphate-buffered saline (PBS). The standard curve ranged between 1.6 and 50 ng/ml.

IgA assay. Fecal IgA concentrations were measured by enzyme-linked immunosorbent assay (Chondrex) in samples diluted 1:1,000. After normalizing fecal weight to 1-mg/μl solutions, samples were diluted 1,000× in PBS. The standard curve ranged between 1.6 and 50 ng/ml.

Oxidative stress biomarkers in urine. Biochemical assays for AOPP and MDA were carried out on collected urine specimens according to methods previously established (13, 47). AOPP was measured spectrophotometrically according to a previously described method (89), using a microplate reader (Tecan Infinite M200; Tecan, Switzerland) with the following modifications. Briefly, 200-μl amounts of urine diluted 1:5 in PBS or chloramine-T standard solutions (0 to 100 μmol/liter) were mixed with 20 μl of acetic acid and 10 μl of 1.16 M potassium iodide (KI; Sigma). The absorbance was immediately read at 340 nm against a blank containing 200 μl of PBS, 20 μl of acetic acid, and 10 μl of KI. AOPP concentrations were expressed in μmol/liter of chloramine-T equivalents.

The free radical-induced lipid peroxidation level in urine was determined by measuring MDA with an assay modified from the thiobarbituric acid-reactive-substance method (90). Briefly, 600-μl amounts of urine or 1,1,3,3-tetraethoxypropane standard solutions (0.15, 0.30. 0.60, 1.20, 2.40, 5.0, and 10.0 nmol/ml) were mixed with 300 μl of 37% hydrochloric acid. After centrifugation (800 × g for 10 min), 500 μl of 0.65% N-methyl-2-phenylindole (MPI) (acetonitrile-methanol [3:1] diluent; Sigma) and 500 μl of deproteinized serum were added, followed by a 45-min incubation at 45°C. The resulting blue-colored chromophore was measured at an absorbance of 586 nm in a microplate reader (Tecan Infinite M200; Tecan, Switzerland) using 1,1,3,3-tetraethoxypropane as a standard and water as the blank. Concentrations were expressed as nmol/ml.

Serum IL measurement. Serum was extracted from 5 ml of peripheral blood of each individual to measure IL-2, IL-4, IL-6, IL-10, TNF, IFN-γ, and IL-17A protein levels according to the manufacturer's recommendations for the BD cytometric bead array (CBA) human Th1/Th2/Th17 cytokine kit (BD Biosciences, San Jose CA, USA).

Statistical analysis. Differences in frequencies for categorical and continuous variables between cases and controls were evaluated using the chi-squared and Student's t test, respectively. We assessed fecal microbial diversity and the relative abundances of bacterial and eukaryotic taxa using phyloseq (91), along with additional R-based computational tools (92–97). PCoAs were conducted using phyloseq (Bray-Curtis dissimilarities as distance metric) on both variance-stabilizing-transformed and rarefied OTU matrices and then statistically confirmed by a permutational multivariate analysis of variance (PERMANOVA) to confirm our results were not a consequence of heteroscedastic dispersion between groups (38). The Shannon and Chao1 alpha diversity indices were calculated using phyloseq and statistically confirmed by the Mann-Whitney test (GraphPad Prism software, version 5c). The R packages DESeq2 (98) and MaAsLin (35) were used to calculate differentially abundant OTUs. Correlation analysis was performed using the bicor method in the R package microbiome to correlate the 100 most abundant OTUs from the 16S and 18S rRNA gene data sets. Features in the analysis were included as OTUs and as OTUs combined into taxonomic families.

To confirm that the effects of *Blastocystis* on the gut bacterial community structure were not due to the presence and interaction of other protozoans or influenced by variation in sequencing depth, we used PERMANOVA models. We built three models, one each for *Blastocystis* with *Hymenolepis nana*, *Blastocystis* with *Debaryomyces hansenii*, and *Blastocystis* with sequencing. Neither protozoan interacted significantly with the effect of *Blastocystis* on the gut bacterial community structure, and *Blastocystis* remained a significant driver of community structure in both models. For the 18S rRNA gene data set, a PERMANOVA on gut eukaryotic community structure shows that the sequencing depth appears to interact significantly with the *Blastocystis* species colonization effect on community structure. This result is biologically intuitive, since the presence of *Blastocystis* species in itself is contributing to the number of sequences detected. However, to further control for this result, we carried out the DESeq2 analysis using variance-stabilizing-transformed data. This analysis yielded the same number and identity of differential OTUs between *Blastocystis*-colonized and -noncolonized individuals as we obtained with nontransformed data.

The statistical analyses for calprotectin, IgA, serum cytokines, SCFA, and urine oxidative stress markers were performed with GraphPad Prism software, version 6.00 (GraphPad Software, Inc., San Diego, CA, USA). For comparison between two groups, Student's *t* tests and Mann-Whitney tests were used for normally and abnormally distributed data sets, respectively, and no samples were excluded from statistical analysis. Statistically significant differences were set at a *P* value of ≤0.05. A *P* value of >0.05 was considered not significant.

Data availability. Sequences are publicly available at https://doi.org/10.6084/m9.figshare.6359306. The R code supporting the results of this article is available in the Xoxocotla Gut Microbiome project repository on figshare at https://figshare.com/s/a99310b6d9be4e9de4fe.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/mSystems.00007-18.

FIG S1, TIF file, 2.3 MB.
FIG S2, TIF file, 2.5 MB.
FIG S3, TIF file, 2.4 MB.
TABLE S1, DOCX file, 0.04 MB.
TABLE S2, XLSX file, 0.1 MB.
TABLE S3, XLSX file, 0.03 MB.
TABLE S4, DOCX file, 0.1 MB.
TABLE S5, DOCX file, 0.04 MB.
TABLE S6, DOCX file, 0.01 MB.
TABLE S7, DOCX file, 0.1 MB.

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ACTA PROTOZOOLOGICA

Population Dynamics of Amoeboid Protists in a Tropical Desert: Seasonal Changes and Effects of Vegetation and Soil Conditions

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Abstract. In arid environments, protist diversity is higher in soil covered by vegetation than in bare soil and is also likely to vary in line with the marked seasonal patterns; however, these patterns have not been explored in detail in arid zones. Herein, we used culture – and morphological-based approaches to describe patterns of amoeboid protist diversity in vegetated and bare soil areas from the intertropical desert of Tehuacán, Mexico, during dry and wet seasons. Overall, 27 protist species belonging to Amoebozoa, Discoba and Rhizaria were retrieved using culture-dependent methods. Among the soil protist groups found, Discoba (principally represented by Heterolobosea) was always the most prevalent taxa. Protist diversity was different between soil with vegetation and bare soil, principally during the dry season. Moreover, the electrical conductivity and pH of the soil were correlated with the protist species during the wet season. Our results support the hypothesis that soil protist diversity patterns exhibit a seasonal variation between dry and wet seasons. This seasonal variation likely relies on water availability, although the role of other environmental factors cannot be completely ruled out. In addition, the soils with vegetation could be a refuge for the amoeboid protists during the harsh soil conditions of dry seasons.

Keywords: Heterolobosea, seasonal variation, soil microhabitat, soil protist, Tehuacán desert

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INTRODUCTION

Amoeboid protists constitute the most diverse functional category in soils (Geisen et al. 2018); they play a major role in nutrient cycling by preying on bacteria (Bonkowski 2004), fungi (Geisen et al. 2016) and algae (Seppey et al. 2017), subsequently releasing labile nutrients available for other microorganisms and plants into the soil (Geisen et al. 2017).

Although the protists are dependent on the availability of water (Anderson 2000, Griffiths et al. 2001), desert soils host many amoeboid protists despite their low moisture and nutrient contents (Robinson et al. 2002, Perez-Juárez et al. 2017). While many soil factors drive their distribution at a local scale (e.g., pH and nutrients) (Geisen et al. 2014, Lanzén et al. 2016), water availability seems to always be the most limiting factor in arid soils (Whitford 2002). This is why the harshness of arid soils is exacerbated during dry seasons, and many amoeboid protists withstand the challenges of low water availability and UV radiation prevailing in dry seasons only in soils covered with vegetation, as plants contribute to keeping water and nutrient levels relatively constant in the arid soil (Barness et al. 2009, Li et al. 2011, Sylvain and Wall 2011). Indeed, evidence shows that in arid soils, unicellular diversity tends to be higher in soils covered by vegetation than in bare soils (Robinson et al. 2002; Rodríguez-Zaragoza and Steinberger 2004; Rodríguez-Zaragoza et al. 2005 a, b; Bamforth 2008; Barness et al. 2009; Fernández 2015). Additionally, many protists simply cannot survive in an active state without water (Bamforth 1963); therefore, arid soils in dry seasons filter only taxa with physiological and morphological traits adequate to withstand the lack of water (Austin et al. 2004, Geisen et al. 2014, Fernandez 2015). Most of these species, however, can withstand long periods of dormancy spent as cysts, a coccoid form that can withstand adverse conditions, including drought (Geisen et al. 2014). Protist cysts from various species differ in their structure, ontogeny and surface properties (Foissner 2011) and probably also in their resistance to the harsh conditions encountered during the dry season.

This study was conducted in the intertropical desert of Tehuacán in Mexico. This desert is regarded as a biodiversity hotspot (Davila et al. 2002), harboring even an endemic soil protist species (i.e., a testate amoeba species, Pérez-Juárez et al. 2017). In this desert, the vegetation is dominated by *Prosopis laevigata* and *Parkinsonia praecox* (Fabaceae). Both species exhibit a patchy distribution interspersed with wide areas of bare soil. The two shrubs have ecophysiological features that may affect soil in different ways and can create microenvironments in the soil under their canopies, protecting it also from the climate of the desert that includes long periods of drought, typically interrupted by a short wet season (Barness et al. 2009; Serrano-Vázquez et al. 2013)

In this context, we described the patterns and underlying causes driving amoeboid protist diversity in soils with vegetation and in bare soil areas during the dry and wet season in Tehuacán, an intertropical desert in Central Mexico. To do this, we used culture – and morphological-based approaches. The morphological-based approaches are valid for identifying ameboid protists because amoeboid protists are a polyphyletic group of eukaryotic unicellular organisms that use pseudopodia (i.e., extensions of cytoplasm) for movement and feeding. Pseudopodia vary in shape among taxa and, therefore, along with differences in patterns of locomotion, are regarded as valuable morphological traits to classify these protists into different groups (Fahrni et al. 2003; Pawlowski and Burki 2009).

Therefore, we posit that the temporal dynamics of soil amoeboid protist populations can follow two patterns to be tested in our research: (1) Species are homogeneously distributed during the dry season, and less resistant populations decrease considerably in the bare soils but persist underneath plants, which act as a refuge. (2) Alternatively, all species present may have developed similar resistance mechanisms, and consequently, biodiversity remains unaltered. In this manuscript, we tested the likeliness of both scenarios and whether the first was retained. Additionally, we determined whether there was an effect of other related variables, such as soil characteristics and soil depth, on amoeboid protist species.

MATERIAL AND METHODS

Study area

Tehuacán Valley is a semiarid desert formed by the rain shadow effect of the Sierra Madre Oriental (Villaseñor et al. 1990). Alluvial terraces deposited by the Salado River constitute local landscapes with relatively flat surfaces and deep soils (López-Galindo et al. 2003). The predominant soil units are calcareous regosols and fluvisols, according to FAO-WRB (López-Galindo et al. 2003). Our study site is located in Zapotitlán Salinas between 18°12' and

18°25' N and 97°24' to 97°25' W. The annual mean temperature is 21 °C, and the precipitation is between 400–450 mm. The vegetation in the alluvial terraces is dominated by shrubs of *Pr. laevigata* (Humb. & Bonpl. ex Willd.) MC Johnst and *Pa. praecox* (Ruiz & Pav.) Hawkins (Osorio-Beristain et al. 1996, Valiente-Banuet et al. 2000, Serrano-Vázquez et al. 2013). Both species form shrub patches that alternate with large areas of bare soil (Fig. 1). *Prosopis laevigata* and *Pa. praecox* play key ecological roles because they provide a suitable habitat for other plants, microorganisms, birds, rodents, and insects that are critical for such processes as organic matter decomposition, seed dissemination, pollination, and plant establishment in the Tehuacán desert (Valiente- Banuet et al. 2000).

Sampling

Three individual organisms of *Pr. laevigata* and three of *Pa. praecox* with similar morphological characteristics (3–3.5 m height, 2.5–2.9 m canopy diameter, and 0.2–0.3 m basal diameter), along with three bare soil sites in an alluvial terrace were selected (hereafter referred to as microhabitats). For each microhabitat, we collected soil samples during the wet season (September 2008; 27 samples) and dry season (May 2009; 27 samples). The soil samples were collected using a stainless steel corer (10 cm in diameter) beneath each selected shrub and from bare soil areas. Soil samples were collected from 0 to 30 cm deep in soil layers of 10 cm (0 to 10, 10 to 20 and 20 to 30 cm). A total of 54 soil samples were collected (three microhabitats by three replicates by three depths by two seasons = 54) and stored in self-sealing bags. Bags were deposited in a camp cooler to avoid overheating and stored at 4 ºC, after measuring the soil moisture and obtaining subsamples for soil amoeboid protists, until processing for the next analyses.

Physical and chemical soil properties

To examine the roles of two legume shrubs on amoeboid protist communities, we evaluated how *Pr. laevigata* and *Pa. praecox* modify the soil conditions in the first 30 cm of depth (in 10 cm intervals). All soil samples were analyzed separately for each physical and chemical property. The soil moisture content was determined by the gravimetric method after arriving at the laboratory (weighing samples before and after drying them at 110 ºC for 72 h; Ortiz and Ortiz 1980). The soil composition (percentage of sand, silt, and clay) was determined using a hydrometer with a method described by Bouyoucos (1962). This method takes into account the precipitation time of the particles and temperature (Medina et al. 2007). The pH was determined in a 1:2.5 soil solution in distilled water (Bates 1954, Willard et al. 1958) using a potentiometer (Conductronic pH 120, pH Electrode BA17; Boeco, Hamburg, Germany). Electrical conductivity was determined from a soil extract with a soluble salt tester (Mark Kelway, model SST, Kel Instruments Co., Inc., Wyckoff, NJ, USA) following the manufacturer's instructions. Organic matter content was measured using humid combustion as described by Walkley and Black (1934) and available phosphorus (orthophosphates) based on Olsen's (1954) extraction method, using $NaHCO_3$ as the extractant (Cajuste 1986).

Isolation and identification of amoeboid protists

We obtained the amoeboid protists in triplicate for each soil sample immediately after arriving at the laboratory. All soil samples were analyzed separately. One gram of dry soil for each sample was added to 10 ml of 1:5 soil extract, and the suspension was thoroughly mixed by vortexing for five 15-s pulses in screw-capped glass test tubes. This suspended sample was then left untouched for 15 min to allow the sedimentation of heavy particles (Rodríguez-Zaragoza et al. 2005a). All the supernatant was then gently transferred onto bacteria-free nonnutritive agar plates (Rodríguez-Zaragoza et al. 2005a).

The amoeboid protists were allowed to settle on the agar for 2 h before withdrawal of the excess water, and both trophozoites and cysts were completely isolated with a Pasteur pipette, pulling out the end after heating it on a Bunsen flame to capture cells or cysts one by one, as described in Smirnov and Brown (2004). The same person carried out a intentional, nonbiased and exhaustive sampling to select the amoeboid protists. All picked cells or cysts were individually transferred to new nonnutrient agar plates with soil extract to allow for their proliferation, and they later were identified after seven days of incubation at 28.5 °C. Amoeboid protist species were placed under a coverslip and morphologically identified using an Olympus CH2 phase contrast microscope based on classical amoeboid protist identification textbooks. The main bibliographic sources used in this study were Brown and De Jonckheere (1999), Page (1976), Page (1988), and Smirnov and Brown (2004). It is important to note that the optical resolution of the method used was lower than that of others; however, it is a practical method for cultures of amoeboid protists (Smirnov and Brown 2004).

We prepared the soil extract by suspending 200 g of soil collected at each site in 1000 mL of distilled water and heating the mixture to 60 °C in a water bath for 6 h, then filtering it through a Whatman paper number 41 and autoclaving it for 15 min at 121 °C and 1.1 kg cm^{-2} pressure. The final solution was then stored at 4 °C until use (Rodríguez-Zaragoza et al. 2005b).

Data analyses

The soil parameters such as moisture, organic matter content and percentage of sand, silt and clay were arcsine square root transformed to meet the assumptions of normality and equality of variances. The variance in soil data was analyzed with nested linear mixed effects models, in which season and habitat within season were considered fixed factors and soil depth within habitat was considered as a random factor. The models were fitted with the JMP statistical software version 8.0 (SAS Institute Inc., Cary, North Carolina, USA).

Based on morphological identification, we constructed a presence-absence matrix with all amoeboid species recorded in all samples analyzed. These data were used to evaluate the following parameters: 1) variation in richness of amoeboid protists by microhabitat and 2) relationship between richness of amoeboid protists and physical and chemical parameters of soil in each microhabitat. The richness of amoeboid protists was compared among microhabitats by constructing accumulation curves for each season. We estimated these curves with confidence intervals of 95% with the program EstimateS version 9 (Colwell 2013).

The Sørensen (2c/a+b) method was employed to compare the similarity in the communities of amoeboid protists in the two seasons analyzed.

We analyzed the correlation between amoeboid protist composition in the three previously selected microhabitats and the soil parameters using canonical correspondence analysis (CCA). For each season, we constructed one matrix of microhabitat samples

by amoeboid protists and another matrix of microhabitat samples by soil parameters. Amoeboid protists recorded only once were excluded from the analysis to avoid bias by rare protists. Analyses were performed using the envfit function implemented in the vegan package using R software version 2.9 (Oksansen et al. 2013).

RESULTS

Physical and chemical soil parameters

The physical and chemical soil parameters varied according to season, microhabitat and soil depth. In general, soil had a loamy clay texture. Soil moisture was lower than 20%. The electrical conductivity showed values less than 1 mmhos cm^{-1}; and the pH remained slightly basic. The contents of organic matter and available phosphorus were poor in all microhabitats. There were significant differences in the soil moisture, pH, sand and silt content between microhabitats during both dry and wet seasons, while organic matter was only significantly different in the dry season (Tables 1–2).

Richness of amoeboid protists

Amoeboid protist was composed of species belonging to Amoebozoa, Discoba and Rhizaria. This composition varied according to season and microhabitat. However, species of Discoba (i.e., eruptive amoebae *sensu* Smirnoff and Brown 2004; Adl et al. 2018) were observed in all microhabitats in two seasons (Table 3).

Considering soil microhabitats, we did not observe clear significant differences in the number of species,

with the exception of bare soil during the dry season, where the species of amoeboid protists decreased below detection level (P < 0.05; Fig. 2). Additionally, when comparing morphotypes, it was observed that during the wet season, the number of protist species with eruptive morphotypes decreased, and only during this season did the lingulate morphotype appear (Fig. 2).

The rarefaction curves showed that during the dry season, the richness was higher in soils protected by the canopy of shrubs than in bare soil (Fig. 3a). This protist diversity pattern, however, was absent during the wet season because amoeboid protist richness was similar in both soils protected by the canopy of shrubs and bare soils (Fig. 3b).

The Sørensen analysis showed a similarity of 80% between the species of protists existing in the dry and wet seasons. We found 25 species during the dry season and 20 during the rainy season. Of all species, 18 were detected in both seasons, 7 were present only during the dry season, and only 2 were detected during the wet season.

Correlation analyses

The CCA showed that the correlation between amoeboid protists and soil parameters varied depending on the season (Fig. 4, Table 4). In the dry season, the first and second canonical axes explained 21–33% of the total variance. In the wet season, the first and second canonical axes explained 17–28% of the total variance (Fig. 4). The electrical conductivity (P = 0.011) and pH (P = 0.037) were the only soil parameters significantly correlated with protists in the wet season (Fig. 4).

Table 1. Mean and standard deviation of physical and chemical soil parameters (moisture, sand, silt, clay, pH, electrical conductivity (EC), organic matter and orthophosphates under *Pr. laevigata* (PL), *Pa. praecox* (PP) and bare soil (BS) during dry and wet seasons. Means that do not share a letter are significantly different (P < 0.05).

Soil property	Dry			Wet		
	PL	PP	BS	PL	PP	BS
Moisture (%)	8.3 ± 0.5a	13.8 ± 2.2b	7.0 ± 0.4a	15.0 ± 2.1b	6.4 ± 0.6a	8.8 ± 0.9a
Sand (%)	43.2 ± 2.1b	36.8 ± 1.6a	32.7 ± 5.2a	39.2 ± 1.5ab	44.6 ± 3.2b	36.2 ± 2.0a
Silt (%)	33.4 ± 2.4b	40.6 ± 1.6a	42.9 ± 4.7a	39.5 ± 1.3ab	35.4 ± 2.9b	41.0 ± 1.9a
Clay (%)	23.4 ± 1.2	22.6 ± 1.4	24.4 ± 1.2	21.3 ± 0.7	20.0 ± 1.2	22.8 ± 0.7
EC (millimhos cm^{-1})	1.1 ± 0.3	1.7 ± 0.4	0.5 ± 0.2	2.3 ± 0.7	4.6 ± 0.9	4.6 ± 1.0
pH	7.7 ± 0.0b	7.6 ± 0.1b	8.3 ± 0.3a	7.7 ± 0.1b	7.7 ± 0.0b	7.4 ± 0.1a
Organic matter (%)	2.7 ± 0.2b	1.7 ± 0.2a	1.6 ± 0.5a	1.7 ± 0.2	1.7 ± 0.1	1.5 ± 0.1
Ortophosphates (mg kg^{-1})	5.2 ± 0.6	4.0 ± 0.3	3.0 ± 0.4	4.2 ± 0.3	3.8 ± 0.1	3.9 ± 0.2

Table 2. Statistical effect [F-value (d. f., P-value)] of season and habitat within season for different soil properties using linear mixed effect models with nesting.

Soil property	Season F (d. f., P)	Microhabitat (Season) F (d. f., P)
Moisture (%)	0.1 (1, 41, 0.7)	8.4 (4, 12, 0.002)
Sand (%)	1.4 (1, 41, 0.2)	3.6 (4, 10, 0.05)
Silt (%)	0.06 (1, 41, 0.8)	5.0 (4, 10, 0.02)
Clay (%)	3.6 (1, 41, 0.06)	0.8 (4, 14, 0.6)
Electrical conductivity (millimhos cm^{-1})	13.6 (1, 41, 0.0006)	1.2 (4, 11, 0.4)
pH	16.6 (1, 41, 0.0002)	5.5 (4, 14, 0.007)
Organic matter (%)	3.3 (1, 41, 0.08)	3.4 (4, 14, 0.04)
Ortophosphates (mg kg^{-1})	0.05 (1, 41, 0.8)	2.5 (4, 14, 0.09)

DISCUSSION

The Tehuacán desert exhibits variation in the amoeboid protist community concomitant with marked seasonal changes. Bare soil amoeboid protists were strongly affected by seasonal changes because during the dry season, there was a drastic decrease in their richness, except for Heterolobosea, whereas amoeboid richness remained constant underneath both *Pa. praecox* and *Pr. laevigata* desert shrubs in wet and drought conditions. It has been shown that desert shrubs modify the soil physical and chemical parameters under their canopies (Serrano-Vázquez et al. 2013).

In this study the pH was the only edaphic factor that was modified by the two shrubs in wet or drought conditions, in contrast to the bare soil. In addition, we found a correlation with the pH and electrical conductivity of soil that likely explains shifts in the diversity of amoeboid protist species. The pH and electrical conductivity could play a key role in the availability of nutrients in the soil (Stewart and Tiessen 1987, Braschi et al. 2003). In soils with alkaline pH, mineral complexes with nutrients may be produced, making them unavailable to plants and many microorganisms (Tunesi et al. 1999), as a consequence of evapotranspiration and salt accumulation that increase the electrical conductivity (Li et al. 2011, Serrano-Vázquez et al. 2013).

Thus, the pH and electrical conductivity are important in the microbial communities distributed in the soil (Avis et al. 2008, Puignare et al. 2004) and, consequently, in the diversity patterns of many microeukaryotes, including amoeboid protists (Li et al. 2018, Shen et al. 2014). In addition, remaining moisture, relatively moderate temperatures, and nutrient input from dead leaves

are certainly favorable conditions for many amoeboid protists that inhabit the microhabitats with vegetation (Robinson et al. 2002).

It can therefore be reasonably assumed that shrub canopies act as refugia for many protist species during the dry season in contrast to the bare soil. During the dry season, the community of protists in bare soils was poor, in contrast to the diversity found underneath shrubs. Additionally, all species found in bare soil samples also occurred in the samples from under shrubs and were almost only Heterolobosea. This pattern of diversity has been previously reported for protists from other hot deserts (Robinson et al. 2002, Bamforth 2008, Fernández 2015). This suggests that this pattern of diversity is the result of nonrandom mechanisms, such as those mediated by a source-sink dynamics process (Fernández 2015). Most likely, during the wet season, bare soils were only colonized by generalist species with particular traits that allowed them to survive (but not to establish permanent populations) in these harsh soils.

It has recently been shown that protist composition also responds to changes in soil moisture, especially regarding larger protists, which quickly disappear with decreasing soil moisture content (Geisen et al. 2014). Most Amoebozoa and Rhizaria follow this pattern; in contrast, Heterolobosea does not seem affected by seasonal changes and can be found both under bare soils and canopies in any season. Heterolobosea includes several thermophilic species and seems phylogenetically well adapted to extreme heat, as is the case for some marine species and soil organisms (Dobson et al. 1997; De Jonckheere et al. 2011a, b). They are known to respond quickly to rain pulses by explosive growth

Table 3. Amoeboid protist species recorded under the canopy of *Prosopis laevigata* and *Parkinsonia praecox* and the bare soil at three different depths (10, 20, and 30 cm) and two seasons: dry (D) and wet (W). ND = not determined, sp = organisms that could not be identified at the species level.

Amoeboid protist (abbreviation)	Taxonomy	Morphotype	*Prosopis laevigata*						*Parkinsonia praecox*						bare soil					
			0–10		10–20		20–30		0–10		10–20		20–30		0–10		10–20		20–30	
			D	W	D	W	D	W	D	W	D	W	D	W	D	W	D	W	D	W
***Acanthamoeba castellanii* (Acaca)**; Douglas, 1930	Amoebozoa; Centramoebidae	Acanthopodial	2	2	2	1	0	0	2	2	0	1	1	1	0	1	0	0	0	0
***Acanthamoeba lugdunensis* (Aclu)**; Pussard and Pons, 1977	Amoebozoa; Centramoebidae	Acanthopodial	3	1	1	1	0	1	0	1	0	1	1	2	0	1	0	0	0	0
***Biomyxa* sp (Bisp)**	Rhizaria; Endomyxa	ND	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
***Echinamoeba exundans* (Ecex)**; Page,1967	Amoebozoa; Tubulinea	Acanthopodial	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
***Echinamoeba silvestris* (Ecsi)**; Page, 1975	Amoebozoa; Tubulinea	Acanthopodial	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
***Filamoeba nolandi* (Fino)**; Page, 1967	Amoebozoa; Variosea	Acanthopodial	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0
***Stachyamoeba lipophora* (Stli)**; Page, 1975	Discoba; Heterolobosea	Flabellate	1	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0
***Rosculus ithacus* (Roit)**; Hawes, 1963	Rhizaria; "sainouroids"	Flabellate	0	0	0	0	2	1	1	0	1	1	1	1	1	0	0	0	0	0
***Vermamoeba vermiformis* (Veve)**; Smirnov et al., 2011	Amoebozoa; Tubulinea	Monotactic	1	1	0	2	0	2	2	1	1	1	0	0	0	1	0	0	0	0
Filose 1 (Fil1)	ND	ND	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
***Korotnevella stella* (Kost)**; Schaeffer, 1926; Goodkov, 1988	Amoebozoa; Dactylopodida	Dactylopodial	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
***Mayorella spatula* (Masp)**; Bovee, 1970	Amoebozoa; Dactylopodida	Mayorellian	0	2	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0
***Naegleria galeacystis* (Naga)**; Napolitano, Wall and Ganz, 1970	Discoba; Heterolobosea	Eruptive	2	0	2	0	3	0	0	1	0	0	2	0	0	1	0	2	0	0
***Tetramitus jugosus* (Teju)**; Page, 1967	Disscoba; Heterolobosea	Eruptive	0	1	1	0	2	0	0	0	2	1	1	0	1	0	0	2	0	0
***Tetramitus rostratus* (Tero)**; Perty, 1852	Discoba; Heterolobosea	Eruptive	1	0	2	0	3	0	1	1	1	1	0	0	0	0	0	0	0	0
***Tetramitus aberdonica* (Teab)**	Discoba; Heterolobosea	Eruptive	3	2	3	1	3	1	3	2	2	2	3	1	0	0	0	1	0	3
Heterolobosea 1 (Het1)	Discoba; Heterolobosea	Eruptive	1	0	0	2	0	0	1	0	0	0	1	0	0	0	0	0	0	0
***Vahlkampfia avara* (Vaav)**; Page, 1967	Discoba; Heterolobosea	Eruptive	3	1	3	2	3	0	2	0	3	0	2	2	1	1	0	0	0	0

Species	Taxonomy	Morphology																			
Tetramitus enterica (**Teen**); (Page) Brown & De Jonckheere, 1999	Disscoba; Heterolobosea	Eruptive	1	1	3	2	3	2	2	0	2	0	1	0	3	0	1	0	1
Heterolobosea 2 (Het2)	Discoba; Heterolobosea	Eruptive	0	0	3	0	0	0	0	2	0	2	0	0	0	0	0	0	0
Vahlkampfia inornata (**Vain**); Page, 1967	Discoba; Heterolobosea	Eruptive	2	0	2	0	1	0	2	0	3	2	1	2	1	0	2	0	0
Heterolobosea 3 (Het3)	Discoba; Heterolobosea	Eruptive	1	0	2	0	1	0	0	1	0	2	0	0	0	0	0	0	0
Heterolobosea 4 (Het4)	Discoba; Heterolobosea	Eruptive	2	0	1	0	1	1	0	0	2	3	1	0	0	0	1	0	0
Parvahlkampfia ustiana (**Paus**); Page, 1974	Discoba; Heterolobosea	Eruptive	2	0	1	0	1	1	0	1	1	2	0	0	0	0	0	0	0
Filose 2 (Fil2)	ND	ND	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Stenamoeba placida (**Stpl**); Smirnov et al., 2007	Amoebozoa; Thecamoebida	Lingulate	0	1	0	1	0	3	0	0	0	0	0	0	1	0	2	0	1
Vannella platypodia (**Vapl**); Glaeser, 1912; Page, 1976	Amoebozoa; Vannellida	Fan-shaped	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	1

episodes (Bass and Bischoff 2001). This strategy seems well suited to desert soils, and these organisms are commonly found in these environments (Ekelund and Ronn 1994, Foster and Dormaar 1991, Bass and Bischoff 2001). Heterolobosea seem therefore to be very competitive in desert environments and to have well-adapted year-round resident populations in deserts (Bass and Bischoff 2001). This idea is supported by the observation that protists with Heterolobosean morphology (i.e., eruptive amoebae) have been recorded in several hot deserts across several continents (Robinson et al. 2002, Mayzlish-Gati and Steinberger 2007, Dumac et al. 2016).

Because Heterolobosea is the group that is more diverse in this desert, we suggest that heat positively selects for it against other protists in desert soils, but experiments and additional studies are needed to explore this idea. However, we suggest that these protists can withstand adverse desert conditions by entering a dormant life stage (i.e., cysts), while their other life states help them to survive during active stages in the dry and heat of desert conditions. Additionally, although many soil protists are capable of producing cysts, these structures have different morphological, physiological and persistence parameters and resist different adverse conditions (Geisen et al. 2018); thus, the cysts of Heterolobosea could be more resistant than the other soil protists. The exact identity of these Heterolobosea still remains to be determined, as this group contains many forms that cannot be distinguished by morphology alone (Anderson et al. 2011, Harding et al. 2013, Geisen et al. 2015). Molecular makers should be helpful to analyze the identities and diversity of this important protist group in future research.

CONCLUDING REMARKS

In strong agreement with previous studies, our study showed that in deserts, soil beneath shrubs exhibit richer soil protist communities than bare soils. This is very likely because shrubs provide shelter and other conditions that promote the development of soil amoeboid protists.

We also found that the species were distributed homogeneously during the wet season; however, the less resistant species died or their populations decreased considerably in the bare soil during the dry season, but

Table 4. Correlation (CCA) between protist amoeboid species and soil properties matrix during wet season (P values based on 999 permutation. * = P < 0.05).

Soil property	CA1	CA2	r2	Pr (> r)
Moisture	−0.69778	−0.71631	0.0973	0.313
Sand	0.94794	−0.31844	0.024	0.738
Silt	−0.68819	−0.72553	0.0514	0.508
Clay	−0.13104	0.99138	0.2244	0.067
Electrical conductivity	0.01926	0.99981	0.3154	0.015 *
pH	0.17476	−0.98461	0.2409	0.039 *
Organic matter	−0.85677	−0.5157	0.1798	0.099
Ortophosphates	−0.4224	−0.90641	0.198	0.095
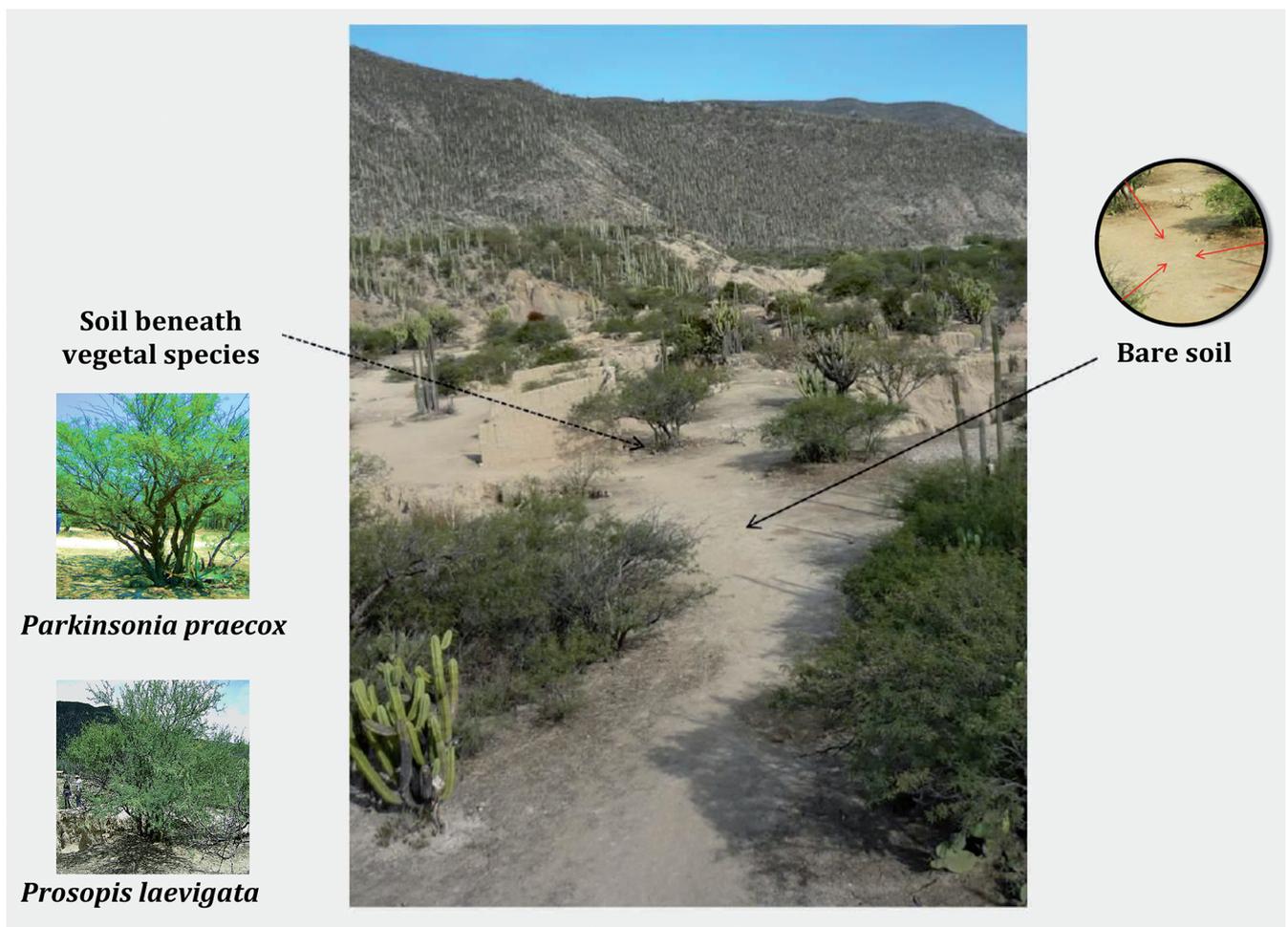
Figure 1. Study area, showing vegetation patches in the desert of Tehuacán, Puebla, Mexico. In addition, the analyzed microhabitats are shown: *Pr. laevigata*, *Pa. praecox* and bare soil.
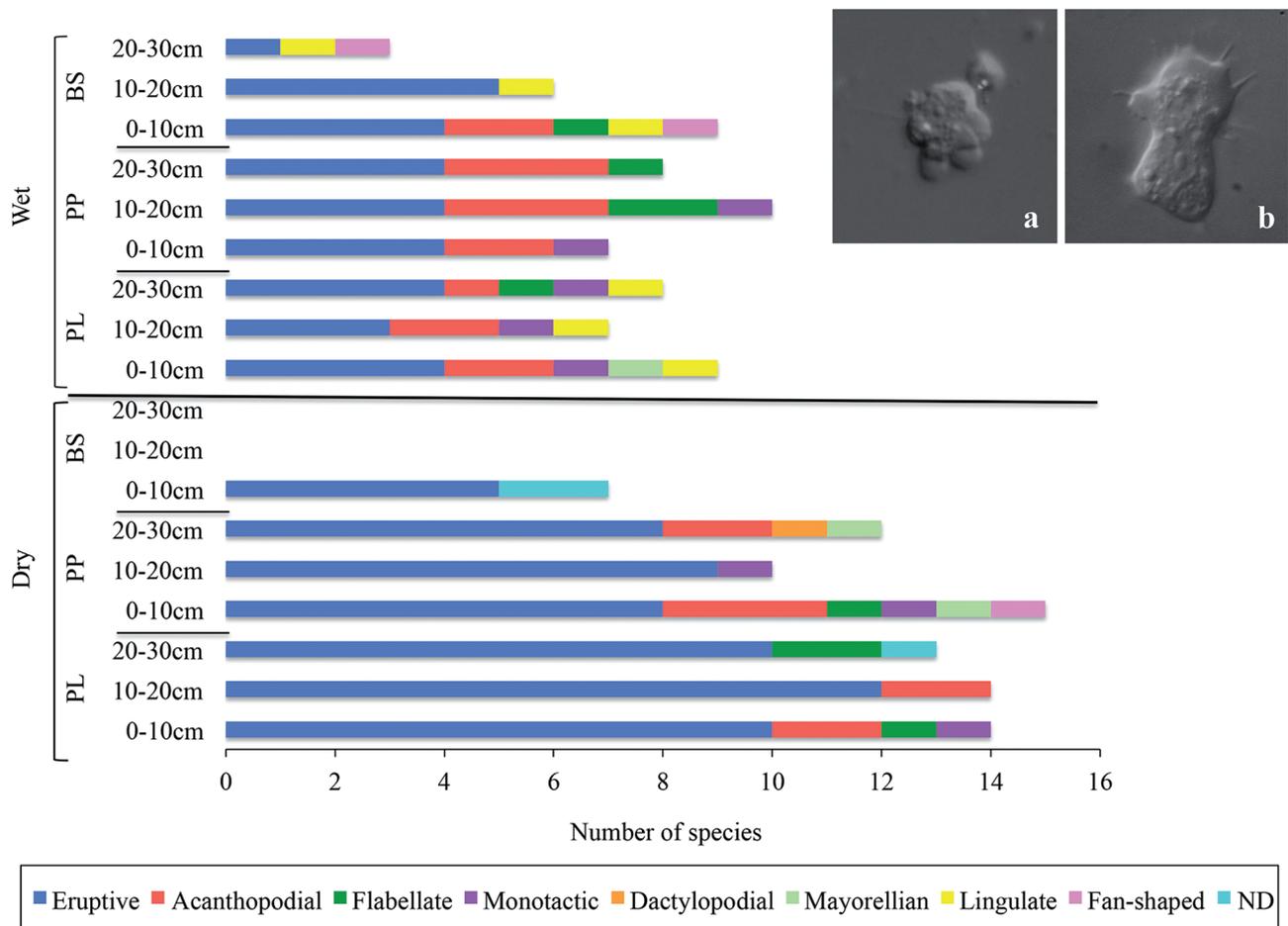
Figure 2. Cumulative richness plots of amoeboid protists present under *Pr. laevigata* (PL), *Pa. praecox* (PP) and bare soil (BS) during dry and wet seasons at 0–30 cm. a) eruptive pseudopods, and b) acanthopodial pseudopods. ND: not determined.

they persisted under two desert shrubs that we investigated, which acted as refugia.

Finally, we remark that thermophilic Heterolobosea can withstand adverse conditions (i.e., heat and low moisture) during all seasons and probably constitute the resident population of heterotrophic protists in many deserts. Given the essential role played by small heterotrophic protists in soils, Heterolobosea are most likely to constitute keystone organisms in arid ecosystems.

◀◀ **Figure** 3. Rarefaction curves of number of species of amoeboid protists by different microhabitats: *Pr. laevigata* (diamonds), *Pa. praecox* (squares), and bare soil (triangles) in a) dry and b) wet season (error bars are 95% CI).
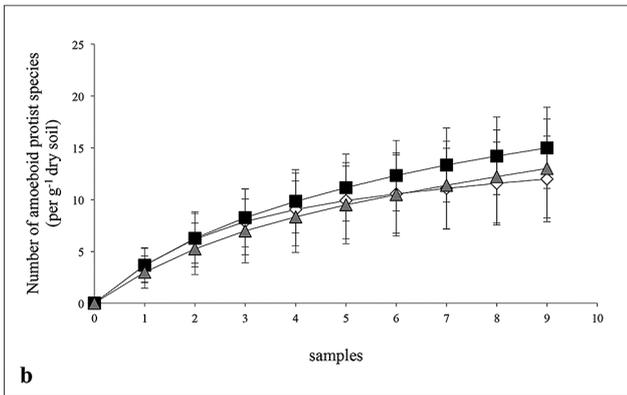
▼ **Figure** 4. Relationship between amoeboid protist richness and soil parameters during the wet season in three microhabitats by CCA: PL: *Pr. laevigata*, PP: *Pa. praecox*, and BS: bare soil. The names and abbreviations of the amoeboid protist species can be found in table 3.
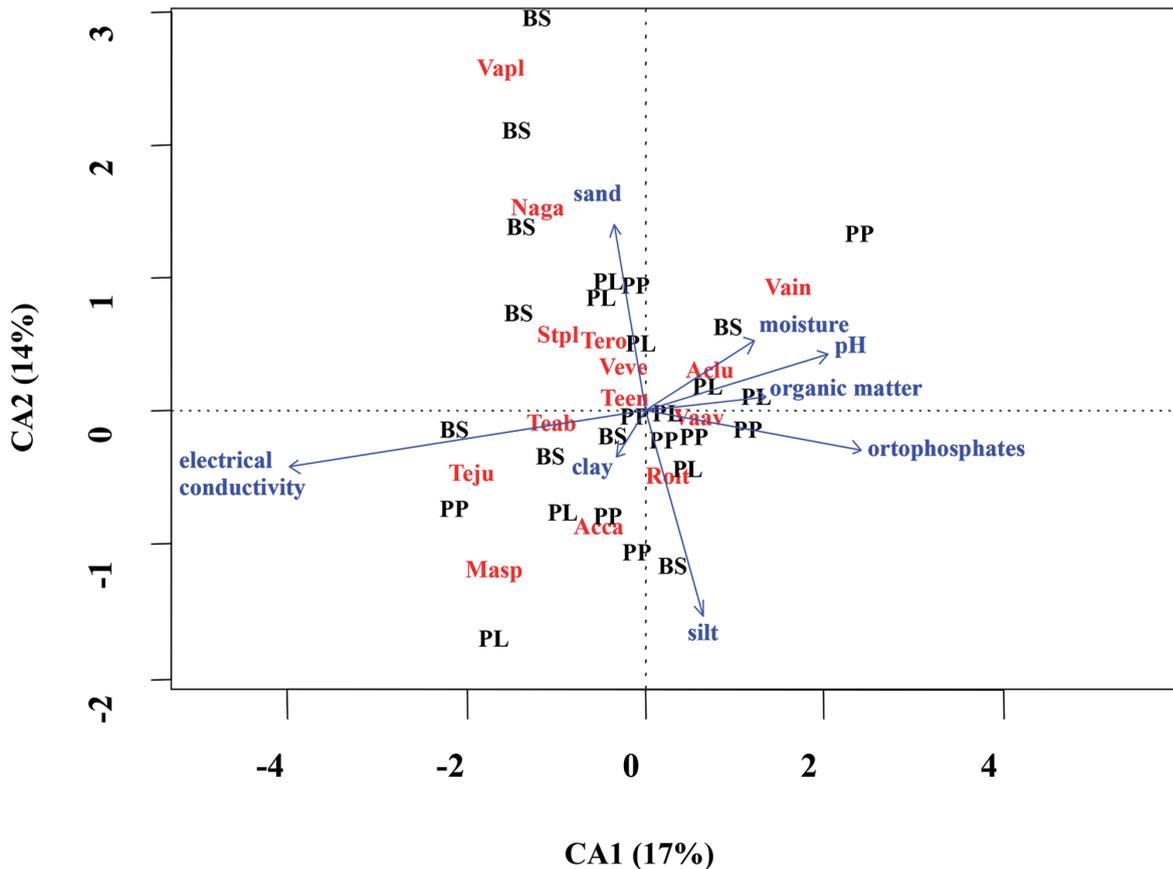
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Chapter 6
Advances in the Study of *Blastocystis* spp. in Mexico: Prevalence, Genetic Diversity, Clinical Association and Their Possible Role in the Human Intestine

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Abstract *Blastocystis* spp. has a worldwide distribution, and it has been associated with gastrointestinal symptomatology; however, its role in health or disease remains unclear. Subtype 3 is the most frequently reported subtype in different populations, with a high haplotype diversity. The recent diversity of this protist may be related to the migration of the human population. The ST3 haplotype network shows that haplotype 1 is ancestor from which the other haplotypes are derived. In the studied community (Xoxocotla, Morelos), a direct association exists between the presence of *Blastocystis* spp. and the changes in the bacterial and eukaryotic intestinal microbiota

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in the absence of gastrointestinal or inflammatory diseases, indicating that *Blastocystis* ST3 favors the diversity and richness of bacterial populations and decrease the inflammatory processes. Thus, there is important evidence that suggests that *Blastocystis* spp. plays an important role as a mutualist in the regulation of the inflammatory response in the studied healthy individuals.

Keywords Blastocystis spp infection · Subtypes · Subtype genetic diversity · Bacterial and eukaryotic microbiota changes

Background

Blastocystis spp. (Heterokonta, Stramenopiles) is and enteric protist with a world-wide distribution that can be inhabiting the digestive tract of several metazoans, such as fishes, amphibians, birds, reptiles, rodents, and humans (Yoshikawa et al. 2007; Silberman et al. 1996; Stechmann et al. 2008; Stenzel and Boreham 1996; Tan 2004). *Blastocystis* spp. has been proposed as an emerging pathogen for humans, replacing traditionally endemic parasites in developing countries, such as *Entamoeba histolytica* and *Giardia duodenalis* (Rodríguez et al. 2008). In the last two decades, there has been an increase in the reported data relative to the frequency of *Blastocystis* spp. worldwide (Tan 2008). However, the microscopic examination of fresh fecal samples is a routine diagnostic method for *Blastocystis* spp. detection; that can lead to erroneous diagnosis and the underestimation or subestimation of the prevalence of this protest (Baldo et al. 2004).

The prevalence of *Blastocystis* spp. varies between 0.5 and 62% (Clark et al. 2013). The real frequency of *Blastocystis* spp. cannot be established based on this range because of two main factors, the predisposition of the individual to be colonized and the diagnostic tool used microscopic analysis or molecular biology through polymerase chain reaction (PCR); studies with both methods were not comparable in sensitivity or specificity, resulting in inconsistent parasite prevalence data (Clark et al. 2013).

This increase in frequency of *Blastocystis* spp. infections has been the most reported for an intestinal protist, and *Blastocystis* spp. infections are more predominant in underdeveloped countries where the hygiene conditions, exposure to domestic and peridomestic animals, as well as the consumption of food and/or contaminated water, represent an ideal environment for Blastocystosis (Clark et al. 2013). However, *Blastocystis* spp. is not exclusive to underdeveloped countries; it is also prevalent in developed countries (Beyhan et al. 2015). The presence of *Blastocystis* spp. has been associated with a variety of symptoms, mainly gastrointestinal symptoms, such as diarrhea and abdominal pain, which in most cases are self-limiting. In addition, irritable bowel syndrome (IBS) has been associated with *Blastocystis* infection (Yakoob et al. 2004).

Studies on the taxonomic classification of *Blastocystis* spp. have helped to better understand this organism; however, knowledge on its genetic diversity, its geography

distribution, the specificity of the host and its role in the human intestine is still lacking. Molecular evidence based on the small subunit ribosomal RNA (SSU rDNA) gene suggests that at least 17 genetic subtypes can be recognized within *Blastocystis* spp. species and nine of these subtypes are found in humans (Silberman et al. 1996; Stenzel and Boreham 1996). Therefore, molecular epidemiology studies of infection by this protist would help us to better understand the role of *Blastocystis* spp. in health and disease, clarifying whether *Blastocystis* spp. is truly a parasite or pathogen or rather a commensal or even a mutualist in the human intestine.

Advances in the Study of *Blastocystis s*pp. in Mexico

Five years ago, clinical studies reported in Mexican individuals with gastrointestinal symptoms or irritable bowel síndrome is associated with *Blastocystis* spp. infection authors suggest that this protist could be the etiological factor (Villalobos et al. 2014; Vargas-Sanchez et al. 2015). To date, there are studies in Mexican populations that consider *Blastocystis* spp. infection as an important factor for the presence of specific symptoms such as chronic abdominal pain (Toro Monjaraz et al. 2018) or consequences of poliparasitism (Galvan-Ramírez et al. 2019; García-Flores et al. 2019).

The massive treatment of the scholar to eradicate helminths infection in Mexico seems to creative an empty ecological niche that has been occupied by other intestinal protozoa including *Blastocystis* spp. (Rodríguez et al. 2008; Rojas et al. 2016). In the last three years, has been reported an important interaction of the intestinal protozoa (Eukaryome) and the intestinal microbiota (Partida-Rodríguez et al. 2017). Eventhough, the mechanisms of interaction between the intestinal microbiota and *Blastocystis* spp. are not well understood, efforts have been made to identify the STs of *Blastocystis* spp. and their potential pathogenic proteins that could be used as genetic biomarkers (Villalobos et al. 2014; Vargas-Sanchez et al. 2015; Rojas-Velázquez et al. 2018). Furthermore, there are reports that mention the posssible role of *Blastocystis* spp. in the modulation of some intestinal bacterial populations and the Immune response (Nieves-Ramírez et al. 2018).

Genetic Diversity and Geographical Distribution of *Blastocystis* Subtypes

Blastocystis spp. is globally distributed, showing a high rate of human infection from underdeveloped to developed countries (Clark et al. 2013; Alfellani et al. 2013). In countries such as Mexico, the phenomenon of epidemiological change of *Blastocystis* spp. increasing the prevalence of infection in the general population (Villalobos et al.

2014; Vargas-Sanchez et al. 2015; Villegas-Gómez et al. 2016; Rojas-Velázquez et al. 2018).

Due to the improvements in molecular biology and the use of polymerase chain reaction (PCR) and sequencing, *Blastocystis* spp. has been studied for its genetic diversity, and currently 17 subtypes have been identified using the small subunit ribosomal RNA gene (SSUrRNA) as a target. Within the 17 subtypes described, with a couple exceptions, only the first 9 subtypes are capable of infecting humans. The molecular analysis of *Blastocystis* spp. showed the genetic variation among *Blastocystis* spp. isolates with 3–5% divergence between subtypes, which increases the possibility that more than one species can infect a host (Silberman et al. 1996; Kukoschke and Múller 1991; Boreham et al. 1992). Despite the great genetic diversity, the distribution of *Blastocystis* spp. subtypes is not homogeneous worldwide. There is a predominant distribution of subtypes; subtypes 1, 2, 3 and 4 have a higher frequency and have been associated with gastrointestinal symptoms (Alfellani et al. 2013; Engsbro et al. 2014; Forsell et al. 2012). In the literature, there are reports that subtypes 1, 2 and 3 have a worldwide distribution, unlike subtype 4, which seems to be more frequent in the European and Asian continent (Alfellani et al. 2013; Ramírez et al. 2016).

Recently, *Blastocystis* spp. subtypes 1, 2 and 3 were identified in five states from Mexico (Ciudad de México, Michoacán, Sonora, Estado de Mexico and Morelos) (Villalobos et al. 2014; Vargas-Sanchez et al. 2015; Villegas-Gómez et al. 2016; Rojas-Velázquez et al. 2018; Alarcon-Valdes et al. 2018). In the last state is where our research group has investigated the genetic diversity, geographic distribution and the possible implications that this protist may have on the human intestine, taking as a study model a rural population of the state of Morelos in Mexico. We collected fecal samples from 182 volunteers (86 men and 96 women) from Xoxocotla, State of Morelos (Mexico), aged between from 2 to 51 years. We found a high frequency of *Blastocystis* spp. in 39.56% of the samples through PCR analysis and sequencing, which also showed the presence of three different subtypes of *Blastocystis*. These three subtypes of *Blastocystis* (ST) were recorded according to the following frequencies: *Blastocystis* ST1, 9.7% ($n = 7$ samples); ST2, 15.3% ($n = 11$ samples); and ST3, 75% ($n = 54$ samples) (Rojas-Velázquez et al. 2018) (Fig. 6.1).

Additionally, we provided new data on the geographic distribution and genetic diversity of *Blastocystis* ST3 of the rural human population in Xoxocotla, Morelos, Mexico and we compare its diversity and structure genetics with what was previously observed in the populations of *Blastocystis* ST3 from other regions of the planet (Rojas-Velázquez et al. 2018) and through the construction of a network of haplotypes of *Blastocystis* subtype 3 isolates from humans, we discovered that this subtype has a high diversity of haplotypes and a high genetic structure in many countries of the world, in contrast to rural populations in Mexico such as the state of Morelos, which seems to have a low diversity of haplotypes. This study also revealed evidence that suggests a recent increase in the diversity of *Blastocystis* ST3 worldwide and may be related to the migration of human populations (Fig. 6.2). One haplotype (Haplotype 1) was the most frequently detected haplotype, and it is perhaps the ancestral type
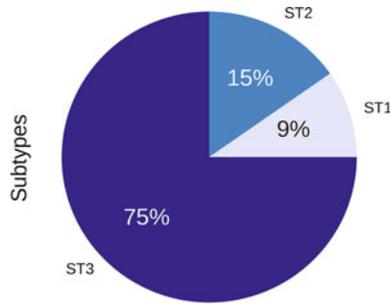
Fig. 6.1 Frequency of *Blastocystis*subtypes in the Xoxocotla, Morelos population. Targeting the SSU-rDNA according to DNA-barcoding. Three *Blastocystis* subtypes (ST) were recorded according to the following frequencies: *Blastocystis* ST1, 9.7% ($n = 7$ samples); ST2, 15.3% ($n = 11$ samples); and ST3, 75% ($n = 54$ samples) (Rojas-Velázquez et al. 2018)

from which all the other haplotypes have been generated recently (Rojas-Velázquez et al. 2018).

Clinical Association or Commensal Behavior of *Blastocystis* spp.

This parasite is often transmitted via the oral-fecal route to people who work directly or indirectly with animals, such as those involved in animal farming or industrial livestock production, and it is estimated that one billion humans are colonized by *Blastocystis* spp. (Tan 2008).

Previously, *Blastocystis* spp. was considered an opportunistic parasite only reported in immunocompromised and immunosuppressed patients, such as HIV-infected patients, who, due to immunodeficiency, could be more susceptible to infection by parasites than general population (Zali et al. 2004; Hailemariam et al. 2004; Gassama et al. 2001). In clinical practice, *Blastocystis* spp. has been considered as non pathogenic infectious agent without clinical importance. However, a number of publications have referred the association between *Blastocystis* spp. and irritable bowel syndrome (Yakoob et al. 2004; Poirier et al. 2012; Beatty et al. 2014; Jimenez-Gonzalez et al. 2012; Ramirez-Miranda et al. 2010). Although *Blastocystis* spp. has been detected in patients with gastrointestinal symptoms such as diarrhea, abdominal pain and distention the prevalence of this infection in asymptomatic individuals is considerably high (Rodríguez et al. 2008; Alfellani et al. 2013; Pandey et al. 2015; Scanlan et al. 2014) in our results, we reported a high frequency of infection in asymptomatic healthy individuals (Rojas-Velázquez et al. 2018).

The existence of genetically diverse infection sources has been recently reported and the human host can be co-colonized with multiple different subtypes or haplotypes. Previous studies have opened the possibility of competition or cooperation

Fig. 6.2 **Haplotype network of *Blastocystis* ST3 of human populations and the statistics data of genetic diversity observed within different geographical populations around the world**. *N*: number of sequences; *S*: number of segregating sites; *h*: number of haplotypes; Hd: haplotype diversity; π: nucleotide diversity; ns: not significant. **$p < 0.01$. Latin America: *Blastocystis* populations of North and South America (i.e., Mexico, Colombia, Brazil, Ecuador, Bolivia, Peru, and Argentina), except that of Morelos. Eurasia: *Blastocystis* populations of Europa and Asia (i.e., Nepal, Switzerland, Iraq, Italy, and France). Haplotype network of *Blastocystis* ST3 of human populations at different regions from Latin America, Europe, and Asia. Each circle represents a haplotype, and each color represents the place where it was obtained. The size of each circle is proportional to the frequency of the haplotype in each population, where it was found.The circles in black stand for missing haplotypes and the short lines show the mutational steps (Rojas-Velázquez et al. 2018)

between *Blastocystis* spp. subtypes for a successful gut colonization (Scanlan et al. 2015). *Blastocystis* mixed infections has been estimated to be 6% worldwide (Alfellani et al. 2013). Scanlan et al. (2015) developed and applied *Blastocystis* ST-specific PCRs for the investigation of the most common subtypes of *Blastocystis* (ST1 to ST4) to a healthy human cohort (n = 50). They reported mixed infections in 22% of the cases, all of which had been identified as single-ST infections in a previous study using state-of-the-art methods (Scanlan et al. 2015).

Recently, had been reported that next generation amplicon sequencing (NGS) was a powerful tool to investigate mixed infections and detect low abundance subtypes of *Blastocystis* (Maloney et al. 2019). Using this technology, we identified mixed *Blastocystis* infections in the aforementioned population (Xoxocotla, Morelos). The study identified 17 mixed infections representing 13.7% of all *Blastocystis* infections. Mixed subtype infections are underrepresented compared to expectations from subtype prevalence (Rojas-Velázquez et al. 2019). However, mixed infections in this study were within the range of observations from other human studies (Alfellani et al. 2013; Scanlan et al. 2015). In our work, we reported co-infections of ST1/ST3, ST2/ST3, and ST1/ST2/ST3. This study provides important information about the epidemiology of *Blastocystis* and represents the first application of a *Blastocystis*-specific NGS protocol to study *Blastocystis* in humans. More studies are needed to characterize mixed subtype infections and intrasubtype variation to understand the transmission dynamics, epidemiology, and potential pathogenicity of *Blastocystis* in humans and animals hostess, and NGS provides a valuable tool for achieving this goal (Rojas-Velázquez et al. 2019).

Interaction of *Blastocystis* spp. and the Gut Microbiota of Asymptomatic Individuals

The relationship between human-associated gut protists and the resident gut bacterial community has only recently begun to be explored (Barash et al. 2017; Burgess and Petri 2016). A number of human-associated microbiome studies have been focused on the role of the microbiome in health and in disease and or the factors that might influence its diversity and composition (Brown et al. 2013). The intestinal microbiota is highly variable among human host, and its diversity is affected by factors such as diet, sociogeographic setting, antibiotic use, disease, age, and to a lesser degree, the human genetics (Yatsunenko et al. 2012; Goodrich et al. 2014; Cho and Blaser 2012). A direct association between intestinal parasites, and human intestinal microbiota both in composition and diversity has been previously reported (Morton et al. 2015; Zaiss et al. 2015). We found that colonization with *Blastocystis* spp. is strongly associated with broad shifts in the gut-resident bacterial community and an increase in bacterial alpha and beta diversity. A correlation analysis between the abundances of the 100 most abundant taxa of the 16S versus 18S genes showed that *Blastocystis* sp. ST3 was positively correlated with members of the *Ruminococcaceae* family and negatively correlated with *Prevotella copri* (Fig. 6.3). However we observed a more discrete correlation between the colonization by *Blastocystis* spp. and eukaryotic microbiota diversity, detecting statistically significant differences in diversity (Shanon) but no in the abundance (Chao). Therefore, the rural community of asymptomatic individuals from the state of Morelos, Mexico, provides an ideal study cohort to better understand how the most commonly found protist in
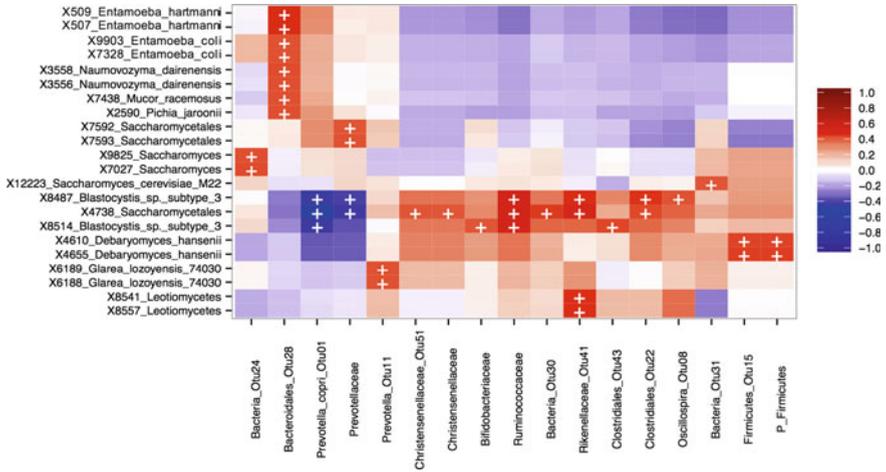
Fig. 6.3 **Heatmap of biweight correlations (bicor method) between top 100 bacterial (x axis) and top 100 taxon (y axis) OTUs in fecal simples from study participants**. Colors denote positive (red) and negative (blue) correlation values. Significant correlations are denoted with a plus sing (p < 0.05, FDR) (Nieves-Ramírez et al. 2018)

the gastrointestinal tract alters the ecology of the bacterial and eukaryotic microbiome in the absence of symptoms of inflammation, which correlates with the most abundant phylotypes in the microbiome of the human intestine. Additionally, this suggests that *Blastocystis* spp. might exert a predatory grazing effect on the more abundant bacteria, as observed with *P. copri* (Nieves-Ramírez et al. 2018). This is supported by food web theory and is an example of macro- and microecology where the increase in community diversity through grazing or predation occurs through a top-down control on the strongest competitors, which consequently allows for the colonization and persistence of weaker competitors in the community (Kato et al. 2018; McDonald-Madden et al. 2016; Paine et al. 2016).

Blastocystis *spp. and Their Effect on the Immune Response*

The role of *Blastocystis* spp. in the intestine is very important since there is a direct interaction between the intestinal epithelium and the underlying immune system (Belkaid and Hand 2015). We found that the immunological effects in individuals without gastrointestinal symptoms colonized by *Blastocystis* spp. displayed lower levels of fecal calprotectin, which is a marker of intestinal inflammation and is derived from the secretion of cytosolic proteins from neutrophils (Walsham and Sherwood 2016), compared to non-colonized individuals. Additionally, individuals colonized with *Blastocystis* spp. show low levels of IgA (the most abundant mucosal antibody) and have a fundamental role in maintaining homeostasis with the microbiome

(Nieves-Ramírez et al. 2018; Gutzeit et al. 2014) by binding and neutralizing invading pathogens and eliminating microbes near the mucus layer (Johansen et al. 1999).

In summary, there are aspects of this parasitosis in the human host that are highly controversial and that, will be resolved as the populations studied increase, both in groups of asymptomatic carriers and in patients with various gastrointestinal pathologies. On the other hand, our results in the population studied, suggest that, colonization by this interesting protist could have a regulatory role of the intestinal bacterial structure, metabolic processes and the immune response that would seem to have a more protective role of intestinal homeostasis than being conditioning of a pathological state.

Compliance with Ethical Standards

Disclosure of Interests All authors declare they have no conflict of interest.

Ethical Approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Approval was granted by the Mexican Commission on Ethics and Research of the Health Ministry of the state of Morelos (Comisiones de Ética y de Investigación del Ministerio de Salud del Estado de Morelos); and the Commission on Ethics in Research of the Facultad de Medicina of the Universidad Nacional Autónoma de México (UNAM) (Comité de Ética de Investigación de la Facultad de Medicina de la Universidad Nacional Autónoma de México).

Informed Consent Informed consent for participation and publication was obtained from all individual participants included in the study.

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