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"PAPEL DE LAS PROTEÍNAS SURFACTANTES EN LA CO-INFECCIÓN DE *Histoplasma capsulatum* Y *Pneumocystis jirovecii* EN PACIENTES CON VIRUS DE LA INMUNODEFICIENCIA HUMANA"

TESIS

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RESUMEN

Histoplasma capsulatum y *Pneumocystis jirovecii* son hongos patógenos que causan enfermedades respiratorias; su co-infección se ha reportado escasamente en pacientes inmunocomprometidos y en mamíferos silvestres. Respuestas inmunes específicas han sido descritas para cada infección, particularmente en modelos animales. Sin embargo, se desconoce la respuesta del huésped cuando están involucrados ambos patógenos. El objetivo de este trabajo fue describir la frecuencia de la co-infección de *H. capsulatum* y *P. jirovecii* en lavados broncoalveolares (BAL) de pacientes internados en un hospital de tercer nivel de la ciudad de México y evaluar la respuesta inmune pulmonar de los pacientes infectados con *H. capsulatum*, *P. jirovecii*, o ambos. La búsqueda de infección por ambos patógenos se realizó en 289 muestras de BAL de pacientes sometidos a una broncoscopia diagnóstica, utilizando la reacción en cadena de la polimerasa (PCR) específica para cada hongo: el marcador Hcp100 para *H. capsulatum* y los marcadores mtLSUrRNA y mtSSUrRNA para *P. jirovecii*. La presencia de estos patógenos se confirmó por el análisis de las secuencias generadas para cada marcador, utilizando los algoritmos BLASTn y neighbor-joining. Se seleccionaron 131 muestras de BAL de los pacientes para analizar las colectinas pulmonares por inmunoensayo enzimático (ELISA) y citocinas por ensayo de citometría con perlas (CBA). Los pacientes se dividieron en ocho grupos en total, con y sin virus de la inmunodeficiencia humana (VIH), infectados con cada uno de los patógenos o ambos, y también se incluyó un grupo de voluntarios sanos (testigos no infectados). Los datos clínicos y de laboratorio de los pacientes se analizaron utilizando un software estadístico; los análisis de las proteínas surfactantes y citocinas se realizaron en un software especializado. Los resultados de las PCR distinguieron tres grupos de pacientes: 60 (20.8%) con histoplasmosis, 45 (15.6%) con pneumocistosis y 12 (4.2%) con co-infección. La prevalencia estimada de co-infección fue mayor en pacientes con VIH. La determinación de proteínas surfactantes y citocinas sustentaron algunos hallazgos novedosos sobre el perfil de moléculas inflamatorias durante las infecciones estudiadas y su co-infección, particularmente en algunos grupos de pacientes con y sin VIH. Igualmente se confirmaron hallazgos previamente descritos en modelos animales o en pacientes, en relación al tipo de respuesta inmune contra los patógenos que causan estas infecciones fúngicas. Se resalta una posible acción inmunomoduladora de *P. jirovecii* cuando ambos parásitos coexisten dentro del mismo huésped. No se encontró una correlación crítica entre la concentración de proteínas surfactantes y la respuesta de citocinas. Sin embargo, el equilibrio de citocinas a lo largo del curso clínico de estas enfermedades fúngicas sigue siendo un campo nuevo para explorar.

ABSTRACT

Histoplasma capsulatum and *Pneumocystis jirovecii* are fungal pathogens that cause respiratory diseases; their coinfection has been scarcely reported in immunocompromised patients and in wild mammals. Specific immune responses have been described for each infection, particularly in animal models. However, the host response to both pathogens in a simultaneous infection is unknown. The aim of this work was to describe the frequency of *H. capsulatum* and *P. jirovecii* coinfection in bronchoalveolar lavages (BAL) from hospitalized patients in a tertiary care hospital in Mexico City, and to evaluate the pulmonary immune response in the patients with *H. capsulatum*, *P. jirovecii*, or their coinfection. Both pathogens' infection was screened in 289 BAL samples from patients who underwent a diagnostic bronchoscopic procedure, using a specific polymerase chain reaction (PCR) for each fungus: the Hcp100 marker for *H. capsulatum*, the mtLSUrRNA and the mtSSUrRNA markers for *P. jirovecii*. The presence of both pathogens was confirmed by the analysis of the generated sequences for each marker, using BLASTn and neighbor-joining algorithms. 131 BAL samples were selected to analyze the pulmonary colectins using enzyme-linked immunosorbent assays (ELISA) and the cytokines using a cytometric bead assay (CBA). Patients were classified in eight groups, with and without human immunodeficiency virus (HIV), infected with each one or both pathogens, and a healthy volunteers group was also included (non-infected controls). The patients' clinical and laboratory data were analyzed using a statistical software; the surfactant proteins and cytokines data were analyzed with a specialized software. PCR results discriminated three groups of patients: 60 (20.8%) with histoplasmosis, 45 (15.6%) with pneumocystosis, and 12 (4.2%) coinfected. The estimated coinfection prevalence was higher in HIV patients. Surfactant proteins and cytokine determination support some new findings on the inflammatory mediator profile during the studied infections and their coinfection, particularly in HIV and non-HIV groups. Moreover, this study confirmed several facts about the immune response to these fungal pathogens, which were previously described on animal models. A possible immunomodulatory activity of *P. jirovecii* was highlighted during the coinfection with *H. capsulatum* in the same host. A critical correlation between the surfactant proteins and the cytokines was not found in this study. However, the cytokine balance through the clinical curse of these fungal infections remains a new field to explore.

CAPÍTULO 1
ANTECEDENTES

- ***Histoplasma capsulatum***

Histoplasma capsulatum es un complejo de especies crípticas, actualmente representado por 11 especies filogenéticas con diferente distribución geográfica (Kasuga et al. 2003; Teixeira et al. 2016), que presenta dos morfotipos: una fase micelial saprobia-geofílica (fase-M, infectiva) y una fase levaduriforme (fase-L, virulenta-parasitaria). Sólo en esta fase se expresan los genes específicos de fase asociados a factores de virulencia. El hongo crece en guano de aves y murciélagos depositado en espacios cerrados o abiertos (cuevas, grutas, patios caseros, etcétera). Los murciélagos son los principales reservorios y dispersores de *H. capsulatum* en la naturaleza, ya que cursan la enfermedad clínica (Taylor et al. 1999), incluso con diseminación. La infección ocurre por inhalación de propágulos fúngicos de fase-M aerosolizados y que se convierten en la fase-L parasitaria en el huésped infectado, en el interior de células fagocíticas de vías respiratorias altas y bajas durante las primeras horas de la infección (Sahaza et al. 2014; Suárez-Álvarez et al. 2019). La fase-L tiene predilección por las células del sistema fagocítico mononuclear, aunque es preferentemente procesada por células dendríticas y también puede ser fagocitada por polimorfonucleares y por células epiteliales. *H. capsulatum* es un parásito intracelular facultativo y en la mayoría de los casos, las levaduras pueden ser destruidas por fagocitos del huésped; sin embargo, este hongo ha desarrollado varias estrategias para evadir los mecanismos citocidas de los fagocitos (Maniscalchi-Badaoui et al. 2006; Taylor y Duarte 1995).

Histoplasma capsulatum es el agente causal de la histoplasmosis, una de las micosis sistémicas más importantes que afecta a humanos y otros mamíferos; es un patógeno primario clase 3 que, ocasionalmente, puede actuar como oportunista (Casadevall y Pirofski 1999). *H. capsulatum* es de amplia distribución mundial y ha sido aislado en más de 25 países en 5 continentes (Kasuga et al. 2003); tiene preferencia por áreas tropicales y subtropicales, sin embargo, se ha documentado su presencia en el ambiente del continente europeo (González-González et al. 2013). Se han descrito brotes autóctonos de histoplasmosis en latitudes extremas, entre los paralelos 54° Norte (Alberta, Canadá) (Anderson et al. 2006) y 38° Sur (Neuquén, Patagonia argentina) (Calanni et al. 2013), que apoyan la amplia dispersión geográfica del patógeno. La enfermedad es endémica en varias regiones de América y la forma epidémica de la histoplasmosis tiene gran impacto en regiones del mundo que exponen poblaciones humanas a alto riesgo de infección, como ocurre en la mayoría de los países latinoamericanos y algunos

asiáticos (Tewari et al. 1998). En México, los trabajadores rurales pueden desarrollar esta forma de la enfermedad por su actividad ocupacional asociada a la minería, al manejo de aves de corral y a la recolección de guano para uso como fertilizante; asimismo, los profesionistas que ejercen actividades laborales relacionadas a la arqueología, geología y espeleología también tienen riesgo de adquirir la forma epidémica de la enfermedad. En la actualidad, la histoplasmosis ha adquirido mayor importancia debido a que el hongo, en ocasiones especiales, puede comportarse como oportunista en individuos inmunocomprometidos, como los pacientes con neoplasias, trasplantes, tratamientos inmunosupresores y particularmente los pacientes con Virus de la Inmunodeficiencia Humana (VIH)/sida, aunque es necesario enfatizar que este hongo no coloniza el hospedero como lo hacen la mayoría de los microorganismos oportunistas (Casadevall y Pirofski 1999). La presentación clínica y la gravedad de la histoplasmosis dependen de las condiciones inmunológicas del huésped, del tamaño del inóculo, de la virulencia y de la especie filogenética de la cepa fúngica (Sahaza et al. 2015; Sepúlveda et al. 2014; Suárez-Álvarez et al. 2019).

- ***Pneumocystis jirovecii***

Las especies de *Pneumocystis* son hongos oportunistas específicos para cada huésped, característica conocida como estenoxenismo (Demanche et al. 2001; Gigiliotti et al. 1993). Actualmente, sólo se reconocen cinco especies que cumplen con las normas del Código Internacional de Nomenclatura Botánica: *P. carinii* y *P. wakefieldiae* en ratas, *P. murina* en ratones, *P. oryctolagi* en conejos y *P. jirovecii* en humanos (Aliouat-Denis et al. 2008). Se ha demostrado co-evolución entre algunos huéspedes mamíferos y sus respectivas especies de *Pneumocystis* (Akbar et al. 2012; Aliouat-Denis et al. 2008; Demanche et al. 2019; Derouiche et al. 2009). El estudio de la morfología, fisiología y genética, así como del ciclo de vida de este hongo ha sido limitado por su incapacidad para crecer en medios de cultivo artificiales (Akbar et al. 2012; Gigliotti y Wright 2012); sin embargo, se ha propuesto un ciclo de vida hipotético donde *Pneumocystis* sp. presenta dos morfotipos, la asca (forma quística) y la forma trófica, donde las primeras son las responsables de la transmisión de la enfermedad de un individuo a otro por vía aérea y contienen ocho formas tróficas, que representan las ascosporas liberadas de las ascas y se adhieren a los neumocitos tipo I del huésped infectado (Aliouat-Denis et al. 2009).

En humanos, se ha descrito un gran número de casos de neumonía intersticial por *P. jirovecii* desde que en 1981 surgió la pandemia de sida y, actualmente, a pesar de la eficacia de la terapia antirretroviral es la principal causa de mortalidad por hongos en pacientes infectados con VIH. La pneumocistosis tiene una amplia distribución mundial y también se diagnostica en pacientes con otras

inmunodeficiencias. Asimismo, la presencia de *P. jirovecii* se ha asociado con exacerbaciones infecciosas de pacientes con enfermedad pulmonar obstructiva crónica y en infecciones asintomáticas de huéspedes inmunocompetentes. Se ha detectado *Pneumocystis* colonizando vías respiratorias, lo que sugiere que los portadores asintomáticos pueden ser la fuente de infección para los individuos inmunocomprometidos (Morris y Norris 2012).

- **¿Qué semejanzas y qué diferencias tienen estos dos hongos?**

Es interesante comparar estos hongos patógenos, ya que se ha visto que pueden infectar al mismo individuo en la naturaleza. Sin embargo, a pesar de que *Histoplasma* y *Pneumocystis* comparten algunas características comunes, las características diferenciales entre ambos patógenos marcan la patogenia y el comportamiento de las enfermedades asociadas.

Características comunes	
Phylum: *Ascomycota*	
Vía de entrada: respiratoria Órgano blanco primario: pulmón	
Forma clínica más frecuente: Pulmonar	
Características diferenciales	
H. capsulatum	*P. jirovecii*
Preferentemente intracelular	Preferentemente extracelular
Una especie infecta diferentes huéspedes mamíferos	Una especie es específica para cada huésped mamífero (estenoxenismo)
Otras formas clínicas asociadas: Diseminada, con manifestaciones en diferentes órganos Crónica	Otras formas clínicas asociadas: Diseminada
No se transmite de un individuo a otro	Se transmite de un individuo a otro
No coloniza la vía aérea	Coloniza la vía aérea superior
Reservorios y dispersores mamíferos	El humano es el reservorio y dispersor
Presencia en la naturaleza: saprobio-geofílico	No se conoce

- **Co-infección de *H. capsulatum* y *Pneumocystis* sp**.

La co-infección pulmonar de *H. capsulatum* y *P. jirovecii* se ha descrito en varios estudios, la mayoría en pacientes con VIH en condición clínica de sida, con una frecuencia estimada entre 0.55 y 13.3% (Almeida-Silva et al. 2016; Baughman et al. 1994; Caceres et al. 2018; Carreto-Binaghi et al. 2019; Gago et al. 2014; Huber et al. 2008; Le Gal et al. 2013; Samayoa et al. 2017; Velásquez et al. 2010; Wahab et al. 2018; Wheat et al. 1985). Ambos patógenos pueden definir la condición de sida en pacientes con un estadio clínico C3, correspondiente a un conteo de $CD4^+$ <200 células/mL. Aunque estos hongos pueden estar asociados a otras inmunosupresiones, la asociación con sida es la más frecuentemente encontrada. Particularmente, se ha reportado que la incidencia de pneumocistosis es de 10 casos por 100 personas/año, siendo 50% de ellos infectados por VIH. Asimismo, se ha descrito que 75% de los pacientes con sida desarrollan un evento de neumonía por *P. jirovecii* durante su vida, por lo que este grupo de pacientes es el más indicado para el estudio de *P. jirovecii*, aún con el inconveniente de su estado inmunológico alterado.

La relevancia de la co-infección fue recientemente analizada por Nevez y LeGal (2019), quienes consideraron que la frecuencia de esta co-infección podría ser mayor que la reportada, ya sea por omisión inadvertida de la búsqueda de ambos patógenos al mismo tiempo y, principalmente, por la baja sensibilidad de los métodos diagnósticos utilizados, en particular para *P. jirovecii* que no es cultivable y su diagnóstico de laboratorio se fundamenta en tinciones que pueden conducir a errores de interpretación. Algunos de los estudios que abordaron la co-infección de *H. capsulatum* y *P. jirovecii* utilizaron métodos moleculares que mejoran la sensibilidad y especificidad diagnóstica para detectar ambas infecciones, tanto en muestras de lavado broncoalveolar (BAL) como en biopsias humanas (Almeida-Silva et al. 2016; Carreto-Binaghi et al. 2019; Gago et al. 2014) o en murciélagos naturalmente infectados (González-González et al. 2014).

- **Proteínas surfactantes**

El surfactante pulmonar es un fluido cuya composición está conformada por 10% de proteínas surfactantes y 90% de lípidos; ambos son producidos por los neumocitos tipo II. Existen cuatro tipos de proteínas surfactantes: SP-A, SP-B, SP-C y SP-D, de las cuales SP-A y SP-D son moléculas hidrosolubles y de gran tamaño, mientras que SP-B y SP-C son pequeñas y muy hidrofóbicas (Han y Mallampalli 2015; Johansson et al. 1994).

SP-A es un polipéptido de 18-mer y 26 kDa, unida a oligosacáridos; estructuralmente puede caracterizarse como un híbrido de colágeno con un dominio tipo lectina. Ésta participa en la conversión del surfactante endógeno (secretado en el lecho alveolar) en mielina tubular. SP-B es un polipéptido de 79

residuos que contiene tres puentes disulfuro intracatenarios; existe principalmente como homodímero con fuerte carga positiva y puede remover selectivamente lípidos aniónicos e insaturados de la superficie alveolar, incrementando la presión de la misma (Chroneos et al. 2010; Johansson et al. 1994). SP-C es un polipéptido hidrofóbico que contiene 35 residuos de aminoácidos y grupos palmitoil unidos por enlaces covalentes. Su porción de α-hélice está insertada en la bicapa lipídica del surfactante, acelerando la adsorción de las bicapas lipídicas de las membranas celulares de las células del epitelio pulmonar formando una monocapa de surfactante (Chroneos et al. 2010; Johansson et al. 1994). SP-D es un polipéptido de 12-mer y 39 kDa, formado por una glicoproteína de colágeno con estructura parecida a las lectinas tipo C (Chroneos et al. 2010; Johansson et al. 1994).

Entre las proteínas surfactantes, SP-A y SP-D (conocidas como colectinas) son las únicas que actúan en la defensa del huésped como moléculas integrantes de la respuesta inmune innata; se unen a diversos patógenos mediante sus dominios de reconocimiento de carbohidratos, dañan sus membranas y regulan la fagocitosis de los mismos, estimulando a los macrófagos alveolares (Chroneos et al. 2010; Hagwood y Clements 1990; Johansson et al. 1994). SP-A tiene preferencia para unirse a manosa y fucosa, mientras SP-D se une a maltosa, glucosa y manosa; ambas también pueden unirse a carbohidratos complejos en las superficies celulares (Hagwood y Clements 1990).

Las proteínas SP-A y SP-D opsonizan virus como herpes simple e influenza y también bacterias Grampositivas y Gramnegativas, favoreciendo la fagocitosis de éstos. Sin embargo, sólo SP-A incrementa la fagocitosis y la muerte bacteriana, destacando el incremento de la adherencia de micobacterias y su subsecuente fagocitosis, mecanismo que parece tener relación con la expresión del receptor de manosa en los macrófagos (Crouch 2000; Hagwood y Clements 1990; Mason et al. 1998). Se conocen algunas interacciones entre estas colectinas y patógenos pulmonares, ya que SP-A y SP-D regulan la producción de citocinas y radicales libres, promoviendo un estado pro o antiinflamatorio (Nayak et al. 2012; Pastva et al. 2007). Por ejemplo, ambas colectinas inhiben la proliferación de células T; SP-A inhibe la maduración de células dendríticas y SP-D estimula la presentación de antígenos por estas mismas células (Haagsman et al. 2008). Además, SP-A y SP-D poseen mecanismos bactericidas, como la inhibición competitiva de la unión del lipopolisacárido (LPS) de bacterias Gramnegativas a su proteína de unión en la membrana externa de la bacteria (Stamme et al. 2002). Gardai et al. (2003) demostraron un aumento de mediadores proinflamatorios en la respuesta inmune innata y adaptativa, a través de la inducción de factores nucleares de transcripción (NF-kB y AP-1) utilizando macrófagos estimulados con LPS y la subsecuente unión de éstos con la región de colágena de SP-A o SP-D; sin embargo, cuando la unión de estas colectinas con los macrófagos se llevó a cabo a través de sus cabezas globulares, se generó un efecto antiinflamatorio mediante la proteína

reguladora SIRPα. Esta función inflamatoria "dual" de estas surfactantes pulmonares participa en los mecanismos inmunológicos que se desencadenan en respuesta a distintos microorganismos (Carreto-Binaghi et al. 2016).
La interacción de las proteínas surfactantes con los receptores tipo Toll (TLR, del inglés Toll-like receptor) y sus moléculas asociadas, como CD14, puede ser un mecanismo para su función de mediador inflamatorio (Pastva et al. 2007). Murakami et al. (2002) demostraron que SP-A se une al dominio extracelular de TLR-2, impidiendo la unión de peptidoglicanos bacterianos a este receptor y, en consecuencia, evitando la activación de NF-kB y la producción de TNF-α, lo cual resulta en una respuesta inflamatoria disminuida. Asimismo, cuando SP-A se asocia con MD-2, una molécula de señalización accesoria de TLR-4, inhibe la unión de LPS bacterianos, favoreciendo la presencia de un evento antiinflamatorio que se ha descrito *in vivo* en pacientes con sepsis neonatal (Haagsman et al. 2008). Asimismo, SP-D a través de su dominio de reconocimiento de carbohidratos también se une a los dominios extracelulares de TLR-2 y TLR-4 (Ohya et al. 2006).
SP-A regula la función de los macrófagos al disminuir la actividad de las cinasas requeridas para la producción de citocinas proinflamatorias; igualmente, "upregulate" la expresión del receptor de IL-1β y "downregulate" TLR-4, e inhibe la producción de TNF-α e IL-6 en respuesta a LPS (Henning et al. 2008; Ketko et al. 2013; Nguyen et al. 2012).
Con respecto a SP-D, puede antagonizar los efectos proinflamatorios de TNF-α en macrófagos y células dendríticas, inhibiendo la producción de esta citocina. Por el contrario, TNF-α incrementa indirectamente la producción de SP-D mediante la inducción de IL-13, lo que se confirma en ratones deficientes de esta citocina. (Hortobágyi et al. 2008).

- **Interacción de las proteínas surfactantes A y D con *H. capsulatum* y *P. jirovecii***

La unión de SP-A y SP-D a una gran variedad de hongos patógenos de vías respiratorias puede resultar en la inhibición directa del crecimiento fúngico y en el favorecimiento de la fagocitosis para su eliminación. Sin embargo, la respuesta inmune activada por estas proteínas surfactantes puede jugar un papel en la patogénesis de la enfermedad asociada.
Datos interesantes con respecto a algunas infecciones micóticas revelan que SP-A y SP-D se unen a *Cryptococcus neoformans* acapsular, pero no al hongo capsulado. SP-D aglutina las levaduras acapsulares de este hongo y muestra una unión más fuerte que SP-A. También se ha descrito que en la infección de ratones knockout para SP-D con *C. neoformans*, éstos desarrollaron menor infiltrado de eosinófilos y niveles más bajos de IL-5 en BAL (Holmer et al. 2014).

Asimismo, SP-A y SP-D se adhieren a las conidias de *Aspergillus fumigatus*, reacción mediada por calcio e inhibida por manosa o maltosa, que incrementa la fagocitosis y subsecuente destrucción del hongo por los macrófagos (Crouch 2000; Hagwood y Clements 1990; Mason et al. 1998).

En cuanto a *H. capsulatum*, pocos estudios han descrito la interacción entre el hongo y las proteínas surfactantes. McCormack et al. (2003) demostraron que la exposición de levaduras de *H. capsulatum* a SP-A y SP-D estimuló una disminución de la incorporación de leucina tritiada, dependiente de la dosis, revelando una incapacidad del hongo para crecer en un medio de cultivo suplementado, debido a un incremento en la permeabilidad de la membrana celular de las levaduras causado por cambios conformacionales en sus proteínas expuestas. Sin embargo, interesantemente, SP-A y SP-D no inhibieron el crecimiento de levaduras de *H. capsulatum* internalizadas en los macrófagos (Brummer y Stevens 2010; McCormack et al. 2003). Además, este mismo estudio también demostró que ninguna de las colectinas agregó levaduras de *H. capsulatum*, ni modificó la fagocitosis de las mismas. No obstante, los ratones knockout para SP-A fueron más susceptibles a la infección que los ratones del grupo control, posiblemente asociado a una disminución en el número de células T $CD8^+$ pulmonares y esplénicas en los ratones knockout (McCormack et al. 2003). Los escasos estudios realizados de interacción de *H. capsulatum* con colectinas sólo utilizaron la fase-L del hongo y no contemplaron la fase-M infectiva presente en el primer contacto con la respuesta de defensa del huésped, siendo esto un campo abierto para estudios de estas moléculas tan importantes en la primera fase de la defensa mediada por la respuesta inmune innata a patógenos respiratorios.

Con respecto a *Pneumocystis*, su glicoproteína mayor de superficie (MSG, anteriormente conocida como gp120) rica en manosa, representa una de las proteínas más abundantes de la membrana de este hongo y se conoce que, en las formas tróficas, SP-A y SP-D se unen a MSG por un mecanismo dependiente de calcio, mientras que en las ascas estas colectinas pueden unirse al β-glucano de la pared celular, pero ninguna de estas proteínas surfactantes promueve la fagocitosis del patógeno (Vuk-Pavlovic et al. 2001; Zimmerman et al. 1992). Algunos estudios han demostrado incremento de la producción de SP-A durante la infección con *Pneumocystis*, mediante cuantificación de la proteína y su RNAm (Phelps et al. 1996, Qu et al. 2001).

- **Interacción de citocinas con *H. capsulatum* y *P. jirovecii***

La resolución de la infección por *H. capsulatum* requiere de la participación de células y moléculas efectoras del huésped. En el huésped humano, la respuesta

inmune innata en el proceso infeccioso por *H. capsulatum* ha sido poco explorada; la mayoría de los estudios se han realizado en el modelo murino y se considera que sus actores celulares y moleculares pueden detener la infección en sus primeras etapas (Sahaza et al. 2014). Sin embargo, una vez establecida la infección, los macrófagos, las células dendríticas y los linfocitos T son los principales actores celulares esenciales que actúan en armonía con efectores generados por mecanismos de señalizaciones resultantes de las interacciones de moléculas de superficie del patógeno (PAMPs, del inglés pathogen-associated molecular patterns) con receptores de reconocimiento presentes en las células del huésped (PRRs, del inglés pattern recognition receptors) (Deepe y Gibbons 2009). Los productos de estos eventos son, en general, mediadores químicos de la respuesta inmune y células activadas.

La respuesta inmune protectora desarrollada durante la histoplasmosis experimental y en la adquirida por infección natural es la adaptativa, mediada primordialmente por linfocitos T $CD4^+$, aunque se han comprometido los linfocitos T $CD8^+$ (Deepe y Gibbons 2006). La respuesta Th1 en modelos murinos se caracteriza por la producción de las citocinas IL-12, IFN-γ y TNF-α que son esenciales para la activación de macrófagos, las principales células encargadas de eliminar las levaduras intracelulares (Allendoerfer y Deepe 1998; Allendoerfer et al. 1997; Cain y Deepe 1998; Clemons et al. 2000; Gildea et al. 2001; Huffnagle y Deepe 2003; Newman 1999; Zhou et al. 1995).

La respuesta Th2 caracterizada por la producción de IL-4, IL-5, IL-10 e IL-13 se asocia con una exacerbación de la enfermedad y modula la severidad de la infección. Sin embargo, en la mayoría de los hongos patógenos de humanos, la respuesta Th2 es importante en la regulación y/o equilibrio de la inmunidad mediada por Th1 (Mencacci et al. 1998). Por lo que, en la histoplasmosis, para una buena resolución de la enfermedad es crucial el balance Th1/Th2 ya que la exacerbación de Th1 o la tendencia favorable hacia Th2 puede intervenir en la etiopatogénesis de la enfermedad.

En los últimos años, se ha dado importancia a un grupo especial de células T colaboradoras (Th17), las cuales inician su participación en la interfase innata-adaptativa de la respuesta inmune contra una gama de patógenos, principalmente, por la producción de IL-17 e IL-22, bajo la inducción de IL-23. Deepe y Gibbons (2009) reportaron que IL-17 podría ser necesaria para la eliminación de *H. capsulatum*, puesto que se detectó una sobrerregulación de ésta en pulmón, durante la infección aguda de ratones C57BL/6 inoculados intranasalmente con la fase-L del hongo; experimentos adicionales revelaron que IL-23 podría prolongar la supervivencia de los ratones en la ausencia de la respuesta protectora mediada por IL-12. Este efecto, fue dependiente de IL-17, lo que sugiere que el eje IL-17/IL-23 también favorece la inmunidad protectora durante la infección con *H. capsulatum* (Deepe y Gibbons 2009).

Es conocido que la infección de los macrófagos por *H. capsulatum* induce la formación de granulomas en diferentes tejidos. Como ocurre en otras infecciones, la mayoría de las veces los granulomas son necesarios para contener el crecimiento del hongo, prevenir la diseminación sistémica y proteger los órganos del daño tisular inflamatorio generalizado (Mukhopadhyay y Gal 2010), aunque en ocasiones pueden generar reacciones adversas asociadas a necrosis tisular con o sin facilitación de la persistencia del parásito en el huésped (Heninger et al. 2006). Se ha demostrado que existen diferencias en el infiltrado inflamatorio y la formación de granuloma según el tejido afectado, así como en el perfil de citocinas expresado durante la infección, que variaron con la virulencia y la especie filogenética de la cepa utilizada cuando se inoculan ratones con la fase M o L de *H. capsulatum* (Berry 1969a; 1969b; 1969c; Durkin et al. 2004; Heninger et al. 2006; Sahaza et al. 2015; Sepúlveda et al. 2014).

En la infección por *Pneumocystis* spp. las formas tróficas, que se caracterizan por la ausencia de pared celular, generan una débil activación de células dendríticas y una subsecuente disminución de la proliferación y polarización de células T $CD4^+$ con menor producción de IFN-γ, IL-13 e IL-17A. Las formas tróficas además suprimen la respuesta proinflamatoria inducida por las ascas (Evans et al. 2017). Los macrófagos alveolares, las células dendríticas y las células epiteliales pulmonares (neumocitos) reconocen el β-glucano de la pared celular de las ascas, iniciando la respuesta de linfocitos T $CD4^+$ (Evans et al. 2016).

Recientemente se ha descrito a los macrófagos como los principales responsables de la eliminación de *Pneumocystis*, aunque una respuesta adecuada depende del tipo de macrófagos. Los macrófagos clásicos (M1) que predominan en ausencia de un sistema inmune competente no son eficientes en la eliminación de *P. carinii* en la infección de ratas inmunosuprimidas; en contraste, en ratas inmunocompetentes que tienen una mayor proporción de macrófagos alternativos (M2), se observó una mejor eliminación del patógeno (Deckman et al 2017; Nandakumar et al. 2017).

Se ha demostrado que *Pneumocystis* puede inducir respuestas de células tipo Th1, Th2, Th17 y T reguladoras (Treg), aunque ninguno de estos subtipos celulares se ha asociado con una mejor resolución de la enfermedad (Kelly y Shellito 2010). La pérdida de células T $CD4^+$ que ocurre en algunos padecimientos como la infección por VIH, es crítica para una buena respuesta inmune adaptativa contra *Pneumocystis*. En modelos murinos con inmunodeficiencias, modificaciones en la función de células T $CD4^+$ llevan a la alteración de moléculas coactivadoras, como CD2 y CD28, necesarias para el montaje óptimo de la respuesta de defensa adaptativa (Kelly y Shellito 2010). Se ha relacionado la respuesta Th1 con la disminución en la intensidad de la infección por *P. carinii* en ratas Harlan Sprague-Dawley inmunosuprimidas con corticosteroides, asociada a la producción de TNF-α por los macrófagos después del tratamiento de los animales infectados con IFN-

y aerosolizado (Thomas y Limper 2004). En los pacientes que utilizan fármacos anti-TNF, la pneumocistosis es la infección fúngica más frecuente, confirmando los hallazgos de los modelos murinos; asimismo, en un modelo de macacos inmunizados con una proteína recombinante de *P. jirovecii*, la protección estuvo relacionada con un incremento de las células tipo Th1 de sangre periférica (Kling y Norris 2016).

La participación de Th17 en la infección por *Pneumocystis* se asocia con eliminación del patógeno, lo que se ha descrito con *P. murina* en ratones deficientes de IL-23 (Rudner et al. 2007).

Con respecto a la respuesta tipo Th2, la IL-5, en conjunto con los eosinófilos, aumentan la eliminación temprana de *P. murina*, cuando la IL-5 se administra de manera exógena (Eddens et al. 2015). Recientemente se ha visto que *Pneumocystis* genera una respuesta inflamatoria exagerada cuando se estimula con IL-13 e IL-17A, debido a que se combina una respuesta Th2 con Th17, lo cual se presenta en pacientes con patología pulmonar crónica colonizados con este patógeno (Eddens et al. 2017).

- **Interacción de las proteínas surfactantes A y D con citocinas en modelos fúngicos**

Las proteínas surfactantes, como parte de la respuesta inmune innata, interactúan con células y citocinas en el proceso de inflamación. Esto se ha descrito en varios escenarios, por ejemplo en la inmunorregulación del embarazo (Nayak et al. 2012), rechazo de transplantes de pulmón (D'Ovidio et al. 2013), enfermedades inflamatorias crónicas como artritis reumatoide (Nayak et al. 2012) y asma (Wang et al. 2011), eliminación de células apoptóticas pulmonares (Nayak et al. 2012), y en los cuadros clínicos producidos por alérgenos de polen y de *A. fumigatus*, entre otros (Nayak et al. 2012). En modelos infecciosos de etiología fúngica, esta interacción ha sido poco considerada; algunas excepciones fueron citadas en el subtítulo de "Interacción de SP-A y SP-D con *H. capsulatum* y *P. jirovecii*". Sin embargo, en los modelos infecciosos de la presente tesis, es la primera vez que se aborda este tema, por lo que los resultados obtenidos se encuentran en las publicaciones generadas por la tesis y en algunos aspectos de la discusión.

PLANTEAMIENTO DEL PROBLEMA Y PREGUNTA DE INVESTIGACIÓN

Las infecciones respiratorias por *H. capsulatum* y *P. jirovecii* comparten varias características, lo que hace posible su coexistencia en un mismo paciente. Dada la poca información de la co-infección de ambos patógenos, se justifica su búsqueda en humanos.

En el microambiente pulmonar, las proteínas surfactantes SP-A y SP-D son las principales moléculas de la respuesta inmune innata que participan en la interacción con patógenos respiratorios.

¿Cómo se encuentran SP-A y SP-D y la respuesta subsecuente de citocinas asociada al microambiente pulmonar cuando se presenta la infección por uno u otro hongo o la co-infección por ambos?

Para responder esta pregunta, el presente estudio analizó la producción de SP-A y SP-D y citocinas proinflamatorias en el microambiente pulmonar de pacientes infectados con *H. capsulatum* y *P. jirovecii*. Para su desarrollo, este trabajo contempló diferentes grupos de pacientes hospitalizados a causa de infecciones respiratorias.

CAPÍTULO 2
HIPÓTESIS

En pacientes con co-infección de *H. capsulatum* y *P. jirovecii* la producción de proteínas surfactantes SP-A y SP-D puede alterar la producción de citocinas proinflamatorias de la respuesta antifúngica para estos patógenos.

OBJETIVO GENERAL

Determinar las proteínas surfactantes SP-A y SP-D en muestras de lavado broncoalveolar (BAL) en los grupos de pacientes hospitalizados a causa de infecciones respiratorias, con infección de *H. capsulatum* y *P. jirovecii* o la co-infección de ambos y su efecto en la producción de citocinas proinflamatorias.

OBJETIVOS PARTICULARES

1. Detectar la frecuencia de las infecciones por *H. capsulatum* y *P. jirovecii*, así como la co-infección, por medio de reacciones en cadena de la polimerasa (PCR, del inglés Polymerase Chain Reaction) específicas para cada patógeno
2. Cuantificar SP-A y SP-D en BAL por medio de inmunoensayos enzimáticos (ELISA, del inglés Enzime-Linked Immunosorbent Assay)
3. Cuantificar citocinas proinflamatorias en BAL, utilizando ensayo de citometría con perlas cubiertas con anticuerpos específicos para cada una (CBA, del inglés Cytometric Bead Assay)
4. Analizar y correlacionar los datos de SP-A y SP-D con los de citocinas

CAPÍTULO 3
MATERIAL Y MÉTODOS

- **Muestras de estudio**

Se utilizaron muestras de BAL de pacientes con infección respiratoria aguda (neumonía hipoxémica) que requirieron broncoscopia diagnóstica, hospitalizados en el Instituto Nacional de Enfermedades Respiratorias (INER), bajo los siguientes criterios:

- ✓ Inclusión: Muestras tomadas directamente durante la broncoscopia, transportadas en hielo al Laboratorio de Microbiología Clínica del INER, donde fueron centrifugadas inmediatamente a 3700 rpm por 20 min y separadas en cuatro alícuotas de 600 µl, que se congelaron -80°C en el menor tiempo posible (<1h).
- ✓ Eliminación: muestras con <40% de recuperación de líquido de BAL, durante el procedimiento.

Se recolectaron muestras de pacientes durante dos periodos de tiempo distintos y como testigo se utilizaron muestras de BAL de personas sanas recolectadas en el año 2011 (donadas por el grupo del Dr. Eduardo Sada, Departamento de Investigación en Microbiología del INER).

Los pacientes se clasificaron en ocho grupos:

1. Individuos sanos
2. Pacientes con VIH, no infectados por los hongos estudiados
3. Pacientes con VIH, infectados con *H. capsulatum*
4. Pacientes con VIH, infectados con *P. jirovecii*
5. Pacientes con VIH, co-infectados por ambos hongos
6. Pacientes sin VIH, infectados con *H. capsulatum*
7. Pacientes sin VIH, infectados con *P. jirovecii*
8. Pacientes sin VIH, co-infectados por ambos hongos

Considerando que la presencia de *P. jirovecii* es más frecuente en sujetos inmunosuprimidos, entre los grupos de pacientes con VIH se seleccionaron aquellos en estadio clínico C3.

- **Consideraciones éticas**

El presente estudio se rigió por la Declaración de Helsinki enmendada de su original (1964) en la 64ª Asamblea General de la Asociación Médica Mundial que tuvo lugar en Fortaleza, Brasil (Asociación Médica Mundial 2013), donde se establecieron los principios éticos para las investigaciones médicas en seres humanos; de igual manera, esta investigación se ajustó a los reglamentos

nacionales, conformados por el Reglamento de la Ley General de Salud en Materia de Investigación para la Salud, publicado en el Diario Oficial de la Federación el 06 de enero de 1987.

Se tomaron toda clase de precauciones para proteger la vida, la salud, la dignidad, la integridad, la intimidad y la confidencialidad de la información personal de los pacientes que participaron en el estudio, para reducir al mínimo las consecuencias del mismo sobre su integridad física, mental y social. Se utilizaron las muestras de BAL de pacientes a quienes se realizó un procedimiento diagnóstico de rutina y que hayan sido enviadas al Laboratorio de Microbiología Clínica del INER; no se tomaron muestras *ex profeso* para este estudio.

No se utilizaron animales de experimentación ni se dañó el medio ambiente al realizar esta investigación. No existen conflictos de interés relacionados con la misma.

Se considera una investigación que emplea técnicas y métodos de investigación que no modificaron de manera mal intencionada las variables fisiológicas, psicológicas y sociales de los individuos que participaron en el estudio.

El beneficio para los pacientes fue un diagnóstico más rápido de las infecciones por los hongos *H. capsulatum* y *P. jirovecii*, en comparación con los métodos convencionales (cultivo, tinciones). Asimismo, la reacción en cadena de la polimerasa posee alta sensibilidad y especificidad para el diagnóstico microbiológico.

El proyecto fue aprobado por las Comisiones de Investigación y Ética de la Facultad de Medicina de la UNAM (dictamen 132/2015) y por el Comité de Ética en Investigación del INER (protocolo B13-14).

- **Tamaño de la muestra**

Se realizó un muestreo por conveniencia, de acuerdo con el número de muestras de BAL que llegaron al Laboratorio de Microbiología Clínica en el periodo de estudio. Se estimó el tamaño de muestra requerido, de acuerdo con la siguiente fórmula:

$n = (Z\alpha^2 * p * q) / d^2$

donde:

$Z\alpha^2 = 1.962$ (la seguridad se estima del 95%)

p = proporción esperada, de acuerdo con un estudio previo (Velásquez et al. 2010) en pacientes con VIH donde se ha encontrado una co-infección de *H. capsulatum* y *P. jirovecii* de 11.4% = 0.114

q = 1 – p (1 – 0.114 = 0.886)

d = precisión deseada (3%)

Considerando que durante el año 2013 el Laboratorio de Microbiología Clínica del INER recibió 3474 muestras de BAL, el tamaño de muestra total para este estudio, según la fórmula, fue de 384 muestras por año.
Las muestras de los pacientes recolectadas en los dos periodos de tiempo del estudio permitieron mejorar la confianza del análisis estadístico. Para evitar sesgos, se realizó un análisis entre los datos de las muestras de la primera colecta y los de la segunda colecta, utilizando la prueba de Mann-Whitney (P = 0.3376), que no reveló diferencias estadísticamente significativas entre los datos y permitió analizar al total de muestras como una población única.

- **Obtención de DNA de muestras de BAL**

La extracción de DNA se realizó con el kit comercial "DNeasy Blood & Tissue Kit" (Qiagen, Valencia, CA), según lo descrito en Carreto-Binaghi et al. (2019).

- **Diagnóstico molecular de *H. capsulatum* en muestras de BAL**

Las referencias de base para determinar la presencia del patógeno en las muestras estudiadas vienen mencionadas en Carreto-Binaghi et al. (2019). Se consideró que existe presencia del patógeno en las muestras procesadas al obtener la secuencia del marcador. La descripción del método molecular de diagnóstico específico para *H. capsulatum* se detalla a continuación:

PCR anidada para el fragmento del gen que codifica para la proteína Hcp100
Se utilizaron dos pares de iniciadores: los externos, HcI (5'-GCGTTCCGAGCCTTCCACCTCAAC-3') y HcII (5'-ATGTCCCATCGGGCGCCGTGTAGT-3') y los internos, HcIII (5'-GAGATCTAGTCGCGGCCAGGTTCA-3') y HcIV (5'-AGGAGAGAACTGTATCGGTGGCTTG-3'). Estos últimos delimitan una secuencia específica de 210 pb, única de *H. capsulatum*.
Mezcla de reacción de la primera PCR: 200 mM de dNTPs, 2 mM $MgCl_2$, 100 pM de cada iniciador externo, 1 U Taq DNA polimerasa y 2 µL de DNA. Condiciones de ciclado: un ciclo a 94 °C por 5 min; 35 ciclos a 94 °C por 30 s, 50 °C por 30 s y 72 °C por 1 min; un ciclo a 72 °C por 5 min.
Mezcla de reacción de la segunda PCR (anidada): 200 mM dNTPs, 2.0 mM $MgCl_2$, 100 pM de cada iniciador interno, 1 U Taq DNA polimerasa y 1 µL del producto de la primera reacción. Condiciones de ciclado: un ciclo a 94 °C por 5 min; 30 ciclos a 94 °C por 30 s, 65 °C por 30 s y 72 °C por 1 min; un ciclo a 72 °C por 5 min.
Como testigo positivo, se utilizó el DNA extraído de la cepa de referencia EH-53 de H*. capsulatum* perteneciente al Laboratorio de Inmunología de Hongos,

Departamento de Microbiología y Parasitología, Facultad de Medicina, UNAM. Como testigo negativo, agua Milli-Q.

- **Diagnóstico molecular de *P. jirovecii* en muestras de BAL**

Para determinar la presencia de *P. jirovecii* en las muestras estudiadas también se utilizó PCR anidada para dos marcadores mitocondriales de *Pneumocystis* según lo descrito por Carreto-Binaghi et al. (2019). Se consideró que existe presencia del patógeno en las muestras procesadas al obtener la secuencia de al menos uno de los dos marcadores utilizados. Enseguida se detallan los procedimientos para amplificar los marcadores moleculares que facilitan el diagnóstico confiable de *Pneumocystis* spp.

PCR anidada para el gen de mtLSUrRNA

Se utilizaron dos pares de iniciadores: los externos, pAZ102H (5'-GTGTACGTTGCAAAGTACTC-3') y pAZ102E (5'-GATGGCTGTTTCCAAGCCCA-3') y los internos, pAZ102X (5'-GTGAAATACAAATCGGACTAGG-3') y pAZ102Y (5'-TCACTTAATATTAATTGGGGAGC-3'). Estos últimos iniciadores delimitan una secuencia específica de 267 pb.

Mezcla de la primera PCR: 10 mM de cada desoxinucleósido trifosfato (dNTPs), 25 mM de cada iniciador externo, 0.5 U Taq DNA polimerasa y 2.5 µL de DNA. Condiciones de ciclado: un ciclo a 94 °C por 2 min; 30 ciclos a 94 °C por 30 s, 50 °C por 1 min y 65 °C por 1 min.

Mezcla para la segunda PCR (anidada): 20 mM dNTPs, 50 mM de cada iniciador interno, 1 U Taq DNA polimerasa y 2.5 µL del producto de la primera reacción. Condiciones de ciclado: un ciclo a 94 °C por 2 min; 30 ciclos a 94 °C por 30 s, 55 °C por 1 min y 72 °C por 1 min.

PCR anidada para el gen de mtSSUrRNA. Se utilizaron dos pares de iniciadores: los externos, pAZ112-10F (5'-GGGAATTCTAGACGGTCACAGAGATCAG-3') y pAZ112-10R (5'-GGGAATTCGAACGATTACTAGCAATCCC-3') y los internos, pAZ112-13R1 (5'-GGGAATTCGAAGCATGTTGTTTAATTCG-3') y pAZ112-14R1 (5'-GGGAATTCTTCAAAGAATCGAGTTTCAG-3'). Estos últimos delimitan una secuencia específica de 300 pb.

Las mezclas de reacción para este marcador fueron iguales a las mencionadas para el gen de mtLSUrRNA.

Condiciones de ciclado para la primera PCR: un ciclo a 94 °C por 2 min; 40 ciclos a 94 °C por 30 s, 55 °C por 1 min y 65 °C por 1 min; un ciclo a 65 °C por 5 min. Condiciones de ciclado para la segunda PCR (anidada): un ciclo a 94 °C por 2 min; 10 ciclos a 94 °C por 30 s, 52 °C por 1 min y 65°C por 1 min; 30 ciclos a 94 °C por 30 s, 63 °C por 1 min y 65 °C por 1 min; un ciclo a 65 °C por 5 min.

Para las condiciones de trabajo de cada prueba molecular de *P. jirovecii* se utilizó como testigo positivo el DNA extraído de una muestra clínica positiva para *P. jirovecii* por tinción de metenamina de plata (Grocott) y como testigo negativo, agua Milli-Q.

- **Visualización, secuenciación de los productos de PCR y análisis de las secuencias**

La visualización de los productos de amplificación, su purificación y secuenciación, así como el análisis de las secuencias vienen discriminados en Carreto-Binaghi et al. (2019).

- **Determinación de SP-A y SP-D en muestras de BAL (ver capítulo 5)**

Ambas proteínas fueron cuantificadas por el método de ELISA. Los ensayos se realizaron por duplicado para cada muestra.

SP-A

Se utilizó el kit comercial "Pulmonary Surfactant Associated Protein A (SPA) BioAssay ELISA Kit (Human)" de USBiological Life Sciences (Salem, MA), de acuerdo con lo descrito por Shimizu et al. (1989). Este ensayo puede detectar 5.0-100 ng/mL de SP-A. Para leer este ensayo, se utilizó un lector de microplacas Epoch (Biotek Instruments, Inc., Winooski, VT) con filtro de 450 ± 10 nm.

SP-D

Se utilizó el kit comercial "Surfactant Associated Protein D (SPD) BioAssay ELISA Kit (Human)" de USBiological Life Sciences (Salem, MA), según lo descrito por Honda et al. (1995). Este ensayo puede detectar 6.25-400 ng/mL de SP-D. Para leer este ensayo, se utilizó el lector de microplacas Epoch en las mismas condiciones descritas para SP-A.

- **Determinación de citocinas en muestras de BAL (ver capítulo 5)**

Se utilizó un ensayo de citometría con perlas (CBA, del inglés "Cytometric Bead Assay"). Se procesaron las muestras por duplicado utilizando el kit comercial "LEGENDPLEX Human Inflammation Panel" de BioLegend (San Diego, CA) que emplea perlas carboxílicas conjugadas con un anticuerpo específico marcado con fluorocromo para cada citocina estudiada. El kit posee la ventaja de cuantificar simultáneamente las 11 citocinas humanas seleccionadas para el estudio [IL-1β, IFN-γ, TNF-α, MCP-1 (CCL2), IL-6, IL-8 (CXCL8), IL-12p70, IL-17A, IL-18, IL-23 e

IL-33]. Este ensayo tiene la suficiente sensibilidad para detectar 0.6-0.9 pg/mL de IL-1β, 1.0-1.1 pg/mL de IFN-γ, 1.0 pg/mL de TNF-α, 1.1-1.2 pg/mL de CCL2, 0.8-1.0 pg/mL de IL-6, 0.9-1.0 pg/mL de CXCL8, 0.6-0.7 pg/mL de IL-12p70, 1.9-2.1 pg/mL de IL-17A, 0.7-1.1 pg/mL de IL-18, 1.1-1.2 pg/mL de IL-23 y 0.6-1.1 pg/mL de IL-33. Para la lectura, se utilizó un citómetro de flujo BD FACS Calibur dual laser (BD Biosciences, San Diego, CA, USA) del Laboratorio Nacional de Citometría de Flujo, Instituto de Investigaciones Biomédicas, UNAM.

El kit "LEGENDPLEX" permite personalizar el panel de citocinas a estudiar, por lo que se añadió un panel adicional para determinar dos citocinas que se han relacionado con SP-A y SP-D en la infección pulmonar de otros patógenos fúngicos: IL-5 (Holmer et al. 2014) e IL-13 (Hortobágyi et al. 2008), cuyos límites de detección son 1.1-1.3 pg/mL y 0.8-1.1 pg/mL, respectivamente.

El análisis de los datos obtenidos de la citometría de flujo se realizó con el programa LEGENDPLEX versión 7.0 (http://www.biolegend.com/legendplex), mediante la extrapolación de los valores de la curva estándar de cada citocina, lo que permitió cuantificarlas en cada muestra de BAL.

- **Análisis estadístico**

El análisis estadístico para los resultados de los ensayos de detección molecular por PCR de *H. capsulatum* y *P. jirovecii* se realizó utilizando la prueba de Chi-Cuadrada (χ^2) para detectar diferencias entre los grupos de pacientes. Asimismo se utilizó la razón de momios para la prevalencia, con su respectivo intervalo de confianza al 95% para cada tipo de infección (*H. capsulatum*, *P. jirovecii* y co-infección), como está referido en Carreto-Binaghi et al. (2019).

El análisis de los datos obtenidos de las proteínas surfactantes y citocinas en los diferentes grupos de pacientes se realizó utilizando el programa GraphPad Prism versión 5 (San Diego, CA, USA). (http://www.graphpad.com/scientific-software/prism/), mediante la extrapolación de los valores contra las concentraciones (ng/mL) de la curva estándar de cada proteína surfactante y citocina estudiada; a todas las muestras se les restó el testigo negativo del sistema antes del análisis. Por último, se realizó ANOVA de una cola con prueba de Kruskal-Wallis, con un post-test de Dunns para analizar los datos finales de cada grupo de individuos.

En ambos análisis, se consideraron significativos los valores de $P \leq 0.05$.

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Histoplasma capsulatum and *Pneumocystis jirovecii* coinfection in hospitalized HIV and non-HIV patients from a tertiary care hospital in Mexico

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ABSTRACT

Background: Histoplasma capsulatum and *Pneumocystis jirovecii* are respiratory fungal pathogens that principally cause pulmonary disease. Coinfection with both pathogens is scarcely reported. This study detected this coinfection using specific molecular methods for each fungus in the bronchoalveolar lavage (BAL) of patients from a tertiary care hospital.
Materials and methods: BAL samples from 289 hospitalized patients were screened by PCR with specific markers for *H. capsulatum* (Hcp100) and *P. jirovecii* (mtLSUrRNA and mtSSUrRNA). The presence of these pathogens was confirmed by the generated sequences for each marker. The clinical and laboratory data for the patients were analyzed using statistical software.
Results: The PCR findings separated three groups of patients, where the first was represented by 60 (20.8%) histoplasmosis patients, the second by 45 (15.6%) patients with pneumocystosis, and the last group by 12 (4.2%) patients with coinfection. High similarity among the generated sequences of each species was demonstrated by BLASTn and neighbor-joining algorithms. The estimated prevalence of *H. capsulatum* and *P. jirovecii* coinfection was higher in HIV patients.

Introduction

Histoplasma capsulatum is the causative agent of histoplasmosis, one of the most important systemic mycoses in humans; this disease is particularly related to endemic regions in America, and autochthonous outbreaks have been described from latitude 54° north (Alberta, Canada) (Anderson et al., 2006) to 38° south (Neuquén, Argentina) (Calanni et al., 2013). The epidemic form of histoplasmosis commonly implies an occupational risk, as reported in some Latin American countries, but currently, it represents a human immunodeficiency virus (HIV)-defining condition (Centers for Disease Control and Prevention, 2008). Thus, *H. capsulatum* could be considered a primary fungal pathogen because it infects healthy people, although it also shows an opportunistic behavior since it causes more severe disease in immunocompromised individuals.

H. capsulatum is a dimorphic fungus with two morphotypes, a mycelial infective phase found mainly in bat and bird guano and a yeast virulent phase, which can survive within macrophages and depicts a chronic granulomatous inflammation in host tissues (Köhler et al., 2017; Pomerville, 2018). Infection occurs by inhalation of aerosolized mycelial propagules (mainly microconidia and small hyphal fragments), which become yeasts upon entering the host's respiratory tract.

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Pneumocystis jirovecii, which was first described by Frenkel (1976), is currently accepted as the specific fungal agent of human pneumocystosis (Morris and Norris, 2012). During the acquired immunodeficiency syndrome (AIDS) epidemic in the 1980s, this fungal species was associated with the primary cause of mortality in HIV/AIDS patients, despite the use of antiretroviral therapy. Pneumocystosis is distributed worldwide, and it can also be diagnosed in other immunocompromised patients and in chronic obstructive pulmonary disease exacerbations (Morris et al., 2008). In immunocompetent human hosts, *P. jirovecii* has been reported as an asymptomatic infection or colonization, suggesting it can be the source of infection transmission for other individuals (Morris and Norris, 2012). All *Pneumocystis* spp. depict two morphotypes: the trophic and the asci forms. The asci typically contain ascospores, which are rounded or elongated and can be aerosolized, acting as probable transmission propagules from one individual to another (Hauser and Cushion, 2018). Each *Pneumocystis* sp. is host-specific for one mammal species (a characteristic known as stenoxenism) and cannot be grown in laboratory artificial media (Akbar et al., 2012; Hauser and Cushion, 2018).

H. capsulatum and *P. jirovecii* coinfection was previously reported in a few studies (Baughman et al., 1994; Gago et al., 2014; Huber et al., 2008; Le Gal et al., 2013; Velásquez et al., 2010; Wheat et al., 1985). Most of these studies were developed in samples from hospitalized AIDS patients, where *Histoplasma* was diagnosed by culture growth and *Pneumocystis* by Grocott staining of bronchoalveolar lavage (BAL) or pulmonary biopsies. Three studies have described *H. capsulatum* and *Pneumocystis* sp. coinfection using molecular methods: Gago et al. (2014), who used a multiplex PCR assay in human BAL or biopsy samples; González-González et al. (2014), who used specific nested PCRs in lung samples of randomly captured *Tadarida brasiliensis* bats from Argentina, Mexico, and French Guyana; and Almeida-Silva et al. (2016), who reported an HIV patient with several opportunistic fungal infections detected by nested PCR and multiplex qPCR.

H. capsulatum and *Pneumocystis* sp. share different characteristics that support the possibility of coinfection in the same individual. They are ascomycete fungi that infect through the host's respiratory tract. The lung is the main target organ for both fungi, causing the pulmonary disease representative of their most common clinical form. However, some biological, pathogenic and clinical differences are remarkable between both parasites. For *H. capsulatum*, yeasts are preferentially found in the host's intracellular space (primarily within macrophages). For *Pneumocystis* sp., the two morphotypes are particularly found in the host extracellular space, and the trophic forms develop strong interactions with alveolar epithelial cells. *H. capsulatum* is found in natural habitats, whereas *Pneumocystis* spp. have only been described in infected hosts. *H. capsulatum* does not colonize healthy or immunocompetent individuals, and no possible respiratory transmission from one individual to another has been reported, whereas both features are common for *Pneumocystis* sp. (Köhler et al., 2017; Pomerville, 2018; Skalski et al., 2015). Overall, histoplasmosis patients can develop disseminated disease (rarely seen in pneumocystosis).

The aim of this study was to detect the frequency of *H. capsulatum* and *P. jirovecii* coinfection, using specific molecular methods for each fungus, in BAL samples of patients from a tertiary care hospital focused on respiratory diseases in Mexico.

Materials and methods

Patients

This study was developed in two periods: from June to October 2014, and from May to September 2016. We considered a total of 289 patients hospitalized for acute pulmonary diseases at the Instituto Nacional de Enfermedades Respiratorias "Ismael Cosío Villegas" (INER), in Mexico City, CDMX, Mexico, who required a bronchoscopic diagnostic procedure due to hypoxemic pneumonia. We also analyzed eight samples collected in 2011 from healthy volunteers, which were kindly donated by the team of Dr. Eduardo Sada Díaz, from the "Departamento de Investigación en Microbiología" at INER, as noninfected controls for molecular diagnosis of *H. capsulatum* and *P. jirovecii*. No samples were obtained ex professo for this study.

Clinical data processed from the medical record of each patient were sex, age, place of birth, lactate dehydrogenase (LDH) levels, smoking status, guano exposure, travel history, and main outcome at hospital discharge. Laboratory data evaluated included complete blood count, HIV status (including viral load and $CD4^+$ cell count), and BAL sample laboratory procedures, such as bacterial and fungal cultures (for *H. capsulatum*), Grocott staining (for *P. jirovecii*), as well as multiplex viral PCR and other specific tests for the diagnosis of infectious diseases, which were performed according to physician requests.

BAL samples

From all patients considered for this study, we analyzed one BAL sample. After BAL collection, samples were centrifuged at 2850 × g for 20 min, the supernatant was aliquoted in 600-μl microtubes (Eppendorf, Inc., Enfield, CT, USA) and frozen at −80 °C in less than one hour for other studies, and the pellet was processed for DNA extraction to search for *H. capsulatum* and *P. jirovecii*.

DNA extraction

The pellet of each BAL sample was processed for DNA extraction using a commercial kit (Molecular Biology Kit, Bio Basic Inc., Toronto, ON, CA), according to manufacturer's instructions. DNA samples were quantified in an Epoch microplate spectrophotometer (BioTek Instruments Inc., Winooski, VT, USA) at 260–280 nm and then stored at −80 °C until required. To avoid contamination during molecular screening, all DNA samples were processed in specialized cabinets for each step of the molecular assays performed.

Molecular screening for H. capsulatum *in DNA samples*

Fungal presence was investigated in each extracted DNA sample using a nested PCR for a fragment of the *Hcp100* gene encoding a 100-kDa protein, a molecular marker highly specific for this pathogen (Bialek et al., 2002; Taylor et al., 2005). Two sets of primers were used; the outer primer set included HcI (5′-GCG-TTC-CGA-GCC-TTC-CAC-CTC-AAC-3′) and HcII (5′-ATG-TCC-CAT-CGG-GCG-CCG-TGT-AGT-3′); the inner primers were HcIII (5′-GAG-ATC-TAG-TCGCGG-CCA-GGT-TCA-3′) and HcIV (5′-AGG-AGA-GAA-CTG-TAT-CGG-TGG-CTT-G-3′), delimiting a 210-bp fragment unique to *H. capsulatum*. Details of the amplification are provided in González-González et al. (2012). DNA from the EH-53 *H. capsulatum* strain from a Mexican clinical case was used as a positive control, and milli-Q water (Milli-Q water purifier, Merck KGaA, Darmstadt, DE) was always processed as a negative control.

Molecular screening for P. jirovecii *in DNA samples*

We used nested PCR for the amplification of two genes that are reliable markers for *Pneumocystis* detection: the mitochondrial ribosomal large subunit (mtLSUrRNA) and the mitochondrial ribosomal small subunit (mtSSUrRNA) (Wakefield et al., 2003). For the mtLSUrRNA locus, we used the outer primer set pAZ102-H

(5′-GTG-TAC-GTT-GCA-AAG-TAG-TC-3′) and pAZ102-E (5′-GAT-GGC-TGT-TTC-CAA-GCC-CA-3′). The inner primers, pAZ102-X (5′-GTG-AAA-TAC-AAA-TCG-GAC-TAG-G-3′) and pAZ102-Y (5′-TCA-CTT-AAT-ATT-AAT-TGG-GGA-GC-3′), delimit a 267-bp fragment specific for *Pneumocystis* sp. Nested PCR for the mtSSUrRNA locus was performed with the outer primers, pAZ112-10F (5′-GGG-AAT-TCT-AGA-CGG-TCA-CAG-AGA-TCA-G-3′) and pAZ112-10R (5′-GGG-AAT-TCG-AAC-GAT-TAC-TAG-CAA-CCC-3′). The inner primers, pAZ112-13RI (5′-GGG-AAT-TCG-AAG-CAT-GTT-GTT-TAA-TTC-G-3′) and pAZ112-14RI (5′-GGG-AAT-TCT-TCA-AAG-AAT-CGA-GTT-TCA-G-3′), delimit a 300-bp fragment specific for *Pneumocystis* sp. (González-González et al., 2014). A DNA sample obtained from a pulmonary biopsy from a patient diagnosed by Grocott staining with pneumocystosis was used as a positive control. Milli-Q water (Merck) was always used as a negative control for both *Pneumocystis* molecular markers.

Amplified products (amplicons)

Amplicons from each nested PCR were electrophoresed on a 1.8% agarose gel in 0.5X Tris-borate-EDTA buffer. Electrophoresis was conducted at 120 V for 50 min using a 100-bp DNA ladder (Gibco Laboratories, Grand Island, NY, USA) as a molecular size marker. The amplicons were visualized using a UV transilluminator after GelRed Nucleic Acid Gel Stain (Thermo Fisher Scientific Inc., Waltham, MA, USA) staining (0.5 μg/100 ml). The amplicons were purified using the Nucleotrap PCR Purification Kit (BD Biosciences, Palo Alto, CA, USA) and sent to the High-Throughput Genomics Center (University of Washington, Seattle, WA, USA) for sequencing of the sense and antisense DNA strands; a consensus sequence for each amplified sample was generated.

Sequence analyses

The generated sequences were aligned and manually edited using MEGA software, version 7, http://www.megasoftware.net (Kumar et al., 2016), and their alignments are provided in the electronic supplementary material (Supplementary files 1, 2 and 3) for the readers' reference.

Sequences were analyzed to confirm their high homology for each marker, which was the main criterion for the molecular identification of each fungus in BAL samples, considering for *P. jirovecii* at least one of the two markers used. First, the sequences were analyzed using the BLASTn algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to search in the GenBank database for all homologous sequences corresponding to the nested PCR products of *H. capsulatum* and *P. jirovecii* gene fragments. Afterward, sequence analysis with the neighbor-joining (NJ) method was used to construct the respective genetic relationship trees for each marker using MEGA7 (Kumar et al., 2016). To infer the NJ trees, genetic distances were conducted using the Kimura two-parameter model, considering gaps/missing data and mutation rates between the analyzed nucleotide sites. Bootstrap values (bt) for NJ analyses were based on 1000 replicates (Kumar et al., 2016). To construct the NJ trees, Hcp100 sequences were aligned with the sequence of the G-217B reference strain from Louisiana, USA (GenBank accession no. AJ005963); mtLSUrRNA sequences were aligned with the reference sequence of *P. jirovecii* EH1-PAZ102E (GenBank accession no. JF733748); and mtSSUrRNA sequences were aligned with the reference sequence of *P. jirovecii* (GenBank accession no. HQ228547).

Statistical analyses

The total infection rate was estimated considering all patients. The Chi-Square (χ^2) test was used to detect significant differences between the groups of patients infected with *H. capsulatum*, *P. jirovecii* or both fungi. The prevalence odds ratio test and its corresponding 95% confidence interval (95% CI) were calculated to evaluate the possible strength of the association between each different analyzed variable and the occurrence of histoplasmosis, pneumocystosis or their coinfection. Both statistical analyses were performed using SPSS Statistics version 21.0 (SPSS Inc., Chicago, IL, USA). Values of $P < 0.05$ were considered significant.

Results

We analyzed BAL samples from 289 patients: 84 (29.1%) were from HIV patients and 205 (70.9%) were from non-HIV patients. Seventy-one HIV patients were male and 13 were female with a median age of 34 years, showing a median HIV viral load of 246 852 copies/ml and a median $CD4^+$ count of 23 cells/ml of peripheral blood. One hundred and ten non-HIV patients were male and 95 were female with a median age of 54 years. Most individuals (66.4%) lived in Mexico City and the surrounding conurbation, whereas the rest of patients (33.6%) came from rural areas.

Out of 289 patient BAL samples, the amplified Hcp100 marker diagnosed the presence of *H. capsulatum* infection in 60 patients (20.8%), which included four patients with positive cultures for *H. capsulatum*. The amplification of either mtLSUrRNA or mtSSUrRNA locus diagnosed the presence of *P. jirovecii* infection in 45 cases (15.6%) (Table 1). Regarding *P. jirovecii* detection using two independent molecular markers (mtLSUrRNA and mtSSUrRNA) for its identification, it is important to remark that both markers were amplified in 34 samples, whereas the mtLSUrRNA marker generated more sequences (47) than the mtSSUrRNA (33). None of these markers was amplified in any of the samples from noninfected controls (healthy volunteers). Milli-Q water was always negative in each assay performed.

The BLASTn analysis for Hcp100 sequences showed a range from 94 to 100% similarity between them and the GenBank reference sequence from *H. capsulatum*, whereas mtLSUrRNA and mtSSUrRNA sequences showed a range from 98 to 100% similarity between them and the reference sequences from *P. jirovecii*.

All *H. capsulatum* Hcp100 sequences analyzed by NJ are represented in Figure 1, and the cluster of these sequences supports a close genetic relationship among them. Figure 2 shows the sequence trees analyzed by NJ for the *P. jirovecii* mtSSUrRNA (Figure 2a) and mtLSUrRNA (Figure 2b) markers, respectively, and the topologies of these trees sustained a close genetic relationship among the sequences.

The molecular tools used in this study revealed that 12 (4.2%) patients presented both *H. capsulatum* and *P. jirovecii* infections simultaneously. Out of these 12 coinfected patients, nine samples belonged to HIV patients (10.7% of 84 HIV patients) and the other three to non-HIV patients (1.5% of 205 non-HIV patients) (Table 1).

The main data for all studied patients, such as sex, age, HIV status, LDH levels, smoking status, guano exposure, mechanical ventilation, and lethality, are provided in Table 1. The last column shows the results of the χ^2 test for each variable to detect significant differences among the groups of patients infected with *H. capsulatum*, *P. jirovecii* or both fungi, and *P* values are shown in Table 1. Demographic and clinical data such as sex, LDH levels, mechanical ventilation, and lethality did not show significant differences between groups. Two important risk factors were assessed for the fungal infections studied, such as smoking status (for *H. capsulatum* and *P. jirovecii*) or guano exposure (for *H. capsulatum* only). Significant differences were found between smoking and nonsmoking patients ($P = 0.002$), considering the *H. capsulatum*, *P. jirovecii* and coinfected groups of patients. No significant differences ($P = 0.270$) were found among patients exposed to guano in the environment in regard to *H. capsulatum*

Table 1
Main data from histoplasmosis, pneumocystosis and coinfected patients.

Demographic and clinical data	*H. capsulatum* infection only n = 60	*P. jirovecii* infection only n = 45	Coinfection n = 12	*P*
Age[a] (years)	46 [32–66]	37 [30–47]	34 [30–44]	
Sex				0.212
Female	21 (35%)	15 (33.3%)	2 (16.7%)	
Male	39 (65%)	30 (66.7%)	10 (83.3%)	
HIV patients	16 (26.7%)	32 (71.1%)	9 (75%)	<0.0001
Viral load[a] (copies/ml)	293 479 [24 064–848 244]	427 572 [34 951–851 138]	462 080 [228 922–933 417]	
CD4+ count[a] (cells/ml)	42 [10–56]	21 [7–42]	25 [7–42]	
Non-HIV patients	44 (73.3%)	13 (28.9%)	3 (25%)	
LDH[a](UI/l)	186 [140–273]	227 [152–341]	279 [140–396]	0.070
Smoking				0.002
Active	26 (43.3%)	19 (42.2%)	3 (25%)	
Passive	12 (20%)	4 (8.9%)	0 (0%)	
None	16 (26.7%)	16 (35.6%)	8 (66.7%)	
No data	6 (10%)	6 (13.3%)	1 (8.3%)	
Guano exposure				0.270
Yes	17 (28.3%)	11 (24.5%)	2 (16.7%)	
No	37 (61.7%)	28 (62.2%)	9 (75%)	
No data	6 (10%)	6 (13.3%)	1 (8.3%)	
Mechanical ventilation				0.129
Yes	17 (28.3%)	14 (31.1%)	6 (50%)	
No	37 (61.7%)	25 (55.6%)	5 (41.7%)	
No data	6 (10%)	6 (13.3%)	1 (8.3%)	
Lethality				0.088
Yes	10 (16.7%)	6 (13.3%)	4 (33.3%)	
No	44 (73.3%)	33 (73.4%)	7 (58.4%)	
No data	6 (10%)	6 (13.3%)	1 (8.3%)	

LDH: Lactate dehydrogenase.
Percentages in parentheses were estimated considering the number of patients in each infected group.
[a] Median values of these clinical data are shown, with the corresponding interquartile range (IQR 25–75) in brackets.

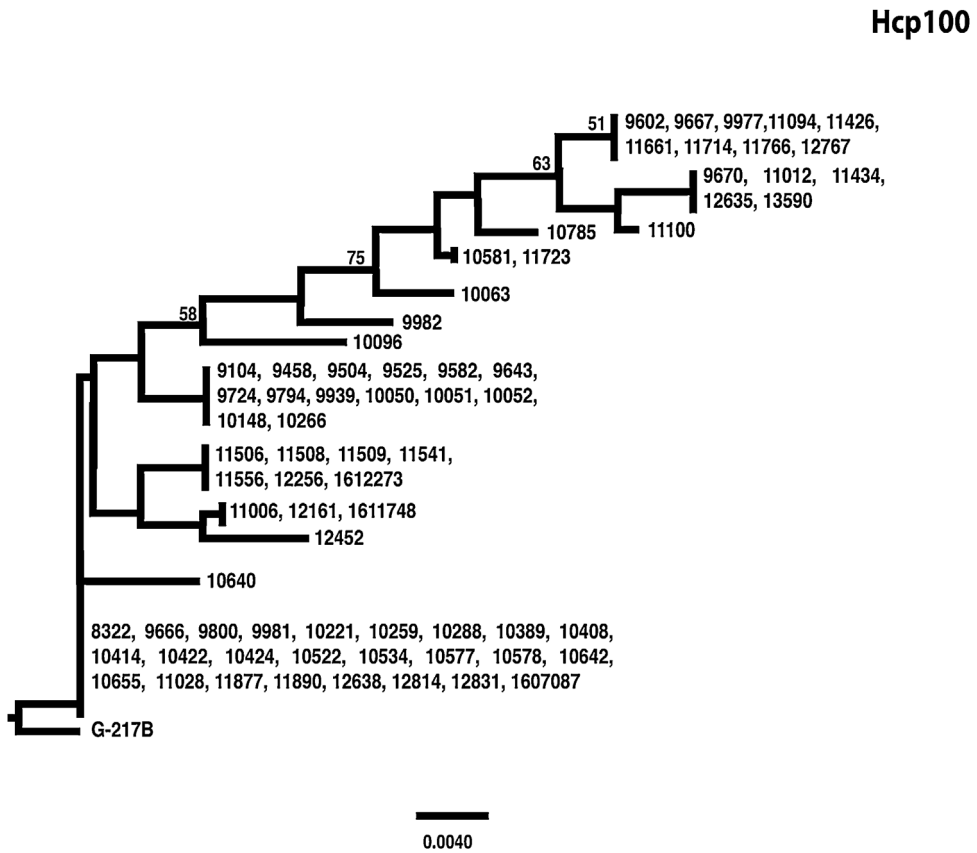

Figure 1. NJ analysis of the *H. capsulatum* sequences. The NJ tree was constructed using a matrix of 142-nt of the Hcp100 gene fragments. The NJ analysis was conducted as described in Materials and methods. Supporting bt values ≥50% are indicated on their corresponding tree branches.

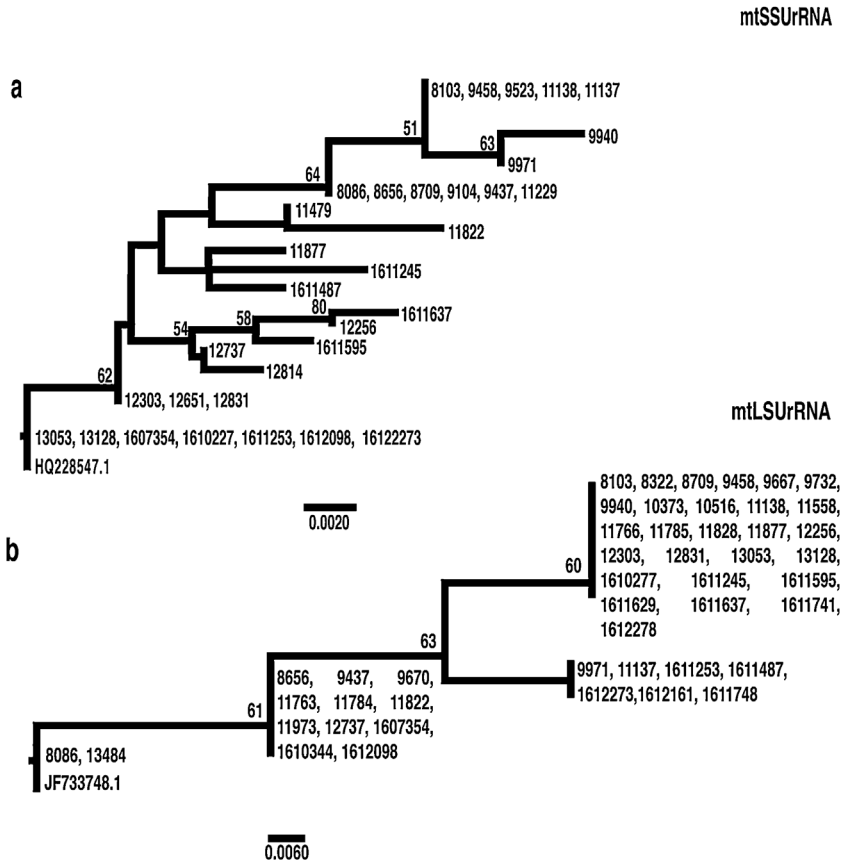

Figure 2. NJ analyses of the *P. jirovecii* sequences. (a) For the mtSSUrRNA sequences, the NJ tree was constructed using a matrix of 274-nt. (b) For the mtLSUrRNA sequences, the NJ tree was constructed using a matrix of 176-nt. The NJ analyses were conducted as described in Materials and methods. Supporting bt values ≥50% are indicated on their corresponding tree branches.

and coinfected groups of patients. Based on additional information from the hospital records, we found that *H. capsulatum* was detected in 25.5% of the patients from urban areas and in 23.7% of the patients from rural areas, whereas *P. jirovecii* was detected in 18.2% of the patients from urban areas and in 22.6% of the patients from rural areas, and no significant differences were detected. Other laboratory data (hemoglobin, hematocrit, total leukocyte count, and platelet count) for each group of patients studied revealed no significant differences among patients with histoplasmosis, pneumocystosis and coinfection (data not shown).

According to laboratory reports, *H. capsulatum* cultures were positive only in four BAL samples of HIV/AIDS patients, corresponding to 5.6% of all the *H. capsulatum*-positive samples detected by PCR, whereas *P. jirovecii* was observed by Grocott staining only in 14 BAL samples, representing 24.6% of all the *P. jirovecii*-positive samples detected by PCR.

Microorganisms distinct from the studied fungi were detected only in 44 out of 289 patients, by culture (for bacteria and other fungi) and by multiplex PCR (for viruses), as reported in the clinical records. Eight patients were infected with *M. tuberculosis*, 14 with Gram-negative bacteria and four with Gram-positive bacteria. Mixed infections with different microorganisms (virus-bacteria, bacteria-bacteria, bacteria-fungi or virus-fungi) were found in 19 patients; 11 patients presented more than two types of microorganisms. Interestingly, out of the 12 patients coinfected with *H. capsulatum* and *P. jirovecii*, three were infected with other microorganisms that could be part of the normal microbiota of the upper respiratory tract: one patient had *Pseudomonas aeruginosa*, the second had *P. aeruginosa* and *Klebsiella pneumoniae*, and the third had *P. aeruginosa*, parainfluenza 2 virus and rhinovirus. Details about the different microorganisms found in the studied groups are available in the Supplementary Table.

Figure 3 shows a detailed description of the statistical analyses for histoplasmosis, pneumocystosis and coinfection in HIV vs. non-HIV patients.

The prevalence of histoplasmosis infection in the whole studied population was 20.8% (60 of 289 patients), whereas the prevalence in HIV patients was slightly lower (19%) than in non-HIV patients (21.5%); however, this difference was not significant ($P = 0.64$) (Figure 3a).

The prevalence of pneumocystosis infection in the whole studied population was 15.6% (45 of 289 patients), and it was significantly higher in HIV patients with $P < 0.0001$ (38.1%) when compared to non-HIV patients (6.3%). Moreover, the estimated prevalence odds ratio for pneumocystosis was 9.08 (95% CI, 4.45–18.56), which suggested that infection with *P. jirovecii* was 9.08-fold more possible in HIV patients (Figure 3b).

The prevalence of coinfection with both fungi was 4.2% (12 of 289 patients), and the frequency of coinfection in HIV patients (10.7%) was significantly higher than in non-HIV patients (1.5%) with $P = 0.001$. Therefore, the prevalence odds ratio estimated for coinfection was 8.08 (95% CI, 2.13–30.6), revealing that HIV patients had an 8.08-fold greater probability of becoming infected with both fungi (Figure 3c).

In particular, it is remarkable that the lethality rate for patients in the histoplasmosis-pneumocystosis coinfection group (33.3%, 4 of 12 patients) was higher than the lethality rate for patients with only histoplasmosis infection (16.6%, 10 of 60 patients) or only pneumocystosis infection (13.3%, 6 of 45 patients), even though these results were not statistically significant (see Table 1).

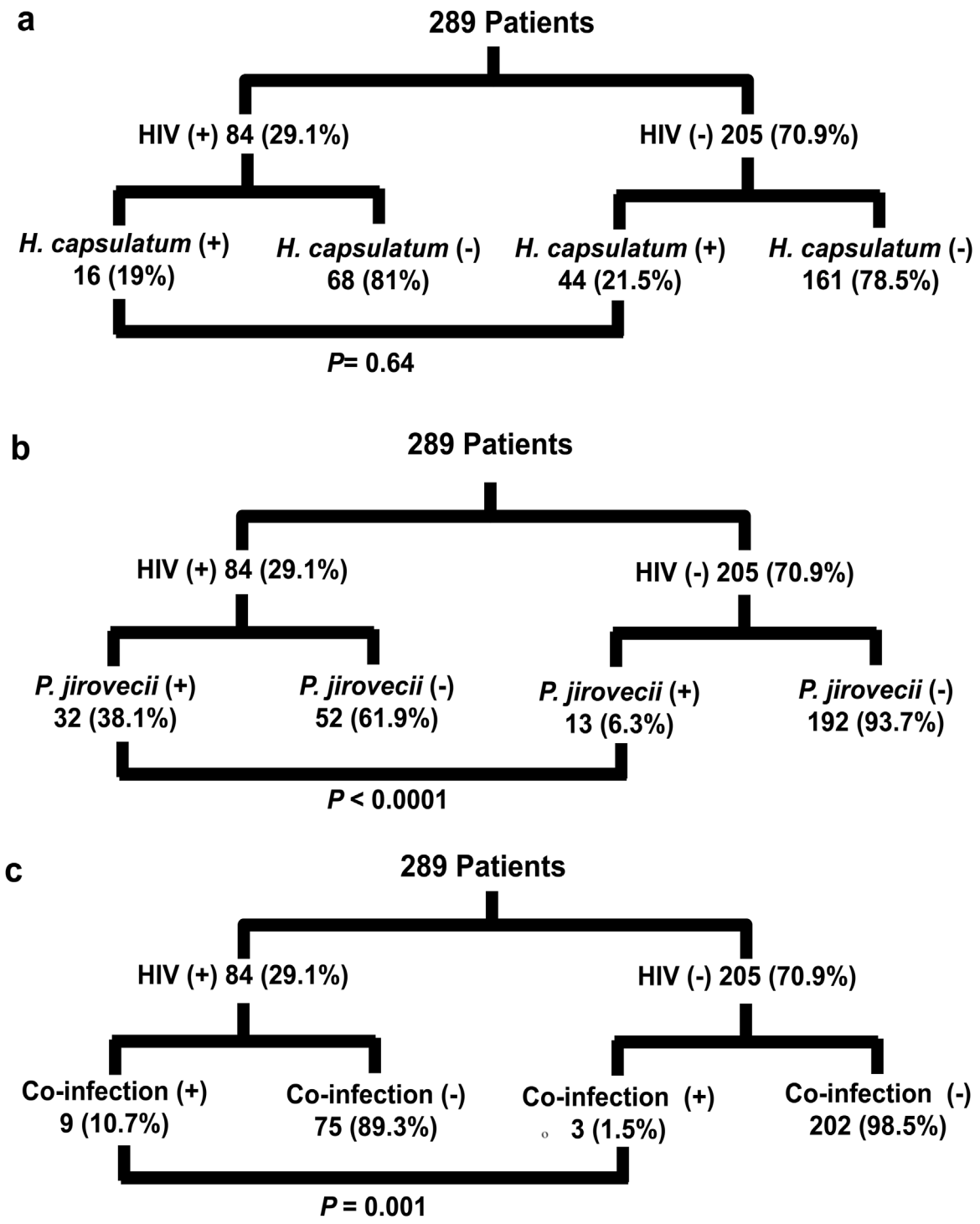

Figure 3. Statistical analyses. Within the studied population, χ^2 and prevalence odds ratio tests were calculated to compare the prevalence of (a) histoplasmosis, (b) pneumocystosis, and (c) coinfection of *H. capsulatum* and *P. jirovecii*, in HIV versus non-HIV patients.

Discussion

The present manuscript highlights the importance of coinfection with two respiratory fungi (*H. capsulatum* and *P. jirovecii*) in a tertiary care hospital, where most individuals were HIV patients, although coinfection was also found in three non-HIV patients without an immunocompromised condition or any other remarkable data in their clinical records. The diagnosis of this coinfection is critical for the establishment of adequate therapeutic strategies to eliminate both fungi.

In previous years, the diagnosis of coinfection with *H. capsulatum* and *P. jirovecii* has been unintentionally neglected because it has not specifically been evaluated previously. The real frequency of this coinfection is unknown, but in a few studies, coinfection has been serendipitously reported, mostly while describing opportunistic infections in HIV patients. When the HIV epidemic started, Wheat et al. (1985) described the first 15 cases of AIDS in Indianapolis, where seven patients developed disseminated histoplasmosis, and two of them were also diagnosed with pneumocystosis; based on these authors' data,

we estimated a coinfection rate of 13.3%. Later, Baughman et al. (1994) screened opportunistic infections in 894 BAL samples from AIDS patients; they found 420 (46.97%) patients with *Pneumocystis,* five of whom were coinfected with *H. capsulatum* (0.55% according to our estimation). In French Guiana, Huber et al. (2008) described 200 cases of AIDS-associated histoplasmosis over 25 years; seven were coinfected with *Pneumocystis* (3.5% according to our estimation). Velásquez et al. (2010) studied 44 HIV/AIDS patients with histoplasmosis and found concomitant pneumocystosis in 11.4% of the cases. Caceres et al. (2018) reported three cases of pneumocystosis in 45 HIV patients with histoplasmosis (6.7% with coinfection, according to our estimation). Interestingly, Le Gal et al. (2013) described the intracellular coexistence of *H. capsulatum* and *P. jirovecii* in an alveolar macrophage from a BAL sample of one AIDS patient.

The abovementioned reports were entirely focused on HIV patients, and most of the frequencies of coinfection that we estimated from their results were similar to our reported rate of 10.7% (9 of 84 HIV patients). Coinfection could be explained by the virus-related immunocompromised condition, which makes the patients more susceptible to concomitant infections.

In regard to the LDH levels, which is a marker associated with the prognosis of patients with disseminated histoplasmosis (Butt et al., 2002; Ramos et al., 2018) and more frequently used to distinguish bacterial from *Pneumocystis* pneumonia (Sun et al., 2016), our results showed that the serum LDH levels were not significant to support the diagnosis of either histoplasmosis or pneumocystosis.

Other risk factors assessed in this study were smoking and guano exposure. Smoking has been previously reported as a risk factor for complications in the ocular histoplasmosis syndrome (Chheda et al., 2012; Ganley, 1973). Additionally, smoking is a presumed risk factor for presenting *P. jirovecii* pneumonia in HIV patients (Blount et al., 2013; Miguez-Burbano et al., 2005) and in non-HIV patients (Santos et al., 2017). Regarding the lack of significance in the analysis concerning guano exposure related to *H. capsulatum* infection, it is important to emphasize that histoplasmosis outbreaks might occur both in rural and urban areas because the fungus has been found in several niches where people could be inadvertently exposed. (Corcho-Berdugo et al., 2011; Muñoz et al., 2010; Taylor et al., 2005). However, other risk factors associated with histoplasmosis or pneumocystosis, such as previous treatment with immunosuppressive drugs, were scarcely referred for the studied patients.

It would be fascinating to identify the influence of one pathogen in regard to the other on the coinfection outcome; however, it was not possible to detect this association because some patients' medical records were incomplete. It is crucial to underline the higher lethality rate associated with coinfection, suggesting a contribution of both pathogens in the fatal clinical course of the patients (Table 1). In general, most of the causes of death referred for histoplasmosis, pneumocystosis and coinfected patients were pneumonia with sepsis and/or septic shock.

An important feature to consider from most studies is the diagnostic procedure used for histoplasmosis (culture) and pneumocystosis (microscopic observation), which require specialized supplies and technical experience for successful diagnosis. Therefore, new methods should be implemented to detect coinfection with these fungi. To date, only a few studies have used molecular methods. Gago et al. (2014) reported one of 14 HIV patients with *H. capsulatum* and *P. jirovecii* coinfection (7.14% according to our estimation) while developing a multiplex PCR assay for fungi. González-González et al. (2014) described coinfection with *H. capsulatum* and *Pneumocystis* spp. in 122 randomly captured bats (35.2% of cases), supporting a high frequency of both pathogens in this particular host (González-González et al., 2014). Thus, we chose molecular methods for histoplasmosis and pneumocystosis diagnosis due to their rapid, specific and accurate detection, emphasizing that these methods were very useful to reveal *H. capsulatum* and *P. jirovecii* in BAL samples. We would like to emphasize that the four HIV/AIDS patients with positive *H. capsulatum* cultures also amplified the specific marker Hcp100, which highlights the efficiency of the molecular method used. Fungal sequences generated by PCR, using DNA extracted from BAL samples, reflected active mycotic disease, and the specificity of the molecular markers used in the present study makes misdiagnosis very improbable. In contrast, immunological diagnostic procedures sometimes are unable to differentiate past from present infection. For both fungi, we selected nested PCR protocols, which have been demonstrated to be very sensitive, reproducible for the identification of pathogens in different clinical samples, and able of avoiding molecular contamination under well-controlled laboratory conditions. Moreover, nested PCR is a useful tool for epidemiological studies, where this method serves as a great screening technique when compared to real-time PCR (Seo et al., 2014; Sharifdini et al., 2015).

We considered the generated sequence of each marker as a unique criterion for diagnosis because the single visualization of the amplified PCR products could lead to misinterpretation or overdiagnosis of the results and because non-specific products could sometimes be produced. Although our criterion is very strict, it is undoubtedly precise and reliable, as demonstrated by BLASTn and NJ genetic analyses of the generated sequences. Thus, the close genetic relationship among the sequences of Hcp100 for *H. capsulatum* and among the sequences of mtLSUrRNA and mtSSUrRNA for *P. jirovecii* confirmed their respective fungal molecular identification. The scientific literature has reported mtLSUrRNA marker as a better diagnostic tool for *P. jirovecii*; however, based on our previous experience (González-González et al., 2014), we also selected the mtSSUrRNA marker because the fungus was identified in a few samples in which only this marker was amplified.

In conclusion, statistical analyses support the highest prevalence of pneumocystosis and coinfection in HIV patients, and based on our findings, *H. capsulatum* and *P. jirovecii* coinfection is a more common medical problem than expected, not only in immunocompromised patients. Searching for both pathogens at the initial stages of disease should be routinely performed to establish adequate treatments for both fungi to improve patient outcomes and diminish the risk for complications. Basic research studies on this coinfection are still needed to explain the interaction of the two pathogens in vivo.

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Ethical approval

Patients' and volunteers' BAL samples were obtained in accordance with the ethical standards of the Helsinki Declaration (1964, amended in 2008). Written consent was obtained and kept in the hospital's individual medical records. This work was approved by the School of Medicine Research and Ethics Committee (UNAM, report 132/2015) and by the INER Ethics Research Committee (protocol B13-14).

Conflict of interest

The authors declare that they have no conflicts of interest.

Authors' contributions

LECB and MLT conceived the study, participated in its design and coordination and helped to draft the manuscript. LECB and FRMV collected the samples. LECB and JAR performed the experimental procedures. MLT, LECB, GGT, TVG, and JAR analyzed the data. EMA and JAMO drafted the manuscript and revised it critically for important intellectual content. JAMO and MLT share the academic responsibility for this manuscript. All authors read and approved the final manuscript.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.ijid.2019.06.010.

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CAPÍTULO 5
ARTÍCULO ENVIADO

Carreto-Binaghi LE, Tenorio EP, Morales-Villarreal FR, Aliouat EM, Zenteno E, Martínez-Orozco JA, Taylor ML. Inflammatory mediators produced in the lung of patients during *Histoplasma capsulatum* and *Pneumocystis jirovecii* infection. J Clin Immunol.

Inflammatory mediators produced in the lung of patients during *Histoplasma capsulatum* and *Pneumocystis jirovecii* infection

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Running head: Lung immune response in histoplasmosis and pneumocystosis

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ABSTRACT

Histoplasmosis and pneumocystosis coinfection has been reported in wild animals and in humans, especially in immunocompromised patients. Immunological responses for each infection have been described particularly in animal models; however, the host response involving simultaneously both fungal pathogens is unknown. The aim of the present work was to evaluate the pulmonary immunological response of patients with *Histoplasma capsulatum* (Hc) and *Pneumocystis jirovecii* (Pj), either against a single pathogen or coinfection. We analyzed pulmonary collectins, cytokines and chemokines profiles of 131 bronchoalveolar lavage (BAL) samples, from HIV and non-HIV patients infected with Hc, Pj or coinfected, as well as from healthy volunteers group (non-infected control). Both fungi induced the production of SP-A in non-HIV patients, whereas in HIV patients only Pj increased SP-A levels. Non-HIV patients developed higher SP-A mean values than HIV patients in Hc infection. SP-D values decreased in all patients when compared with the healthy volunteers. The inflammatory cytokines (IL-1β and TNF-α) increased, particularly in the Non-HIV-Hc group when compared with the HIV-Pj and healthy groups; IL-10 was only detected in the HIV-Pj group. The evaluation of cytokines from the IFN-γ/IL-12 axis showed that all non-HIV groups developed higher mean values of IFN-γ, and that IL-12p70 was higher in all Hc-infected groups. Cytokines from the IL-17/IL-23 axis demonstrated a Th17 response against both fungi. Granuloma-associated cytokines (IL-5, IL-13, and IL-33) were barely detected in the BAL samples. Chemokines (CXCL8 and CCL2) were abundantly found in the non-HIV groups.

This study shows a brief overview of the human pulmonary immune response against Hc, Pj or their coinfection, which is mainly associated with a proinflammatory Th17 response with a huge local chemokine response, which is more prominent in non-HIV patients.

Keywords. *H. capsulatum*, *P. jirovecii*, SP-A and SP-D, cytokines and chemokines, bronchoalveolar lavage

INTRODUCTION

Histoplasma capsulatum is a dimorphic fungus that causes histoplasmosis, which is one of the most frequent human respiratory mycoses.[1] The aerosolized infective mycelial phase propagules of *H. capsulatum* are the main infection source for mammal hosts, and in the infected host, the fungus converts into a parasitic and virulent yeast phase.[1]

Pneumocystis species are opportunistic fungi that cause disease in several mammals, affecting their own host specifically,[2] and in humans, the associated species is *P. jirovecii*.[3] All *Pneumocystis* spp. have trophic and asci forms, the latter act as aerosolized propagules in the transmission from one individual to another.[4]

H. capsulatum is an intracellular facultative parasite, mainly located inside macrophages, while *P. jirovecii* is preferentially located over the surface of type 1 pneumocytes.[5]

The initial clinical feature of histoplasmosis and pneumocystosis is pneumonia, but disseminated and chronic diseases are frequent in histoplasmosis[6] and rare in pneumocystosis.[7] However, *P. jirovecii* might cause these clinical forms in immunocompromised patients.[8]

Histoplasma capsulatum and *Pneumocystis* sp. co-infection has been reported in wild animals[9] and in humans, especially in immunocompromised patients,[10-16] which are more susceptible to lung diseases; both mycoses are AIDS-defining conditions.[17]

In the alveolar microenvironment, both pathogens interact with the surfactant proteins A and D (SP-A and SP-D), which are pulmonary collectins involved in microbial recognition and innate immune response.[18-20] McCormack et al.[21] described a macrophage-independent collectin fungicidal activity against the yeast phase of *H. capsulatum*, and a biosynthesis inhibition of the fungus, meaning the abolition of yeast reproduction inside the host cells. The exact mechanism of adherence of SP-A and SP-D to *H. capsulatum* is unknown, whereas SP-A and SP-D bind to *Pneumocystis* through the major surface glycoprotein. They can also bind to the β-glucan on the cell wall of this fungus, but none of these proteins seems to induce the phagocytosis of *Pneumocystis* organisms.[22,23] SP-A production increases during *Pneumocystis* infection, both at protein and mRNA levels in alveolar cells.[24,25]

Following *H. capsulatum* mycelial phase infection, innate immunity cellular effectors sense the fungus. After 2-3 hours, the mycelial phase converts to yeast phase in the upper respiratory tract.[26] The innate response is relevant to orchestrate the first inflammatory environment. Type I interferon (IFN-I) is produced after the infection of bone marrow derived macrophages with conidia from the mycelial phase,[27] probably through the participation of the Toll-like receptors (TLR-7 and TLR-9), which are critical for the IFN-I response and host survival to *H. capsulatum* infection.[28] In histoplasmosis, inflammatory molecules have been preferentially studied in murine models, processing as inoculum the yeast phase, which could interfere in the time response of the innate and adaptive performances. The production of chemokines CCL3 (MIP-1α), CCL4 (MIP-1β), CCL5 (RANTES), and CCL11 (eotaxin) is associated with innate and adaptive responses, when mice were inoculated with the yeast phase of the fungus.[29]

H. capsulatum infection induces the release of pro-inflammatory cytokines (mainly TNF-α, IL-1β, IL-6, IL-23, and IL-17)[30,31] and chemokines that activate macrophages and stimulate granuloma development through the production of IL-17.[32] However, the immunity against *H. capsulatum* infection is mainly controlled by a Th1 adaptive response, whereas Th2 response contributes to a worse prognosis of the disease.[33,34]

The immune response to *P. jirovecii* has been less explored; most data came from mouse models using the species *P. carinii* (now renamed as *P. murina*), which suggest that the pathogen's clearance inversely correlates with the degree of inflammation.[35-37] The innate response mediated by IFN-I and TNF-α has a role in complications of *Pneumocystis* pneumonia, contributing to lung injury.[38-42] However, IL-1β and IL-6 recruit lymphocytes, macrophages and neutrophils for *Pneumocystis* clearance.[43,44]

Pneumocystis infection appears to be associated to a dysfunction of the IL-23/IL-17 axis, with larger lung fungal burdens and a reduced production of IL-17 and the chemokines CXCL10 (IP-10), CCL3, CCL4, and CCL5,[45] which are crucial for infection resolution.[42] Th1 cytokines also have a critical role in clearance of *Pneumocystis* sp. infection.[46,47] IL-12 plays a very important role in *Pneumocystis* clearance, favoring inflammation through TNF-α and IFN-γ production from alveolar macrophages.[48]

In regard to Th2 response, it has been reported that IL-10 deficient mice develop more lung damage,[49,50] highlighting the detrimental role of excessive inflammation in *Pneumocystis* sp. infections.

The aim of the present work was to evaluate the local immunological response of patients with histoplasmosis and pneumocystosis, either as single pathogen infection or a co-infection of both fungi. By analyzing bronchoalveolar fluids we gained insights into their pulmonary collectins, cytokines and chemokines profiles. Considering that these mycoses

are described as AIDS-defining conditions and are frequently diagnosed in HIV/AIDS patients, this study included a population of HIV patients infected with *H. capsulatum* and *P. jirovecii*.

MATERIALS AND METHODS

Individuals

We studied 131 individuals from a previous report by Carreto-Binaghi et al.[16] 104 patients were diagnosed with *H. capsulatum*, *P. jirovecii* or coinfection with both pathogens, and as control groups 19 HIV patients without these studied fungi and eight healthy volunteers (see Table 1).

Bronchoalveolar lavage (BAL) samples

BAL samples were collected from patients admitted with pneumonia at the Instituto Nacional de Enfermedades Respiratorias Ismael Cosío Villegas (INER), in Mexico City, CDMX, Mexico. BAL samples from healthy volunteers were donated by the "Departamento de Investigación en Microbiología" at INER. No samples were taken ex professo for this study.

A total of 131 BAL samples of the studied groups of individuals discriminated in Table 1 were analyzed. After collection, BAL samples were immediately processed by centrifuging at 2800 × g at 4 ºC for 20 min and each supernatant was aliquoted in 600 µL low protein binding microcentrifuge tubes (Eppendorf North America, Inc., Hauppauge, NY, USA) and frozen at −196 ºC, until required.

Ethics approval and consent to participate

This work was approved by the School of Medicine Research and Ethics Committee (UNAM, report 132/2015) and by the INER Ethics Research Committee (protocol B13-14). In accordance with the ethical standards of the Helsinki Declaration (1964, amended in 2008), each patient and volunteer signed a written consent form for collecting their BAL samples.

SP-A and SP-D screening in BAL samples

Human Surfactant Associated Proteins A and D (SP-A and SP-D) were quantified using BioAssay ELISA Kits (USBiological Life Sciences, Salem, MA, USA), following the manufacturer's instructions. These ELISA assays detect a range of 5.0-100 ng/mL of SP-A and 6.25-400 ng/mL of SP-D. Both assays were read in an Epoch microplate spectrophotometer (Biotek Instruments, Inc., Winooski, VT, USA) with a filter of 450 nm.

Cytokine and chemokine screening in BAL samples

Cytokines and chemokines were determined using the LEGENDPLEX Human Inflammation Panel (BioLegend, San Diego, CA, USA), following the manufacturer's instructions. Panels containing a fluorescent dye, allophycocyanin (APC)-labeled beads conjugated to a monoclonal antibody specific for each target cytokine or chemokine, were used to quantify IL-1β, IFN-γ, TNF-α, IL-5, IL-6, IL-10, IL-12p70, IL-13, IL-17A, IL-18, IL-23 and IL-33 cytokines, as well as MCP-1 (CCL2) and IL-8 (CXCL8) chemokines. Samples were acquired in a BD FACS Calibur dual laser flow cytometer (BD Biosciences, San Diego, CA, USA) at the Laboratorio Nacional de Citometría de Flujo, Instituto de Investigaciones Biomédicas, UNAM. Flow cytometry data were analyzed with the software

LEGENDPLEX version 7.0 (BioLegend), to obtain the concentration of cytokines and chemokines in the samples. Assays were performed in duplicates.

The panel has a high sensitivity level to detect the cytokines: 0.6-0.9 pg/mL of IL-1β; 1.0-1.1 pg/mL of IFN-γ; 1.0-1.0 pg/mL of TNF-α; 1.1-1.3 pg/mL of IL-5; 0.8-1.0 pg/mL of IL-6; 0.7-0.8 pg/mL of IL-10; 0.6-0.7 pg/mL of IL-12p70; 0.8-1.1 pg/mL of IL-13; 1.9-2.1 pg/mL of IL-17A; 0.7-1.1 pg/mL of IL-18; 1.1-1.2 pg/mL of IL-23; and 0.6-1.1 pg/mL of IL-33; and the chemokines: 1.1-1.2 pg/mL of CCL2 and 0.9-1.0 pg/mL of CXCL8.

Statistical analyses

Kruskal-Wallis test with Dunns post-test was performed to conduct all the analyses between groups of individuals, using the software GraphPad Prism version 5.00 for Windows (San Diego, CA, USA). p values ≤ 0.05 were considered significant.

RESULTS

SP-A and SP-D screening

For convenience, the data of SP-A and SP-D measurements from each group of individuals studied are summarized in Table 2. In general, SP-A values increased in most of the groups of patients infected with the studied fungi, and a statistical significance ($p = 0.0007$) was found among the eight groups of individuals studied. Both fungi induced the production of SP-A in non-HIV patients (Non-HIV-Hc and Non-HIV-Pj), whereas in HIV patients infected with *P. jirovecii*, the groups HIV-Hc-Pj and HIV-Pj showed lower SP-A mean values (Figure 1). Notably, Non-HIV-Hc, Non-HIV-Pj and HIV-Pj groups developed higher SP-A mean values than HIV-Hc patients ($p < 0.05$), which presented the lowest

value (Figure 1). Control groups (Healthy and HIV w/o fungi) showed similar levels of SP-A. In contrast, SP-D values decreased in all the groups of patients when compared with the healthy volunteers, and no significance ($p = 0.4397$) was found among the eight groups studied (Figure 1).

Cytokine and chemokine determination

Data concerning the quantification of cytokines and chemokines in the BAL samples of all the studied groups of individuals are reported in Table 3. This table underlines that most cytokines and chemokines were undetectable or developed their lowest mean values in the control groups (Healthy and HIV w/o fungi).

In regard to pro-inflammatory cytokines, IL-1β was significantly increased ($p = 0.0002$) in all the groups of patients studied. In general, in the groups of patients with *H. capsulatum* infection, either alone or with *P. jirovecii*, the values of IL-1β were always higher, predominantly in the Non-HIV-Hc group ($p < 0.05$) when compared with the Healthy and the HIV-Pj groups, as can be seen in Figure 2. This figure also shows a significant difference ($p = 0.0045$) among the mean values of TNF-α in all the studied groups; however, in the individual analyses, although there were no significant differences between the groups of patients, the Non-HIV-Hc group showed the highest TNF-α mean value, whereas in the other groups of patients, it was scarcely detected. It is important to emphasize that IL-1β and TNF-α were undetectable in the Healthy group (Figure 2).

In regard to the axis IFN-γ/IL-12, we determined the participation of IFN-γ, IL-12p70 and IL-18 and statistical analyses revealed significant differences for IFN-γ and IL-18 ($p = 0.0003$ and $p = 0.0420$, respectively) between the mean values of all groups (Figure 3). A significant increase ($p < 0.05$) of IFN-γ was found between Non-HIV-Hc or Non-HIV-Pj

groups when compared with the Healthy group, as well as between Non-HIV-Hc and HIV-Pj groups. It is noteworthy that the non-HIV groups of patients developed higher mean values of IFN-γ and that this cytokine was undetectable in the Healthy group. Overall, most groups of patients infected with *H. capsulatum* (HIV-Hc, Non-HIV-Hc, and Non-HIV-Hc-Pj groups) showed the highest mean values of IL-12p70, whereas the infected individuals with *P. jirovecii* (HIV-Pj and Non-HIV-Pj groups) developed lower IL-12p70 mean values than the control groups (HIV w/o fungi and Healthy), as can be shown in Table 3. In regard to IL-18, individual comparisons between groups did not show differences, although the Non-HIV-Hc group showed the highest mean value (Figure 3).

Regarding the axis IL-17/IL-23, we determined the participation of IL-6, IL-17A and IL-23, and significant differences between all groups ($p = 0.0118$) were only found in the analysis of IL-17A mean values (Figure 4). For IL-6, it is remarkable that the non-HIV patients (Non-HIV-Hc, Non-HIV-Pj, and Non-HIV-Hc-Pj groups) showed higher values than the other groups. A similar pattern was observed for IL-17A, where the individual analyses revealed significant differences ($p < 0.05$) between Non-HIV-Hc or Non-HIV-Pj and the Healthy group. Even though IL-23 developed high mean values in HIV and non-HIV patients infected with *H. capsulatum* (HIV-Hc and Non-HIV-Hc groups) and, in particular, it increased in the HIV-Pj group, no differences were found in the individual analysis for each group (see Figure 4).

Among cytokines related to granuloma formation, IL-33, IL-5, and IL-13 were measured in the BAL samples, showing significant differences for IL-33 and IL-13 in the analyses of all groups ($p = 0.0201$ and $p = 0.0018$, respectively). The highest mean value of IL-33 was found in the Non-HIV-Hc group, and decreased levels were associated with all HIV groups, although the only significant difference ($p < 0.05$) was found between in the Non-HIV-Pj

and Healthy groups (Figure 5). Concerning IL-5, the HIV-Pj group and most non-HIV groups (except Non-HIV-Hc-Pj group) developed high mean values, in contrast with the Healthy group, where this cytokine was not detected; however, no differences were found in the individual analyses for each group. A significant difference ($p < 0.05$) was only detected between the Non-HIV-Hc or Non-HIV-Pj groups and the Healthy group and, in general, most groups of patients showed decreased IL-13 levels when compared with the Healthy group (Figure 5).

Among the anti-inflammatory cytokines, we only detected IL-10 and results revealed that most groups developed low values of IL-10, except for the HIV-Pj group, when compared with the healthy volunteers group, although no statistical differences ($p = 0.1609$) were found (Figure 6).

In regard to chemokines, we detected CXCL8 and CCL2. Figure 7 shows a significant difference ($p < 0.0001$) among the mean values of all the studied groups only for CXCL8, since no differences ($p = 0.4348$) were found for CCL2 values. The mean values of this chemokine were significantly increased ($p < 0.05$) in all non-HIV groups when compared with the Healthy group, which was undetectable. Interestingly, CXCL8 mean values were higher in non-HIV patients that in HIV groups. However, those HIV groups infected with *H. capsulatum* (HIV-Hc and HIV-Hc-Pj) developed higher CXCL8 values than the HIV-Pj group, although significance was not reached. Significant differences ($p < 0.05$) were also found for CXCL8 mean values between the Non-HIV-Hc or Non-HIV-Pj groups and the HIV-Pj group, which developed the lowest values within the groups of patients (Figure 7). Similarly to CXCL8, CCL2 was increased in the non-HIV groups infected with one single fungal pathogen (Non-HIV-Hc and Non-HIV-Pj groups), and remarkably, in the HIV-Hc

group, although no significant differences were found in the individual analyses for each group of patients (Figure 7).

DISCUSSION

This study describes the collectin, cytokine and chemokine profiles in histoplasmosis, pneumocystosis or their coinfection, showing the human pulmonary immune response against both mycoses. The results reached here were similar to those previously reported from animal models, but one of the most remarkable features of this study was the analysis of human BAL samples from HIV and non-HIV patients with a pulmonary disease, infected or not with *H. capsulatum* and *P. jirovecii*. Unfortunately, in human patients, it is hard to precise the timing of infection, unless there is a strong epidemiological evidence; thus, these incognita might contribute to bias on data interpretation about the course of the host immune response.

Several inflammatory mediators have been described in the immune response to histoplasmosis and pneumocystosis, including different settings of both mycoses, as their diverse clinical forms and distinct mammal hosts.[51,52] Because SP-A and SP-D play important roles in lung immunity we investigated whether human *H. capsulatum* and *P. jirovecii* infections affected the production of these collectins.

Collectins SP-A and SP-D interact with pathogens through their carbohydrate recognition domain, damaging the pathogen's cell wall or membrane and stimulating alveolar macrophages to induce phagocytosis.[53] These collectins have been scarcely studied in *H. capsulatum* and *P. jirovecii* infections and their simultaneous detection in coinfected patients was never considered before. We analyzed SP-A and SP-D in BAL samples from

HIV and non-HIV patients infected with *H. capsulatum*, *P. jirovecii*, and for the first time, they were studied in patients coinfected with both fungi. Previous reports emphasized an enhanced SP-A production during *Pneumocystis* sp. infection in mouse[24] and rat models,[54] as well as in humans,[55] and for human *H. capsulatum* infection our result is a novel finding. In general, we found an increase of SP-A in non-HIV patients both with histoplasmosis or pneumocystosis and, in these patients, it was possible to discriminate the participation of each fungus in inducing the host SP-A production. Interestingly, when coinfection was present SP-A levels decreased considerably, suggesting an *in vivo* antagonism of both fungi in the induction of SP-A or, possibly, most of the generated SP-A molecules were adhered to the surfaces of both fungi, which masked its determination in BAL fluid. In contrast, in HIV patients, a lower SP-A detection could suggest an HIV-associated dysfunction in SP-A production or the presence of high fungal burden, which is frequently found in HIV/AIDS patients, where the fungal surfaces are plenty adhered with SP-A molecules, making undetectable the soluble SP-A.

In regard to SP-D, previous reports showed an increase of SP-D levels during *P. carinii* (now *P. murina*) infection.[54] Here, the decreased values of SP-D, either in HIV or non-HIV patients, contrasted with a previous report in AIDS patients without pulmonary disease.[56] The participation of pro-inflammatory and anti-inflammatory cytokines, as well as chemokines, was accurately detected in BAL samples collected in well-standardized conditions. In regard to cytokines detection, significant differences for IL-1β, IFN-γ, IL-18, IL-17A, IL-33, and IL-13 were found between all the groups studied, suggesting a probable role of these cytokines in the inflammatory process associated with histoplasmosis and pneumocystosis.

In general, we found increased levels of IL-1β and TNF-α in non-HIV patients infected with *H. capsulatum*. It is well known that this fungus promotes a Th1 response in murine models,[57] favoring IL-1β, TNF-α, GM-CSF, and IFN-γ production in the host for histoplasmosis control.[58] TNF-α is also the most prominent proinflammatory mediator in the response to microorganisms, including fungi.[59] Although we found no significant differences between individual groups for TNF-α mean values, the contrasting increased values of TNF-α from the Non-HIV-Hc group could be supported by the pro-inflammatory Th1 response for histoplasmosis.[57,60]

Particularly, among the cytokines related to the IFN-γ/IL-12 axis (IFN-γ, IL-12 and IL-18), the *H. capsulatum* infected groups of patients showed the highest mean values in the BAL samples, especially in the Non-HIV-Hc group. These results match with the local production of a Th1 inflammatory response against *H. capsulatum* in non-HIV patients; although in HIV patients IFN-γ, IL-12 and IL-18 levels were also detectable. In contrast, the inflammatory response to human pneumocystosis has been mainly studied within its association with HIV/AIDS patients.[61] Clinical studies show that lung inflammation becomes part of the complicated and chronic disease caused by *P. jirovecii*.[61,62] In this study, among the cytokines of the IFN-γ/IL-12 axis, we only found an increase in IFN-γ levels in HIV and non-HIV *P. jirovecii* infected groups, and even though Th1 response is absent in HIV patients, IFN-γ could be produced by natural killer cells. Cytokines related to the IFN-γ/IL-12 axis showed lower levels in the groups of patients co-infected with both fungi, suggesting an antagonistic interaction between *H. capsulatum* and *P jirovecii* for their production.

Concerning the IL-17/IL-23 axis, it has been reported that IL-6 is produced in mice lung homogenates in response to *H. capsulatum* infection[65] and also in type II pneumocytes

(human cell line A549) when exposed to *Pneumocystis'* proteins.[66] These findings were similar to those described here in the non-HIV groups of patients infected with *H. capsulatum* and *P. jirovecii*, respectively, although no significant differences were found. However, a significant increase of IL-17A in these groups of patients was present when compared to the Healthy group, suggesting the establishment of a local inflammatory response to both fungi in non-HIV patients (see Figure 4). Considering that a Th17 response is elicited after *H. capsulatum* infection,[57] that IL-17A has been detected in experimental histoplasmosis infection,[65] and that this cytokine could also be produced by other cell types,[67] a higher concentration of IL-17A was found in the groups of patients with histoplasmosis, mainly in the Non-HIV-Hc group, as expected. A remarkable finding in our study was the important pulmonary immune response to *P. jirovecii* associated with IL-17A production.

Cytokines involved in granuloma development were evaluated, primarily considering the chronic inflammation depicted in *H. capsulatum* infection. In this study, IL-33, IL-5 and IL-13 were selected based on previous data involving fungal infections.[68-70] In particular, IL-33 plays an important role in granuloma formation during the late adaptive immune response to *H. capsulatum* in a murine model[68] and according to our results the Non-HIV-Hc group showed high levels of IL-33, which could associate it with granulomas production in this group of patients. In contrast, IL-33 was low in the HIV-Hc group, which suggests an interference of the HIV condition with the production of IL-33, avoiding the chronic granulomatous reaction in these patients. In general, granulomas are not related to *P. jirovecii* infection, although some case reports describe an atypical granulomatous reaction to pneumocystosis in non-HIV patients cursing different immunosuppressive conditions.[71-75] In the histoplasmosis infection, IL-5 was elevated in an *Histoplasma-*

induced granuloma in C57BL/6 mice,[76] and even though we found increased levels of this cytokine in the BAL samples of the Non-HIV-Hc group, it was not significant. Increased levels of IL-5 in serum have been reported in human[77] and murine pneumocystosis;[78] although we found increased levels of IL-5 in BAL samples of the *P. jirovecii* infected groups (HIV and non-HIV) no significance was reached. Although low levels of IL-13 were reported in the *Histoplasma*-induced granuloma in mice,[76] significant levels of this cytokine detected in the present study in the Non-HIV histoplasmosis and pneumocystosis patients (Non-HIV-Hc and Non-HIV-Pj groups) could be related to its role in the development of granulomas in these individuals.

In regard to the anti-inflammatory IL-10, a cytokine of Th2 immune response, which participates in the lung damage in the *H. capsulatum*[57] and *P. jirovecii*[36] infections, its absence in most of BAL samples could be explained by the clinical-immunological status of the studied patients. Recently, Rodriguez-Ramirez et al.[79] have suggested that the presence of IL-10 inside the coccidioidal granulomas could be associated to the fatal outcome of coccidioidomycosis.

A remarkable finding in most cytokines response to *H. capsulatum* and *P. jirovecii* infection was related to the coinfection with both pathogens, where high levels of cytokines were found for histoplasmosis patients whereas pneumocystosis patients showed lower cytokine levels. Interestingly, in coinfected patients, the cytokine response was similar to pneumocystosis alone, suggesting an immunomodulation ability of *P. jirovecii* over *H. capsulatum* response, irrespective of the presence of HIV condition.

Among the two chemokines determined for this study only CXCL8 showed significant levels in the non-HIV histoplasmosis and pneumocystosis patients (Non-HIV-Hc, Non-HIV-Pj and Non-HIV-Hc-Pj groups), suggesting the development of a local inflammatory

response to these fungal infections. This is the first time that this chemokine is reported in human histoplasmosis; however, when exposed to *Pneumocystis* surface proteins, the human-pneumocytes cell line A549 produces CXCL8 and CCL2.[80] In our study, most HIV-patients showed low levels of CXCL8 and CCL2, a finding that might correlate to the HIV/AIDS condition of these patients. In histoplasmosis murine models, CCL2 was associated with IL-4 production through a regulated action of its receptor (CCR2),[81] whereas in pneumocystosis murine models, CCL2 and other chemokines (CCL3, CCL5 and cytokine-induced neutrophil chemoattractant) promoted a hyperinflammatory state conducting more lung damage after infection;[82] however, in immunocompromised mice these chemokines remained at low levels during *Pneumocystis* infection.[83]

In conclusion, this study reveals novel findings on the profiles of several inflammatory molecules during the infection of *H. capsulatum*, *P. jirovecii* and their coinfection, which are important in different groups of patients (HIV and non-HIV); moreover, a probable immunomodulatory action of *P. jirovecii* has been suggested according to our results when both parasites coexist within the same host. However, the balance of cytokines and chemokines along the clinical course of these fungal diseases remains as a naïve field to be explored.

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AUTHOR CONTRIBUTION

LECB and MLT conceived and designed the study, and coordination and helped to draft the manuscript. LECB and FRMV collected the samples. LECB and EPT performed the experimental procedures. MLT, LECB, and EPT analyzed the data. EMA, EZ and JAMO drafted the manuscript and revised it critically for important intellectual content. JAMO and MLT share the academic responsibility for this manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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TABLE 1. Studied groups of individuals

GROUP	NUMBER
Healthy volunteers (Healthy, control)	8
HIV-positive without the studied fungi (HIV w/o fungi, control)	19
HIV-positive with histoplasmosis (HIV-Hc)	12
HIV-positive with pneumocystosis (HIV-Pj)	32
HIV-positive with co-infection (HIV-Hc-Pj)	7
HIV-negative with histoplasmosis (Non-HIV-Hc)	35
HIV-negative with pneumocystosis (Non-HIV-Pj)	15
HIV-negative with co-infection (Non-HIV-Hc-Pj)	3
TOTAL	131

Abbreviations in parenthesis are the same as illustrated in all figures.

TABLE 2. Detection of SP-A and SP-D in BAL samples.

GROUP OF INDIVIDUALS	SP-A in ng/mL Mean value (range)	SP-D in ng/mL Mean value (range)
Healthy, control	1.692 (1.208-2.151)	210.4 (63.22-311.0)
HIV w/o fungi, control	1.428 (0-4.042)	147.1 (0-304.9)
HIV-Hc	0.3841 (0-1.950)	159.6 (32.55-310.3)
HIV-Pj	3.312 (0-22.13)	177.4 (0-1573.0)
HIV-Hc-Pj	2.296 (0-4.705)	107.8 (0-211.9)
Non-HIV-Hc	20.45 (0-498.3)	140.8 (0-320.8)
Non-HIV-Pj	4.1 (0-12.48)	117.6 (35.06-322.2)
Non-HIV-Hc-Pj	0.9296 (0-1.859)	113.5 (0-227.1)

Abbreviations of each group of individuals are the same as illustrated in all figures. SP-A and SP-D were determined by separate BioAssay ELISA Kits in BAL samples of hospitalized patients (details in Methods).

TABLE 3. Detection of cytokines and chemokines in BAL samples.

GROUP OF INDIVIDUALS	IL-1β (pg/mL) Mean value (range)	TNF-α (pg/mL) Mean value (range)	IFN-γ (pg/mL) Mean value (range)	IL-12p70 (pg/mL) Mean value (range)	IL-18 (pg/mL) Mean value (range)	IL-6 (pg/mL) Mean value (range)	IL-17 (pg/mL) Mean value (range)
Healthy, control	Undetectable	Undetectable	Undetectable	9.001 (0-33.24)	Undetectable	6.046 (0-16.29)	1.091 (0-7.67)
HIV w/o fungi, control	1.298 (0-3.35)	0.05436 (0-0.6388)	0.9466 (0-5.946)	3.583 (0-67.17)	3.168 (0-50.68)	7.089 (0-65.69)	14.99 (0-91.52)
HIV-Hc	134.5 (0-1592.0)	Undetectable	0.6183 (0-3.741)	13.35 (0-89.47)	2.234 (0-13.71)	12.03 (0-53.82)	37.2 (0-304.4)
HIV-Pj	4.445 (0-88.23)	0.5006 (0-14.4)	1.444 (0-12.32)	0.06805 (0-0.8387)	3.313 (0-19.68)	8.191 (0-39.7)	17.38 (0-131.8)
HIV-Hc-Pj	19.67 (0-124.5)	0.5393 (0-3.269)	1.716 (0-6.862)	0.2037 (0-0.6646)	0.6534 (0-2.253)	7.598 (0-34.57)	25.47 (0-72.63)
Non-HIV-Hc	21.89 (0-354.8)	38.63 (0-1132.0)	8.431 (0-94.2)	33.83 (0-386.7)	31.24 (0-885.3)	163.2 (0-1710.0)	108.1 (0-1700.0)
Non-HIV-Pj	1.615 (0-5.665)	1.153 (0-12.68)	3.053 (0-3.870)	0.6786 (0-9.144)	3.715 (0-15.21)	435.9 (0-5944.0)	25.17 (0-82.48)
Non-HIV-Hc-Pj	6.209 (0.7491-15.53)	4.74 (0-14.18)	2.498 (0-7.493)	36.86 (0-110.5)	0.4064 (0-1.219)	47.52 (0-140.8)	12.66 (0-37.99)

TABLE 3. Detection of cytokines and chemokines in BAL samples (continued).

GROUP OF INDIVIDUALS	IL-23 (pg/mL) Mean value (range)	IL-33 (pg/mL) Mean value (range)	IL-5 (pg/mL) Mean value (range)	IL-13 (pg/mL) Mean value (range)	IL-10 (pg/mL) Mean value (range)	CXCL8 (pg/mL) Mean value (range)	CCL2 (pg/mL) Mean value (range)
Healthy, control	Undetectable	7.809 (7.774-7.864)	Undetectable	7.809 (7.774-7.864)	13.56 (0-60.51)	Undetectable	4.602 (3.75-6.196)
HIV w/o fungi, control	9.771 (0-146.3)	3.221 (0-7.907)	1.946 (0-32.4)	5.587 (0-40.56)	0.01971 (0-0.2381)	160.8 (0-1705.0)	45.62 (0-624.0)
HIV-Hc	230.3 (0-2631.0)	2.083 (0-8.256)	0.4487 (0-2.903)	2.918 (0-8.256)	Undetectable	573.1 (0-3909.0)	130.6 (0-1188.0)
HIV-Pj	1001.0 (0-16209.0)	3.725 (0-8.256)	23.01 (0-377.3)	3.823 (0-8.256)	111.2 (0-3558.0)	2.942 (0-40.3)	46.21 (0-1188.0)
HIV-Hc-Pj	394.8 (0-2631.0)	2.373 (0-8.256)	0.3792 (0-2.481)	2.821 (0.1403-8.256)	0.1118 (0-0.5719)	1082.0 (0-7119.0)	663.1 (2.23-2443.0)
Non-HIV-Hc	258.9 (0-8961.0)	10.92 (0-112.3)	172.6 (0-3956.0)	2.147 (0-26.83)	14.18 (0-396.8)	1966.0 (0-12752.0)	1166.0 (0-10814.0)
Non-HIV-Pj	1.507 (0-12.18)	2.805 (0-40.0)	169.9 (0-2371.0)	0.6418 (0-1.773)	0.01045 (0-0.1499)	2171.0 (2.058-9771.0)	1554.0 (0-12040.0)
Non-HIV-Hc-Pj	1.149 (0-2.795)	7.698 (0-22.73)	0.02881 (0-0.08642)	25.52 (0.3631-74.67)	0.005122 (0-0.01537)	2678.0 (46.52-7072.0)	15.16 (10.4-23.73)

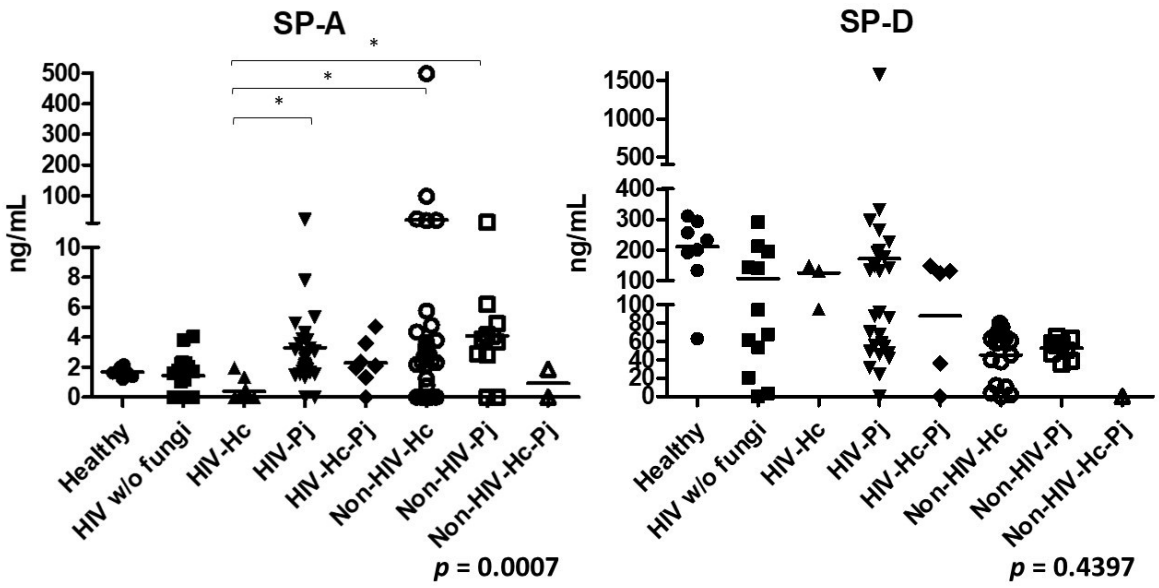

Figure 1. Surfactant proteins determination. Pulmonary SP-A and SP-D were measured by ELISA in the BAL samples of hospitalized patients. Mean values and standard deviation are shown for A) SP-A and B) SP-D. *$p < 0.05$.

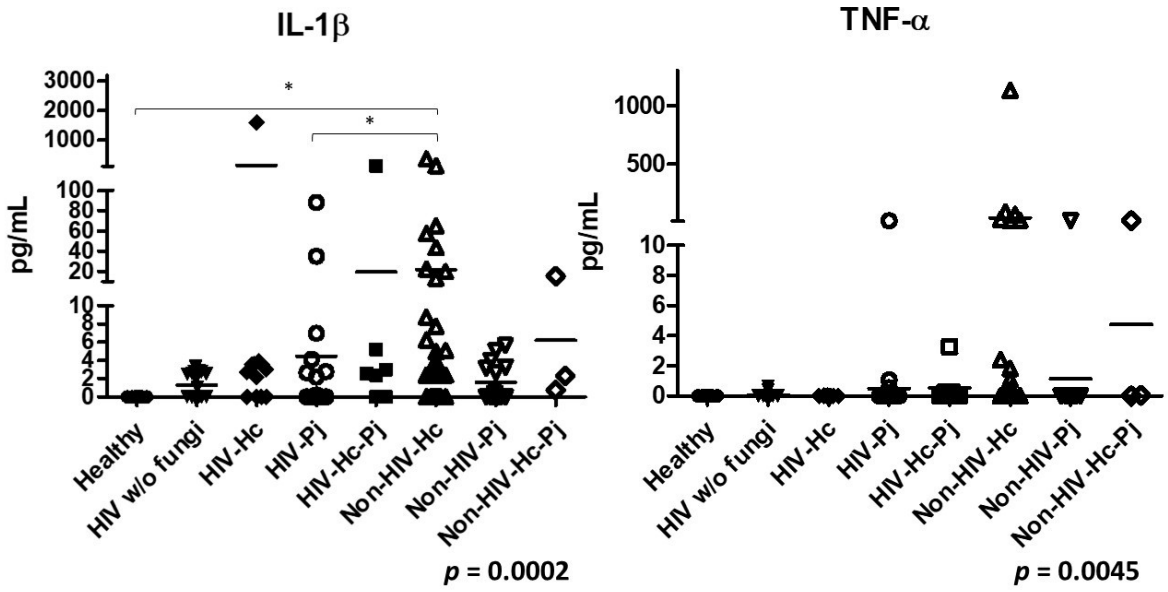

Figure 2. IL-1β and TNF-α pro-inflammatory cytokines determination. These cytokines were detected by a cytometric bead assay in the BAL samples of hospitalized patients. Mean values and standard deviation are shown for A) IL-1β and B) TNF-α. *$p < 0.05$.

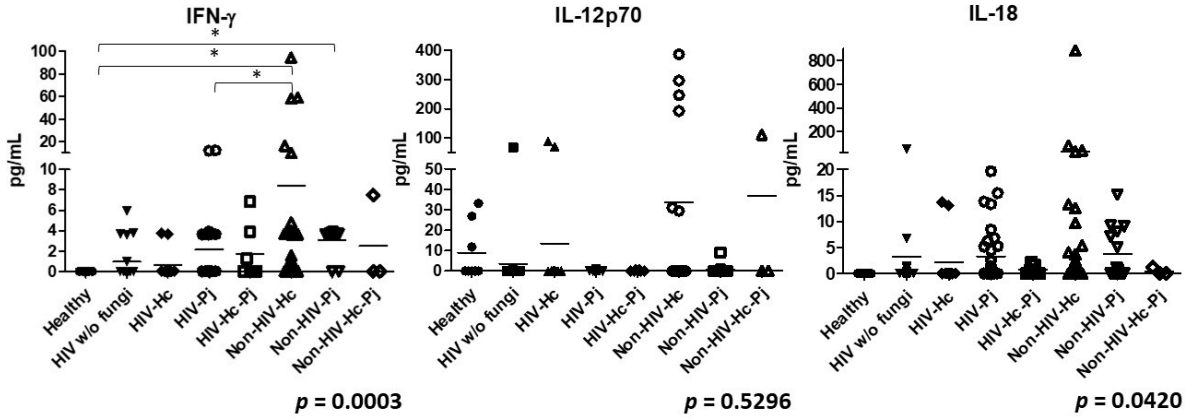

Figure 3. Determination of cytokines that belong to the axis IFN-γ/IL-12. IFN-γ, IL-12p70, and IL-18 were detected by a cytometric bead assay in the BAL samples of hospitalized

patients. Mean values and standard deviation are shown for A) IFN-γ, B) IL-12p70, and C) IL-18. *$p < 0.05$.

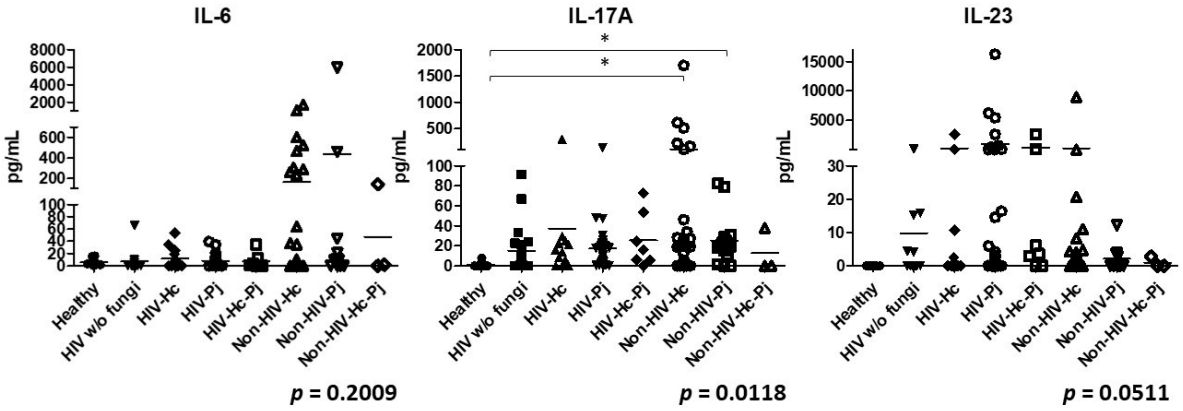

Figure 4. Determination of cytokines that belong to the axis IL-17/IL-23. IL-6, IL-17A, and IL-23 were detected by a cytometric bead assay in the BAL samples of hospitalized patients. Mean values and standard deviation are shown for A) Il-6, B) IL-17A, and C) IL-23. *$p < 0.05$.

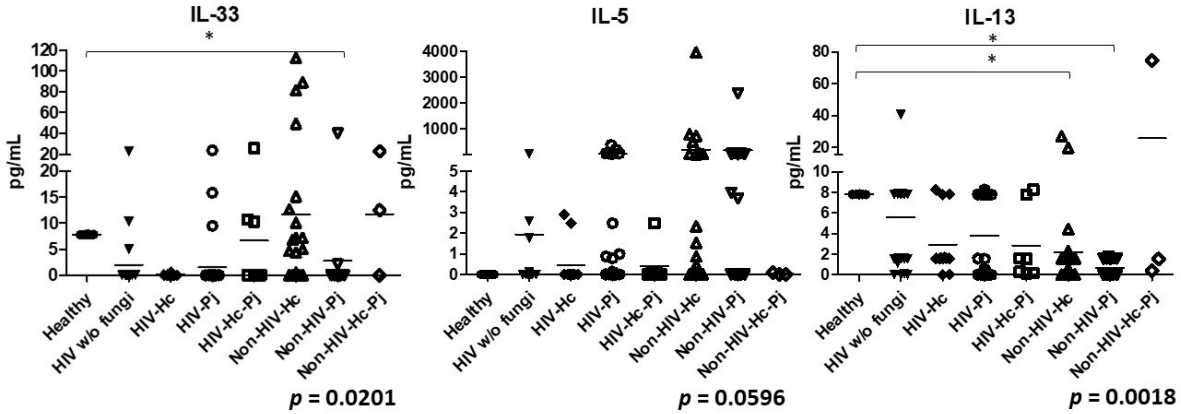

Figure 5. Determination of cytokines associated to granuloma formation. IL-33, IL-5, and IL-13 were detected by a cytometric bead assay in the BAL samples of hospitalized patients. Mean values and standard deviation are shown for A) IL-33, B) IL-5, and C) IL-13. *$p < 0.05$.

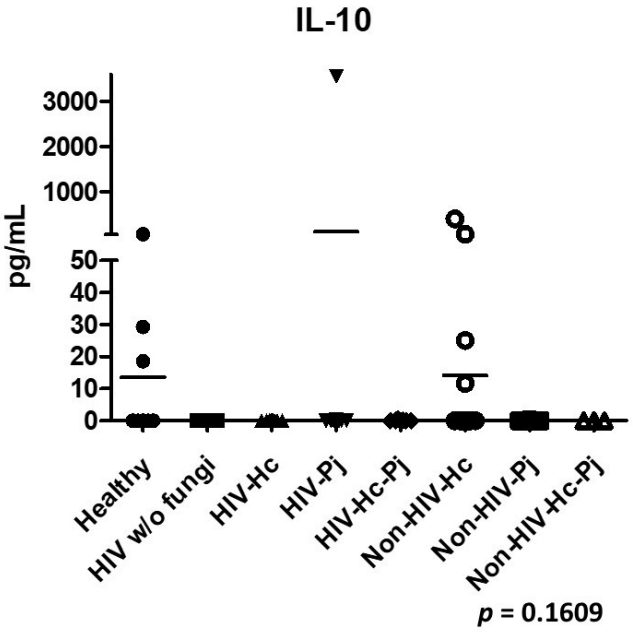

Figure 6. Determination of IL-10. This anti-inflammatory cytokine was detected by a cytometric bead assay in the BAL samples of hospitalized patients. Mean values and standard deviation are shown.

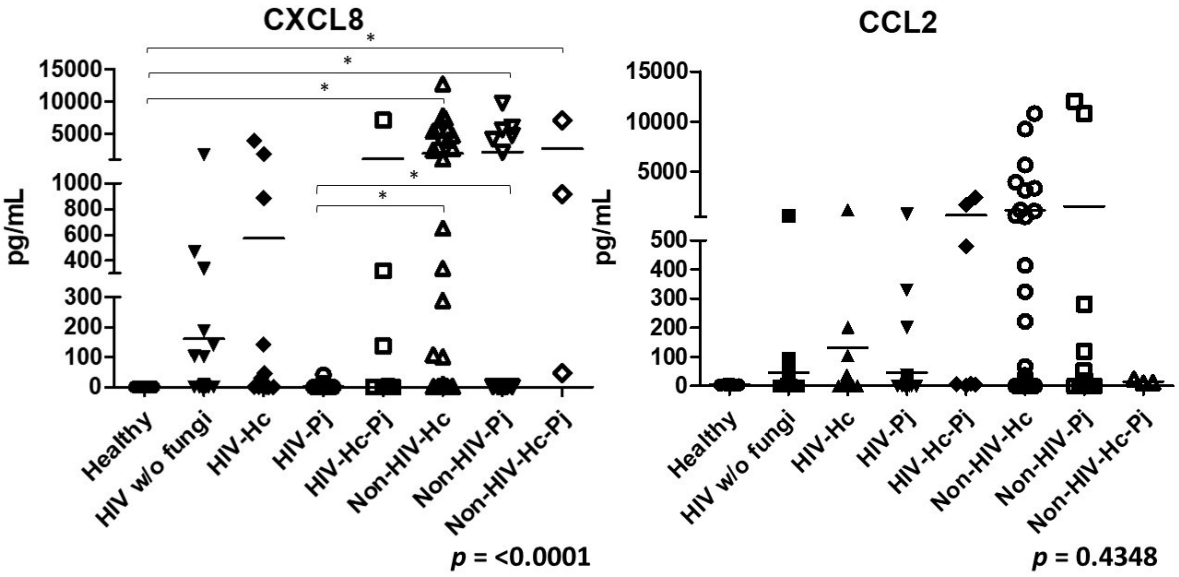

Figure 7. Chemokines determination. CXCL8 and CCL2 were detected by a cytometric bead assay in the BAL samples of hospitalized patients. Mean values and standard deviation are shown for A) CXCL8, and B) CCL2. *$p < 0.05$.

CAPÍTULO 6
DISCUSIÓN

Este trabajo destaca la importancia de la co-infección con dos hongos que causan padecimientos respiratorios (*H. capsulatum* y *P. jirovecii*) en una población de pacientes internados en un hospital de tercer nivel. La mayoría de los pacientes co-infectados eran VIH positivos, aunque también se detectó co-infección en pacientes sin VIH que no manifestaban alguna condición de inmunocompromiso. Este estudio es el primero en buscar de manera intencionada a *H. capsulatum* y *P. jirovecii* infectando al mismo individuo. Es importante resaltar el gran número de muestras analizadas, que permitió encontrar un número considerable de ambas infecciones de manera individual, aumentando la probabilidad de co-infección. Los hallazgos reportados en la presente tesis demuestran que esta co-infección es un problema médico más común de lo esperado, no sólo en pacientes inmunocomprometidos (Carreto-Binaghi et al. 2019; Nevez y LeGal 2019). Por tal motivo, se considera que el diagnóstico de esta co-infección es crítico para el establecimiento de estrategias terapéuticas adecuadas para eliminar ambos patógenos. En este estudio se resalta la importancia del diagnóstico molecular de las infecciones por *H. capsulatum* y *P. jirovecii*, considerando que pocos estudios han utilizado estas técnicas como un tamizaje de estos patógenos. Sin embargo, se debe mencionar que los métodos de PCR utilizados tienen una alta sensibilidad, especificidad y reproducibilidad, que los hacen ideales para el diagnóstico de estas micosis, llegando a detalles más precisos como la secuenciación de los amplicones y su análisis filogenético para corroborar la presencia de cada hongo con certeza (Carreto-Binaghi et al. 2019).

Previamente, la co-infección de *H. capsulatum* y *P. jirovecii* fue detectada por casualidad, principalmente al describir infecciones oportunistas en pacientes con VIH; en estos casos, la co-infección podría explicarse por el inmunocompromiso ocasionado por el virus, que hace a los pacientes más susceptibles a infecciones concomitantes. Carreto-Binaghi et al. (2019), estimó la prevalencia de esta co-infección en pacientes con VIH, con base en datos publicados en la literatura, la cual varió desde 0.55 hasta 13.3%. Los resultados aquí encontrados están dentro de este rango, considerando que la tasa de co-infección reportada en este estudio fue de 10.7% (9 de 84 pacientes con VIH). Además, es indispensable subrayar que la tasa de mortalidad asociada con la co-infección fúngica fue mayor que la encontrada para la histoplasmosis y la pneumocistosis en forma individual, aunque no hubo diferencias estadísticamente significativas entre los grupos, la presencia de co-infección sugiere una probable contribución de ambos patógenos en el curso fatal de la enfermedad.

Se han descrito varios mediadores inflamatorios de la respuesta inmune a la histoplasmosis y la pneumocistosis, tanto en las diferentes formas clínicas como en distintos huéspedes mamíferos. Este estudio destaca, en la respuesta inmune pulmonar, los perfiles de colectinas y citocinas de los pacientes hospitalizados con infección de *H. capsulatum*, *P. jirovecii* o ambos patógenos. Una de las características más importantes a señalar fue la inclusión, en el análisis de muestras de BAL, de pacientes sin VIH que cursaban con una enfermedad pulmonar asociada y que fueron discriminados en diversos grupos mediante las pruebas de PCR ya descritas. Los resultados reportados aquí para la respuesta inmune pulmonar fueron similares a los que se han registrado en modelos animales (ver artículo del capítulo 5); desafortunadamente, en humanos, es difícil precisar el momento de la infección de ambos patógenos, excepto para el caso de *H. capsulatum* cuando existen fuertes datos epidemiológicos que respaldan el origen y fecha de infección; aun así, estás incógnitas podrían contribuir a un sesgo en la interpretación de datos sobre el curso de la respuesta inmune del huésped.

En este trabajo se investigó si las infecciones humanas por *H. capsulatum* y *P. jirovecii* afectaban la producción de las colectinas pulmonares SP-A y SP-D, debido a que éstas juegan un papel importante en la inmunidad pulmonar al interactuar con patógenos a través de su dominio de reconocimiento de carbohidratos. Estas colectinas pueden generar un daño directo a la pared o membrana celular de los patógenos, pero también estimulan a los macrófagos alveolares para inducir fagocitosis. Previamente, se reportaron algunos datos sobre estas colectinas en las infecciones por *H. capsulatum* (Brummer y Stevens 2010; McCormack et al. 2003) y *P. jirovecii*; (Vuk-Pavlovic et al. 2001; Zimmerman et al. 1992); sin embargo, la detección simultánea de estas proteínas surfactantes en pacientes co-infectados nunca había sido considerada. Estudios previos han referido una mayor producción de SP-A durante la infección por *Pneumocystis* sp. en modelos animales (rata y ratón) y en la enfermedad humana (Phelps et al. 1996, Qu et al. 2001), de modo similar a lo descrito en este trabajo en el modelo humano; sin embargo, los resultados de incremento de SP-A en pacientes sin VIH con histoplasmosis aquí reportados representan un hallazgo novedoso en la infección humana por *H. capsulatum* (ver artículo del capítulo 5). Es interesante resaltar que, en pacientes co-infectados por ambos hongos, los niveles de SP-A disminuyeron considerablemente, lo que sugiere un antagonismo *in vivo* de ambos hongos en la inducción de SP-A o una adhesión masiva de las moléculas de SP-A a las superficies de ambos hongos, lo que evitaría su determinación en la muestra de BAL. En general, en los pacientes con VIH con o sin infecciones con ambos hongos se detectaron menores cantidades de SP-A (ver artículo del capítulo 5), lo que podría sugerir una disfunción en la producción de SP-A asociada al virus del VIH o la posible presencia de una alta carga fúngica, donde las moléculas solubles de SP-A se adhirieron a las superficies fúngicas, haciéndola indetectable. Estudios

previos en ratas mostraron un aumento de los niveles de SP-D durante la infección por *P. carinii* (ahora *P. murina*) (Atochina et al. 2001); sin embargo, en el presente trabajo se encontraron valores disminuidos de SP-D en pacientes con y sin VIH que presentaban infección por *P. jirovecii* (ver artículo del capítulo 5).

Con respecto a la participación de las citocinas en las infecciones por *H. capsulatum* y *P. jirovecii*, este trabajo destaca de modo inusitado la posible influencia de la co-infección en la manifestación del perfil de algunas citocinas de los pacientes con esta condición. Los presentes resultados reportan diferencias significativas para IL-1β, IFN-γ, IL-18, IL-17A, IL-33 e IL-13 entre todos los grupos estudiados (ver artículo del capítulo 5), lo que sugiere un papel de estas citocinas en el proceso inflamatorio asociado con las infecciones por *H. capsulatum* y *P. jirovecii*. En general, se reportaron mayores niveles de IL-1β y TNF-α en pacientes sin VIH infectados con *H. capsulatum* (ver artículo del capítulo 5), lo que concuerda con la respuesta Th1 de modelos murinos previamente descrita para este patógeno, donde se favorece la producción de IL-1β, TNF-α, GM-CSF e IFN-γ (Kroetz y Deepe 2012). Aunque el presente trabajo no reveló diferencias significativas entre los grupos estudiados para los valores de TNF-α, el grupo de pacientes sin VIH infectados con *H. capsulatum* mostró un aumento de los valores de esta citocina (ver artículo del capítulo 5), lo que corresponde al perfil Th1 previamente descrito por Allendoerfer y Deepe (1998), considerando que TNF-α es el mediador proinflamatorio más destacado en la respuesta a microorganismos, incluidos los hongos. Entre las citocinas relacionadas con el eje IFN-γ/IL-12, se analizaron IFN-γ, IL-12 e IL-18, y los grupos de pacientes infectados por *H. capsulatum* desarrollaron valores más altos, especialmente en los pacientes sin VIH y con histoplasmosis (ver artículo del capítulo 5), resultados que coinciden con la producción local de una respuesta inflamatoria mediada por Th1 contra *H. capsulatum*. Por otro lado, la respuesta inflamatoria asociada al eje IFN-γ/IL-12 en la pneumocistosis humana muestra que la inflamación pulmonar es parte de la enfermedad complicada y crónica. En este estudio, se demostró un aumento en los niveles de IFN-γ en los grupos con y sin VIH infectados por *P. jirovecii* (ver artículo del capítulo 5), aunque se ha descrito que la respuesta Th1 está ausente en pacientes con VIH, sin embargo, la producción de esta citocina podría estar asociada a otras células, como las NK. Interesantemente, en los grupos de pacientes con co-infección de ambos hongos se detectaron niveles más bajos de las citocinas del eje IFN-γ/IL-12 (ver artículo del capítulo 5), lo que sugiere una interacción antagónica entre *H. capsulatum* y *P. jirovecii* para su producción. Con respecto al eje IL-17/IL-23, se ha reportado en modelos animales la producción de IL-6 asociada a este eje en respuesta a la infección por *H. capsulatum* (Sahaza et al. 2015) y por *Pneumocystis* (Pottratz et al. 1998). En el presente estudio se encontró un aumento de IL-6 en los grupos de pacientes sin VIH infectados con *H. capsulatum* y *P. jirovecii*, respectivamente, aunque no presentaron diferencias

significativas. Sin embargo, un aumento significativo de IL-17A fue detectado en estos mismos grupos de pacientes, lo que coincide con las descripciones previas de una respuesta Th17 ante la infección experimental por *H. capsulatum* (Kroetz y Deepe 2012; Sahaza et al. 2015). Por otro lado, es novedoso el hallazgo de un incremento significativo en la producción de IL-17A en la respuesta pulmonar contra *P. jirovecii* (ver artículo del capítulo 5). Otras citocinas evaluadas en este estudio fueron las involucradas en el desarrollo de granuloma, considerando principalmente la inflamación crónica de la infección por *H. capsulatum*. En modelos murinos, IL-33 juega un papel importante en la formación de granuloma durante la respuesta inmune adaptativa tardía a *H. capsulatum* y, en los resultados de este trabajo, el grupo sin VIH que cursó con infección por *H. capsulatum* reveló altos niveles de IL-33, lo que podría asociarse con la producción de granulomas en este grupo de pacientes. En contraste, los niveles de IL-33 fueron bajos en el grupo de pacientes con VIH infectados por *H. capsulatum* (ver artículo del capítulo 5), lo que sugiere una alteración en la producción de IL-33 debido a la condición de VIH, evitando la formación de una reacción granulomatosa crónica en estos pacientes. Con respecto a *P. jirovecii*, los granulomas no se relacionan generalmente con esta infección, aunque algunos reportes de casos describen una reacción granulomatosa atípica a la pneumocistosis en pacientes sin VIH (Gal et al. 2002; Lauffer et al. 2004; Leroy et al. 2000; Miller et al. 1998; Oki et al. 2001). Con relación a IL-5, se encontraron niveles aumentados de esta citocina en los pacientes sin VIH infectados por *H. capsulatum* (ver artículo del capítulo 5), lo cual se ha descrito en granulomas murinos (Heninger et al. 2006); igualmente se reportaron niveles aumentados de IL-5 en los grupos con y sin VIH infectados por *P. jirovecii* (ver artículo del capítulo 5), lo cual también se ha descrito previamente en modelos murinos y en pacientes con pneumocistosis (Eddens et al. 2015; Fang et al. 1994). También entre las citocinas asociadas a la formación de granuloma, se encontró significancia para los niveles elevados de IL-13 en pacientes sin VIH infectados por *H. capsulatum* y por *P. jirovecii* (ver artículo del capítulo 5), lo que podría relacionarse con el posible desarrollo de granuloma, aunque en el caso de *Pneumocystis*, IL-13 e IL-5 podrían estar más bien relacionadas con células productoras de moco y con eosinófilos (Otieno-Odhiambo et al. 2019). Entre las citocinas antiinflamatorias, se buscó principalmente la presencia de IL-10 en las muestras de BAL, por su papel inmunoregulador ya que es importante en el balance Th1/Th2. Sin embargo, no fue posible detectar esta citocina en la mayoría de los individuos estudiados (ver artículo del capítulo 5), probablemente debido al estado inmunológico de ellos. Además, se sabe que esta citocina de la respuesta tipo Th2 participa en el daño pulmonar durante las infecciones por *H. capsulatum* y *P. jirovecii* (Kelly y Shellito 2010; Kroetz y Deepe 2012).

Un hallazgo importante de este estudio en la respuesta de la mayoría de las citocinas a la infección por *H. capsulatum* y *P. jirovecii* se relacionó con la co-

infección de ambos patógenos, considerando que en los pacientes con histoplasmosis se detectaron altos niveles de citocinas, mientras que los pacientes con pneumocistosis mostraron niveles más bajos. Curiosamente, en pacientes co-infectados, la respuesta de las citocinas fue similar a la infección única por *P. jirovecii* (ver artículo del capítulo 5), lo que sugiere una capacidad de inmunomodulación de este hongo sobre la respuesta de *H. capsulatum*, independientemente de la presencia de la condición de VIH.

Finalmente, dos quimiocinas fueron determinadas para este estudio, y sólo CXCL8 mostró un incremento significativo en los pacientes sin VIH infectados con *H. capsulatum*, *P. jirovecii* o ambos (ver artículo del capítulo 5), lo que apoya el desarrollo de la respuesta inflamatoria a nivel pulmonar contra estas infecciones fúngicas. Es importante destacar que es la primera vez que se reporta esta quimiocina en la histoplasmosis humana; sin embargo, su producción ha sido descrita en una línea de neumocitos humanos expuesta a *Pneumocystis* (Benfield et al. 1999). En este estudio, la mayoría de los pacientes con VIH mostraron niveles bajos de CXCL8 y CCL2 (ver artículo del capítulo 5), un hallazgo que podría asociarse con datos experimentales de ratones inmunocomprometidos (Wright et al. 1999).

Es crítico remarcar que en este trabajo se describen algunos resultados negativos para ciertas citocinas de algunos grupos de pacientes, las cuales no fueron detectables en las muestras de BAL a pesar de la utilización de un método muy sensible (CBA) con límites de detección muy bajos. La explicación para estos hechos puede ser la ausencia real de estas citocinas a nivel local (microambiente pulmonar) o por el tiempo de evolución clínica de los pacientes que ya no presentaban su producción. Estos hechos son variables involuntarias.

Por último, en relación con la interacción entre las proteínas surfactantes y citocinas durante la defensa inmune, se conoce que SP-A regula la función de macrófagos a través de la disminución de la actividad de cinasas necesarias para la producción de citocinas proinflamatorias; también regula y aumenta la expresión del receptor de IL-1β, asociado a la cinasa M (reguladora negativa de TLR-4); por lo que SP-A suprime la producción de TNF-α e IL-6 que se desencadena en respuesta a LPS (Nguyen et al. 2012). También se ha descrito que SP-A induce la regulación transcripcional y postraduccional de TLR-2 y TLR-4 durante la diferenciación de monocitos a macrófagos; disminuye la fosforilación del factor IκBα (regulador de la actividad de NF-κB), lo cual resulta en disminución de la secreción de TNF-α en respuesta a los ligandos de TLR. Asimismo, SP-A disminuye la fosforilación de las proteínas de señalización de NF-κB, incluyendo a la familia MAPK (Henning et al. 2008). SP-A juega un papel importante en el desarrollo de células Treg, descrito en ratones con deficiencia de SP-A, que presentaron alteración en la expresión de Foxp3 y menor producción de células Treg $CD25^{+}Foxp3^{+}$ (Mukherjee et al. 2012). En procesos infecciosos, los ratones

knockout para SP-A infectados con *Pseudomonas aeruginosa* desarrollaron menores niveles de IL-1β en comparación con los testigos, lo que sugiere que SP-A induce la aparición de IL-1β a través de la vía del inflamasoma (Ketko et al. 2013). Con relación a SP-D, se ha descrito que esta colectina unida a partículas de polen estimula la liberación de CXCL8, con un efecto antiinflamatorio, ya que esta quimiocina inhibe la liberación de histamina en los basófilos y antagoniza la producción de IgE en los linfocitos B (Schleh et al. 2010). Considerando la importancia de las proteínas surfactantes en la adecuada función de la respuesta inmune, como lo referido anteriormente, el presente estudio fue pionero en la búsqueda de una relación de las surfactantes SP-A y SP-D con la respuesta mediada por citocinas en pacientes con infecciones fúngicas asociadas. A pesar de que hubo un incremento en la producción de estas colectinas en algunos de los grupos estudiados, no se encontró una relación estrecha entre ellas y la producción de citocinas asociadas a las infecciones por *H. capsulatum* y *P. jirovecii*.

CONCLUSIONES

Los resultados de este estudio sobre la determinación de proteínas surfactantes y citocinas en pacientes con y sin VIH, infectados con *H. capsulatum* y *P. jirovecii* sustentan desde su inicio que la co-infección de ambos patógenos es más frecuente de lo esperado, no sólo en pacientes inmunocomprometidos. Además, los resultados revelan algunos hallazgos novedosos sobre el perfil de moléculas inflamatorias durante las infecciones estudiadas y su co-infección, particularmente en algunos grupos de pacientes con y sin VIH, destacando el incremento en los niveles de IFN-γ e IL-17A en los pacientes sin VIH con *P. jirovecii*. Asimismo, este trabajo permite confirmar hallazgos previamente descritos en modelos animales o en pacientes, en relación al tipo de respuesta inmune contra los patógenos que causan estas infecciones fúngicas, en grupos de pacientes con inmunocompromiso (VIH) y otros sin esta condición.

Además, de los resultados, trasciende una probable acción inmunomoduladora de *P. jirovecii* cuando ambos parásitos coexisten dentro del mismo huésped. Sin embargo, el equilibrio de citocinas a lo largo del curso clínico de estas enfermedades fúngicas sigue siendo un campo nuevo para explorar.

PERSPECTIVAS FUTURAS

En el contexto de la co-infección de *H. capsulatum* y *P. jirovecii*, sería fascinante explicar la influencia de un patógeno con respecto al otro en los tiempos de la evolución clínica de la infección; sin embargo, el presente estudio en humanos no

permitió identificar qué hongo se estableció primero en el huésped o el tiempo de interacción del primer patógeno con respecto al segundo. Es por ello que se requieren estudios en modelos experimentales en tiempo y forma de esta co-infección que permitan explicar la interacción de ambos patógenos *in vivo*.

CAPÍTULO 7
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CAPÍTULO 8
OTROS ARTÍCULOS RELACIONADOS Y PUBLICADOS DURANTE EL DOCTORADO

Carreto-Binaghi LE, Serra Damasceno L, Pitangui NS, Fusco-Almeida AM, Mendes-Giannini MJS, Zancopé-Oliveira RM, Taylor ML. Could Histoplasma capsulatum be related to Healthcare-Associated Infections? BioMed Res Int 2015; 2015:982429. doi:10.1155/2015/982429.

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Review Article

Could *Histoplasma capsulatum* Be Related to Healthcare-Associated Infections?

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Healthcare-associated infections (HAI) are described in diverse settings. The main etiologic agents of HAI are bacteria (85%) and fungi (13%). Some factors increase the risk for HAI, particularly the use of medical devices; patients with severe cuts, wounds, and burns; stays in the intensive care unit, surgery, and hospital reconstruction works. Several fungal HAI are caused by *Candida* spp., usually from an endogenous source; however, cross-transmission via the hands of healthcare workers or contaminated devices can occur. Although other medically important fungi, such as *Blastomyces dermatitidis*, *Paracoccidioides brasiliensis*, and *Histoplasma capsulatum*, have never been considered nosocomial pathogens, there are some factors that point out the pros and cons for this possibility. Among these fungi, *H. capsulatum* infection has been linked to different medical devices and surgery implants. The filamentous form of *H. capsulatum* may be present in hospital settings, as this fungus adapts to different types of climates and has great dispersion ability. Although conventional pathogen identification techniques have never identified *H. capsulatum* in the hospital environment, molecular biology procedures could be useful in this setting. More research on *H. capsulatum* as a HAI etiologic agent is needed, since it causes a severe and often fatal disease in immunocompromised patients.

1. Introduction

The term healthcare-associated infection (HAI) refers to infections associated with healthcare delivery in any setting (e.g., hospitals, long-term care facilities, ambulatory settings, and home care). This term reflects the inability to determine with certainty where the pathogen is acquired since patients may be colonized or exposed to potential pathogens outside the healthcare setting, before receiving healthcare or during healthcare delivery [1, 2].

In recent years, there has been an overall increase in HAI, which is likely a consequence of the advances in medical and surgical procedures related to specific therapies, in addition to the large number of immunocompromised patients who are hospitalized [3]. It is estimated that every day one out of 25 hospital patients has, at least, one HAI. In 2011, there were 722,000 HAI in the United States' hospitals and about 75,000 hospital patients with HAI died during their hospitalization. More than half of all HAI occurred outside the intensive care unit [4].

HAI commonly occur by direct transmission from individual to individual or through fomites manipulated by healthcare workers, as well as through surfaces and devices contaminated by biofilms (surgical instruments, catheters,

mechanical ventilation systems, and others) [5, 6]. Other mechanisms of transmission are aerial dispersion of opportunistic or environmental microorganisms and endogenous dissemination of commensal or opportunistic pathogens [7–9].

Although the role of the inanimate hospital environment in the spread of HAI has been controversial, nowadays molecular biology methodologies are being used to identify pathogens, measure the quality of environmental and hand hygiene over time, and establish a link between outbreaks and cross-transmission events, according to geographic and temporal variables [8].

Currently, changes in morbidity and mortality patterns due to aging of the world population, treatments with immunosuppressive drugs, and the use of invasive devices (particularly long-term ones) have led to a rise in the need of healthcare facilities for patients who are more susceptible to opportunistic infections [10]. Environmental disturbances associated with construction activities near health institutions pose additional airborne and waterborne disease threats for those patients who are at risk for healthcare-associated fungal infections [2]. Particularly, hospitalized patients could be exposed to infective fungal propagules such as microconidia and small hyphal fragments of *Histoplasma capsulatum* that thrive in bat and bird droppings, deposited in the surrounding hospital recreational areas.

Thus, the aims of this paper were to review the reported cases of *H. capsulatum* infections in healthcare settings, in order to propose the different factors that could be related to healthcare-associated histoplasmosis and discuss the features that could favor the presence of this fungus in the hospital environment.

2. Etiologic Agents of HAI

The etiologic agents of HAI are mainly bacteria (85%) and fungi (13%), in contrast to viruses and parasites that are rarely reported. Some environmental factors have been identified to increase the risk for fungal HAI, particularly the use of medical devices, like central venous and urinary catheters; the presence of severe cuts, wounds, and burns; stays in the intensive care unit, surgery, and hospital reconstruction works [4].

Host factors, such as extremes of age and underlying diseases, human immunodeficiency virus/acquired immune deficiency syndrome (HIV/AIDS), malignancy, and transplants, can increase susceptibility to infection, as well as a variety of medications that alter the normal flora, like antimicrobial agents, gastric acid suppressants, steroids, antirejection drugs, antineoplastic agents, and immunosuppressive drugs [2].

Most HAI associated with fungi are caused by *Candida* spp. These infections usually come from an endogenous source. However, cross-transmission via the hands of healthcare workers or contaminated devices can occur [11]. HAI outbreaks by other yeasts, such as *Malassezia* spp., *Saccharomyces* spp., and *Trichosporon* spp., have also been identified in newborns, patients with hematologic malignancies, and transplant recipients [12–18]. Mechanical ventilation, duration of hospital stay, prolonged use of intravascular catheters, parenteral lipid formulations, and prior exposure to broad spectrum antibiotics (including antifungal therapy) are important predisposing conditions identified in these outbreaks [13, 14, 16–18].

The occurrence of invasive fungal infections (IFIs) depends on several factors like the time of exposure to an infectious agent, the patient's immune status, the pathogen's virulence factors, and the host-pathogen interaction [19]. IFI associated with healthcare is mainly caused by opportunistic fungi, from endogenous or environmental sources, which form biofilms in fomites and abiotic surfaces [9].

Species of filamentous fungi, such as *Aspergillus* spp. [20, 21], *Rhizopus* spp. [22], *Rhizomucor* spp. [22, 23], *Absidia corymbifera* [22, 24], *Fusarium* spp. [25–27], *Paecilomyces* spp. [28, 29], *Curvularia* spp. [30], *Phialemonium* spp. [31–34], and *Scedosporium* spp. [35–37], have been particularly associated with HAI in patients with hematologic diseases. The most common sources reported in the above-mentioned filamentous fungal infections were contamination of medical supplies, like intravenous solutions, contact lens solutions [38, 39], bandages [24], pressure cuffs, and invasive devices (endotracheal tubes) [11, 21–23, 40]. Besides, other species of fungi such as *Aureobasidium* spp. [41], *Trichosporon* spp. [42, 43], *Rhodotorula* spp. [44–46], and *Phaeoacremonium parasiticum* [47] have been implicated in nosocomial pseudooutbreaks through contamination of endoscopes.

A very important opportunistic fungus, *Pneumocystis jirovecii*, has also been associated with HAI by person-to-person airborne transmission [48–58]. Infection by *P. jirovecii* presents as an interstitial pneumonia in immunocompromised hosts, particularly HIV patients; in this group, pneumocystosis is considered an AIDS-definitory condition, when CD4+ T lymphocytes are below 200 cells/μL [59]. Currently, an increase of pneumocystosis in non-HIV patients is being observed, especially in patients with transplants, individuals with autoimmune disorders or malignancies, and those using immunosuppressive treatments, like steroids and immunobiological drugs [50, 55, 60–62]. Molecular biology techniques have detected a high prevalence of colonization (10–55%) in immunocompromised patients and in the general population. Individuals colonized by *P. jirovecii* can be considered reservoirs and therefore contribute to the transmission of this pathogen among immunosuppressed patients in the hospital environment [62].

Other important respiratory pathogens, such as *Blastomyces dermatitidis*, *Paracoccidioides brasiliensis*, and *H. capsulatum*, have never been associated with infections in the hospital environment; however, *B. dermatitidis* has been found in pseudooutbreaks associated with contaminated bronchoscopes [63].

3. *H. capsulatum* Infection

H. capsulatum is a dimorphic fungus with a mycelial saprobe-geophilic morphotype (infective M-phase), usually found in bat and bird guano, and a yeast morphotype (parasitic and virulent Y-phase) preferentially located within phagocytes. Infection occurs through inhalation of aerosolized M-phase

propagules, mainly microconidia and hyphal fragments, accumulated in confined spaces usually inhabited by bats or birds [64].

There are eight genetic populations of *H. capsulatum* distributed worldwide [65], between the latitudes 54° North [66] and 38° South [67], suggesting a broad geographic dispersion of the pathogen. *H. capsulatum* has been found in ecological niches with special conditions: air and soil temperatures (18–28°C), humidity (>60%), and darkness (fosters sporulation). Particularly, this fungus needs the presence of high concentrations of nitrogen and phosphorus for the M-phase growth, in addition to other micronutrients, which are plentiful in bat or bird guano [64, 68–70]. Besides, this fungus' ubiquitous distribution in nature (soil, treetops, yards, and public parks, among others) makes it feasible to find the M-phase in open spaces, either in rural or urban areas around hospitals [64, 70]. In a large outbreak that occurred in Acapulco, Mexico, the presence of the fungus was revealed in ornamental potted plants, containing organic material known as compost supplemented with bat guano that is used as fertilizer [70].

Histoplasmosis is a systemic mycosis preferentially distributed in endemic areas of the Americas. Most *H. capsulatum* infections are asymptomatic. A low number of individuals develop pneumonia, which is the main clinical form in immunocompetent patients (primary pulmonary histoplasmosis) with distinctive histopathological features, like chronic granulomatous infiltrate [69]. Epidemic outbreaks of histoplasmosis are related to occupational exposure or recreational activities and affect individuals worldwide [66, 71–73]. However, this disease is one of the most common opportunistic infections among HIV/AIDS patients with CD4+ T lymphocytes below 150 cells/μL (known as AIDS-definitory condition), who may develop severe and fatal disseminated histoplasmosis [59]; approximately 30% of these patients die from this infection [74–76]. *H. capsulatum* infections have also been described in patients with transplants [77, 78], invasive devices, and/or surgical implants [79–81].

H. capsulatum shares some features with the etiologic agents of HAI, bacteria or fungi, which support the nosocomial involvement of *H. capsulatum* infection: worldwide distribution (facilitated by flying reservoirs), its ubiquity, production of aerosolized infective propagules that spread the fungus in the environment and favor the infection by the respiratory pathway, development of biofilm and quorum-sensing (QS) events, and opportunistic behavior in immunosuppressed hosts.

4. Biofilm Formation

It is estimated that 95% of the microorganisms found in nature are attached in biofilms [82]. Over 60–65% human infections involve the formation of biofilms by normal commensal flora or nosocomial pathogens [83–89]. A biofilm is a complex structured community of microorganisms, surrounded by an extracellular matrix of polysaccharides, adhering to each other over a surface or interface [82]; sometimes protein-like adhesins of the pathogen are also involved in biofilm formation [90]. Biofilms constitute a potential source of chronic, recurrent infections and cross-contamination events [7, 89]. Microorganisms in biofilms are protected from the host's immune system and may be 1,000-fold more resistant to antibiotics than planktonic cells [91], due to poor penetration of drugs, low growth rate, and development of the microorganism's resistant phenotypes within biofilms [84, 92].

Fungal biofilms have been found not only in wild soil and water, but also in urban environments, like piping systems, water reservoirs, and constructions, and in healthcare equipments [8, 85, 93–96]. Among the fungal biofilms found on these surfaces, the medically important fungi, such as *Candida* spp., *Aspergillus* spp., *Cryptococcus* spp., *Rhodotorula* spp., *Penicillium* spp., *Sporothrix* spp., *Acremonium* spp., and *Paecilomyces* spp., must be highlighted [8, 90, 93–98].

In medical devices, *Candida* spp. is the most common fungi associated with biofilm formation, usually with endovascular and urinary catheter-related infections in intensive care units, resulting in invasive candidiasis with high mortality [99–101]. The distribution of *Candida* species is variable and in recent years non-*albicans Candida* species have been frequently found in patients with hemodialysis catheter-related candidemia [102].

The presence of biofilms has also been described in ventriculoperitoneal (VP) shunts in patients with *Candida* spp., *Cryptococcus neoformans,* and *Coccidioides immitis* meningoencephalitis. These biofilms were associated with recurrent peritonitis and meningitis [88, 103]. Various fungi have been able to form biofilms on abiotic surfaces in experimental models, such as *A. fumigatus* [104], *M. pachydermatis* [105], *Blastoschizomyces capitatus* [106], *Candida* spp. [107], *Pneumocystis* spp. [108], *Rhodotorula* spp. [109, 110], *C. neoformans* [111], *S. cerevisiae* [112], *Fusarium* spp. [113], *T. asahii* [114], and zygomycetes [115].

Epidemiological surveillance definitions of HAI include surgical site infections associated with surgical implants or medical indwelling devices, when they occur within 30–90 days after the surgical procedure [4]. Clinically, *H. capsulatum* infections have been identified in individuals with invasive devices or surgical implants, and some authors have described endovascular histoplasmosis in patients with vascular prosthetic or synthetic implants [80, 81, 116–119]. Usually, the diagnosis is made by isolation of the fungus in vegetation or over synthetic materials. In addition, histopathological observation has revealed fibrin, large aggregates of yeast cells, mild chronic inflammatory cell infiltrates (predominantly macrophages) [80, 116, 118], and *H. capsulatum* hyphae (M-phase) in a few cases [117]. Furthermore, *H. capsulatum* endocarditis has also been described in native heart valves [118–120]. The aforementioned factors suggest the ability of *H. capsulatum* Y-phase to form biofilms in vivo (human solid organs and medical devices). Recently, it was described that *H. capsulatum* is able to form biofilms on abiotic surfaces [121]. Besides, *H. capsulatum* yeasts have been found clustered in the cells of bats' spleen, lung, and liver and in the lamina propria of intestine villi [122].

There are some reports of *H. capsulatum* peritonitis associated with infected catheters in patients with end-stage renal disease under continuous ambulatory peritoneal dialysis [123–128]. All of these peritoneal histoplasmosis'

cases occurred in residents from an endemic area, in a period longer than 90 days, in contrast with the epidemiological definition of HAI. Thus, continuous exposure to the fungus' infective M-phase propagules appears to be an important risk factor, since no other epidemiological feature could be associated with these cases.

Veeravagu et al. [129] reported a case of *H. capsulatum* meningitis associated with a VP shunt that was diagnosed two days after surgery. It is noteworthy that the patient did not come from an endemic area. Furthermore, *H. capsulatum* was isolated from the VP shunt tip and the surgical instruments, so this could be considered a nosocomial histoplasmosis.

Currently, it is unknown if *H. capsulatum* is able to form biofilms in its filamentous form, which could contaminate hospital environments, medical devices, and supplies, facilitating the direct inoculation of the infective form through cross-contamination. However, it is not a farfetched idea, because biofilms have been described in filamentous fungi, such as *Aspergillus* spp. [104] and zygomycetes [115].

5. Quorum Sensing (QS)

QS is a mechanism of microbial communication dependent on cell density that can regulate several behaviors in bacteria, such as secretion of virulence factors, biofilm formation, survival, and bioluminescence. Fungal QS systems were first described in the pathogenic fungus *C. albicans*, with important signaling molecules, called farnesol and tyrosol (alcohols derived from aromatic amino acids), which control fungal growth, morphogenesis, and biofilm formation, inducing detrimental effects on host cells and other microbes. The concentration of these alcohols increases proportionally to the microbial population and, after reaching a critical threshold, a regulatory response is triggered leading to the coordinated expression or repression of QS-dependent target genes in the entire microbial population [130].

QS activities have also been described in other fungi, such as *H. capsulatum* [131], *Ceratocystis ulmi* [132], and *Neurospora crassa* [133]; however, the molecules responsible for such activities have not yet been purified. In *H. capsulatum*, regulation of α-(1,3)-glucan synthesis in the Y-phase cell wall has been shown to occur in response to cell density [134].

Albuquerque and Casadevall [130] proposed that fungal QS molecules are not only a product of fungal catabolism, but they should have some characteristics: to accumulate in the extracellular environment during fungal growth at a concentration proportional to the population cell density restricted to a specific stage of growth, to induce a coordinated response in the entire population once a threshold concentration is reached, and to reproduce the QS phenotype when added exogenously to the fungal culture. More research about these molecules is needed to elucidate the QS mechanisms in each fungus model, involving different pathogenic events, including biofilm formation.

6. *H. capsulatum* Infection in Drug-Induced Immunocompromised Individuals

IFIs related to immunosuppression caused by drugs in patients with transplant occur because cellular immunity is modified, usually within the first six months posttransplant. During this period, the IFI acquired an opportunistic nature and emerged as HAI [135–137]. After six months posttransplant, patients usually remain stable and continue receiving immunosuppressive drugs at low doses. Thus, they are susceptible to common infections acquired in the community [79, 135–137].

H. capsulatum infections have been identified in solid organ transplant (SOT) recipients [138, 139]. However, a low frequency of histoplasmosis related to HAI has been observed in the first six months posttransplant. Freifeld et al. [138] identified nine cases of pulmonary histoplasmosis in SOT recipients in a period of 30 months, but only four patients developed the disease in the first six months posttransplant. In a 10-year cohort study, Cuellar-Rodriguez et al. [140] found only three cases of histoplasmosis in SOT recipients in the first six months posttransplant; however, eleven cases were identified after the first year posttransplant.

Other authors evaluated the incidence of IFIs in SOT recipients, in a 5-year cohort study [141, 142]. Of the 1,208 cases of IFI, histoplasmosis was diagnosed in 48 patients, where 18 cases (37.5%) occurred in the first six months posttransplant, particularly in kidney, liver, and kidney-pancreas transplants [141]. In general, these patients were receiving immunosuppressive drugs, like tacrolimus, sirolimus, mycophenolate mofetil, and steroids [138, 140, 142].

A more recent study about IFIs identified an increase in the number of histoplasmosis cases in SOT recipients [142]. Among the 70 cases of IFIs reported, 52 (80%) were diagnosed as histoplasmosis, in a 5-year period. The median time from transplant to the diagnosis of this fungal disease was one year. Five SOT recipients developed histoplasmosis within 30 days of transplant; two patients acquired the infection from their donated organs, and three patients developed pulmonary histoplasmosis irrespectively of the transplanted organs [142]. In rare cases, histoplasmosis has also been diagnosed in patients treated with immunobiological molecules, like different monoclonal antibodies [143, 144].

7. Fungal Respiratory Infections in the Hospital Environment

Inadvertent exposure to opportunistic environmental and airborne pathogens can result in infections with significant morbidity and mortality [9]. Fungal infections can range from mild to life-threatening; they vary among mild skin rashes, fungal pneumonia, meningitis, and IFIs. In the hospital, the most common fungal HAI are caused by *Candida* spp. and *Aspergillus* spp. [19].

Airborne infections in susceptible hosts may result from exposure to environmental microorganisms that are ubiquitous in nature, growing in soil, water, dust, or organic matter [2, 9]. Spores or hyphal fragments of fungi usually lie scattered in the environment, especially near decomposing organic matter. *Aspergillus fumigatus* is the species most often associated with pulmonary IFIs [3]. Infection occurs after inhalation of conidia stirred up from construction or renovation works in the hospital. The main risk factor for this HAI is the concentration of *Aspergillus* conidia in the

air [2, 145–149], and the most susceptible individuals are hematopoietic stem cell transplant recipients, neutropenic patients, and those with hematologic malignancies [136, 145, 150–153].

Infections due to *C. neoformans*, *H. capsulatum*, or *C. immitis* can occur in healthcare settings if the nearby ground is disturbed and a malfunction of the facility's air-intake components allows these pathogens to enter the hospital ventilation system [9]. Several outbreaks of histoplasmosis have been associated with disruption of the environment [67, 72, 154]. *H. capsulatum* contaminated environments related to bat and bird colonies living in abandoned buildings and on treetops could disperse the fungus around the hospital. Under this statement, the dispersion of *H. capsulatum* infective propagules could represent a potential risk factor for hospital-acquired histoplasmosis, especially in individuals hospitalized in units lacking adequate air quality control. *H. capsulatum* has never been identified in air quality studies from hospital settings [152]. This could be explained by the difficulties in this fungus' isolation, including prolonged culture growth in laboratory conditions, special nutritional needs, and culture inhibition by the presence of other fast-growing fungi [64, 68].

8. Molecular Biology as a Diagnostic Tool in HAI

Hospital-acquired pneumonia represents one of the most difficult treatment challenges in infectious diseases. Many studies suggest that the timely administration of appropriate pathogen-directed therapy could be lifesaving. However, results of bacterial cultures and antimicrobial susceptibility testing can take 48 hours or longer, but some fungi may not even be able to grow in the first week after culturing.

Nowadays, physicians rely on clinical and epidemiological factors to choose an initial empiric therapy for HAI. A number of rapid molecular tests have been developed to identify pathogens and the bases for most molecular assays are polymerase chain reaction and nucleic-acid-sequence-based amplification. These methodologies offer the promise of dramatically improving the ability to identify pathogens in respiratory tract specimens with high sensitivity and specificity. Data from such applications can also be electronically integrated into shared molecular databases, where clinicians and epidemiologists could ascertain local, regional, national, and international trends [155].

Molecular identification of fungi in hospitals has been scarcely described [156–158]. Lo Passo et al. [156] reported transmission of *Trichosporon asahii* by an endoscopic procedure, when isolated from an esophageal ulcer. *T. asahii* isolates were genotyped by restriction fragment length polymorphism and random amplification of polymorphic DNA, confirming the endoscopic device as the source of transmission.

Notwithstanding, there are some undefined issues regarding the use of these molecular biology tools.

(i) Molecular assays have been used mainly for bacteria and viruses, leaving aside the importance of other microorganisms, such as pathogenic fungi; however, there are specific markers for almost every fungal pathogen, which have recently improved the molecular diagnostic bundle for HAI [157].

(ii) The significance of finding a pathogen's DNA in respiratory tract specimens, in the absence of a positive culture, will show different airway ecology from what it is known, and it exposes the inability to distinguish between infecting and colonizing organisms [155].

(iii) The complexities of the pulmonary microbiome and its metagenomic diversity represent a great challenge with many unanswered questions remaining [155].

(iv) New procedures combining molecular biology techniques and environmental sampling of air have revealed some fungal pathogens living in the hospital surroundings [158], which may be relevant for the acquisition of respiratory HAI.

9. Conclusions

H. capsulatum infection associated with healthcare has been linked to medical devices and surgical implants. The M-phase of *H. capsulatum* may be present in hospital settings, as this fungus adapts to different types of climates and has great dispersion ability. Although conventional pathogen identification techniques have never identified *H. capsulatum* in the hospital environment, histoplasmosis HAI cases have been reported in the last decades. Molecular biology procedures could be useful in this fungus' identification in the air of hospitals and in the diagnosis of this mycosis. More research is needed about *H. capsulatum* involvement in HAI, since it causes a severe and often fatal disease in immunocompromised individuals.

Conflict of Interests

The authors declare that there is no conflict of interests among them and with any financial organization regarding the material discussed in the present paper.

Authors' Contribution

Maria Lucia Taylor, Laura Elena Carreto-Binaghi, and Lisandra Serra Damasceno contributed equally to the design of this study. Laura Elena Carreto-Binaghi and Lisandra Serra Damasceno contributed equally to draft the paper. Nayla de Souza Pitangui, Ana Marisa Fusco-Almeida, and Maria José Soares Mendes-Giannini contributed with their critical opinion to improve the paper. Rosely Maria Zancopé-Oliveira and Maria Lucia Taylor were supervisors of this study. All of the authors read and approved the final version of the paper. Laura Elena Carreto-Binaghi and Lisandra Serra Damasceno contributed equally to the development of the review.

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Respiratory Research

REVIEW Open Access

Surfactant proteins, SP-A and SP-D, in respiratory fungal infections: their role in the inflammatory response

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Abstract

Pulmonary surfactant is a complex fluid that comprises phospholipids and four proteins (SP-A, SP-B, SP-C, and SP-D) with different biological functions. SP-B, SP-C, and SP-D are essential for the lungs' surface tension function and for the organization, stability and metabolism of lung parenchyma. SP-A and SP-D, which are also known as pulmonary collectins, have an important function in the host's lung immune response; they act as opsonins for different pathogens via a C-terminal carbohydrate recognition domain and enhance the attachment to phagocytic cells or show their own microbicidal activity by increasing the cellular membrane permeability. Interactions between the pulmonary collectins and bacteria or viruses have been extensively studied, but this is not the same for fungal pathogens. SP-A and SP-D bind glucan and mannose residues from fungal cell wall, but there is still a lack of information on their binding to other fungal carbohydrate residues. In addition, both their relation with immune cells for the clearance of these pathogens and the role of surfactant proteins' regulation during respiratory fungal infections remain unknown. Here we highlight the relevant findings associated with SP-A and SP-D in those respiratory mycoses where the fungal infective propagules reach the lungs by the airways.

Keywords: Surfactant proteins, Collectins, Respiratory fungal pathogens, Innate immune response

Background

Pulmonary surfactant is a complex fluid that is composed of phospholipids (90 %) and proteins (10 %). There are four surfactant proteins (SP-A, SP-B, SP-C, and SP-D); each one has different biological functions. SP-A and SP-D are hydrophilic, whereas SP-B and SP-C are hydrophobic. SP-B, SP-C, and SP-D are essential for the lungs' surface tension function and are required for the organization, stability and metabolism of lung parenchyma [1]. SP-A and SP-D are known for their contribution to the host's lung immunity. Due to the role of SP-A and SP-D in the immune response, they have been preferentially studied in infectious diseases. The aim of this paper was to review the available information concerning the correlation between the surfactant proteins SP-A and SP-D and those respiratory fungal pathogens whose airborne infective propagules arrive at the lungs producing a progressive pulmonary disease.

Review

Surfactant proteins are produced by different cell types, and in the lung, the four types are synthesized by type II pneumocytes [1]. SP-A and SP-D are also secreted by non-ciliated bronchiolar cells, submucosal gland and epithelial cells of other respiratory tissues, such as the trachea and bronchi. In the lacrimal apparatus, ductal epithelial cells are responsible for their production [2, 3]. Mucosal and glandular/ductal epithelial cells in the gastrointestinal tract (salivary glands, esophagus, small intestine, colon, pancreas, liver, and mesentery) produce low levels of SP-A and SP-D [2, 3]. These proteins have been found in sebaceous and sweat glands, where they are synthesized by the ductal and glandular epithelium; they are also found in the skin, but there is no evidence of surfactant proteins' RNA transcripts in cutaneous cells [3]. SP-A and SP-D can be detected in both the male and female genitourinary tracts (prostate, testis,

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bladder, kidney, and uterus, even in non-pregnant women), and are apparently secreted by glandular and epithelial cells [2, 3]. Significant levels of SP-D, which has an important pro-atherogenic potential, were found in heart and brain tissues, where they are produced by vascular endothelial cells [3].

The chemical structure of SP-A and SP-D comprises different subunits: the N-terminal non-collagenous domain, a collagenous region, the helical neck, and the C-terminal carbohydrate recognition domain (CRD), each one with different ligand binding affinities [3].

The genomic locus of human SP-A consists of two functional genes, *SFTPA1* (*SP-A1*) and *SFTPA2* (*SP-A2*), and a pseudogene. This locus is situated on the long arm of chromosome 10 and the two functional genes are in opposite transcriptional orientation [4–6].

All mammalian species studied to date have a single-copy gene, except from primates onward, where a gene duplication occurred that gave rise to *SP-A1* and *SP-A2* genes; a recent report revealed additional SP-A sequences in some species, such as the opossum (three genes) and the chicken (*SP-A* and *SP-A*-like gene) [4]. A number of alleles have been characterized for each *SP-A* gene. The most commonly observed alleles for the *SP-A1* gene are *6A*, *6A2*, *6A3*, and *6A4*, and those for the *SP-A2* gene are *1A*, *1A0*, *1A1*, *1A2*, *1A3*, and *1A5*. Splicing variation and/or polymorphisms at the 59 and 39 non-translated regions of these alleles, respectively, point to regulatory differences [5].

Genetic studies have been conducted in human populations of adults, children, and newborns, where single nucleotide polymorphisms, haplotypes, and other genetic variants of *SP-A1* and *SP-A2* genes have been associated with acute and chronic lung diseases, like cystic fibrosis, asthma, allergic rhinitis, and chronic obstructive pulmonary disease [6]. Moreover, these human genetic variations have been considered risk factors for infectious diseases such as tuberculosis [7–9] and, for fungal infections in particular, there are *SP-A1* and *SP-A2* polymorphisms related to allergic bronchopulmonary aspergillosis [10].

The human SP-D locus is linked to the SP-A locus and is located proximal to the centromere at approximately 80–100 kb from the *SP-A2* gene. Several polymorphisms for SP-D and an association between SP-D alleles and lung diseases have also been identified [5].

SP-A and SP-D are classified in the C-type lectin family, commonly known as "collectins", because of their structure. Collectins have a relatively high affinity for oligosaccharides, which suggests that they are important determinants of self/non-self recognition [11]. SP-A preferentially attaches only to monosaccharides, in the following order of binding preference: mannose, fucose, glucose, galactose, and N-acetylglucosamine; in contrast, SP-D binds more avidly to maltose, glucose, mannose, fucose, galactose, lactose, glucosamine, and N-acetylglucosamine [12], and to complex carbohydrates on the surface of different cells [13, 14]. SP-A stimulates the expression of the mannose receptor and the scavenger receptor A on the surface of alveolar macrophages, but these mechanisms are not clear [15].

SP-A and SP-D bind different pathogens, such as viruses, bacteria, fungi [6, 16, 17], and the nematode *Schistosoma mansoni* [18], via their CRD, and act as opsonins to trigger the mechanisms of the host's innate immune response or, in some cases, they display their own microbicidal activity by increasing the permeability of the pathogen cellular membrane [1, 3, 15]. Overall, SP-A and SP-D opsonized-microorganisms (bacteria and fungi) enhance their attachment to phagocytic cells (macrophages and neutrophils), with subsequent pathogen clearance [3, 11].

Some interactions between collectins of the host's defense mechanisms and the pathogen are known. Pulmonary collectins regulate cytokine and free radical productions, according to their environment, and play a pro- or anti-inflammatory role [3, 11]. Surfactant collectins inhibit T cell proliferation. In particular, SP-A inhibits dendritic cell maturation and SP-D stimulates antigen presentation by dendritic cells [15]. Furthermore, SP-A and SP-D possess direct bactericidal and fungicidal activity, but the exact mechanisms involved are still unknown [3, 11, 19]. It was described that SP-A decreases the binding of bacterial lipopolysaccharide (LPS) to the LPS-binding protein (LBP), acting as a competitive inhibitor and avoiding the initiation of the inflammatory cascade of LBP/CD14 [20]. However, Gardai et al. [14] demonstrated the increase of proinflammatory mediators of the innate and adaptative immune responses through the induction of nuclear transcription factors, kβ (NFkβ) and activator protein-1 (AP-1), by using LPS-stimulated macrophages after SP-A or SP-D attachment via their collagen regions to calreticulin/CD91 receptor complex on the host's macrophage surface. In contrast, when the globular heads of these collectins bind to host's macrophages, an anti-inflammatory effect is generated through a signal inhibitory regulatory protein α (SIRPα) that activates the tyrosine phosphatase-1 (SHP-1), which blocks src-family kinases and P38 MAP kinase signaling, therefore suppressing proinflammatory mediators [14]. These dual inflammatory functions of SP-A and SP-D could participate in the host's immunological surveillance mechanisms for different microorganisms.

The interaction of surfactant proteins with Toll-like receptors (TLRs) and TLR-associated molecules, such as CD14, may be one mechanism for their inflammatory mediator function [11]. SP-A binds to the extracellular domain of TLR2 via its neck domain. The incubation of a recombinant soluble form of TLR2 with SP-A reduced the binding to bacterial peptidoglycan and the activation of NFkβ as well as TNF-α secretion by rat alveolar macrophages and U937 cells (a macrophage-like cell line) [21]. This finding suggests that direct interaction of SP-A with

TLR-2 alters peptidoglycan-induced cell signaling, which results in a decreased inflammatory response [15]. In contrast, SP-A also associates with MD-2, a TLR-4 accessory protein and signaling molecule, and inhibits the binding of LPS to TLR-4/MD-2 in HEK293 cells (a human embryonic kidney cell line), which would activate NFkβ whether LPS had been attached to the TLR-4/MD-2 receptor complex. Therefore, SP-A inhibits NFkβ activation, explaining an anti-inflammatory mechanism previously described in vivo [15]. In regard to SP-D, it binds to the extracellular domains of TLR-2 and TLR-4 through its CRD by a different mechanism from those used in regular binding to pathogen phosphatidylinositol and LPS [22].

In regard to the role of SP-A and SP-D surfactant proteins in chronic lung diseases, including those produced by pathogens, is unknown. However, it has been suggested that the SP-D levels, in bronchoalveolar lavage fluid and serum, can vary substantially in different pulmonary conditions, which encourages the use of this protein as a biomarker of lung disease or injury [3, 11].

Interaction of SP-A and SP-D with cytokines involved in the defense to pathogens

The collectins SP-A and SP-D have an important participation in the host's innate immune response, where they interact with inflammatory cells and cytokines. This relationship has been described in different situations, such as immunoregulation during pregnancy [3], lung transplant rejection [23], pulmonary apoptotic cell clearance [3], allergic responses produced by pollen or *A. fumigatus* [3], and chronic inflammatory diseases [3, 24].

SP-A regulates macrophage function by diminishing the kinase activity required for the production of proinflammatory cytokines; it also upregulates the expression of IL-1β receptor, related to M-kinase (TLR-4 downregulator). Thus, SP-A inhibits TNF-α and IL-6 production, which is unleashed as a response to LPS [25]. In *Pseudomonas aeruginosa* infection, SP-A knockout mice show lower levels of IL-1β than do wild-type mice, which suggest that SP-A induces IL-1β production through the inflammasome pathway [26].

SP-A upregulates TLR-2 and TLR-4 transcription and post-translational modifications during monocyte-macrophage differentiation and downregulates Ikβα factor (NFkβ controller), which results in diminished TNF-α secretion in response to the binding of TLRs to their respective ligands [27].

SP-A plays a critical role in the differentiation and production of T regulatory cells (Treg), as described in SP-A deficient mice, which revealed an altered Foxp3 expression and the reduced production of Treg $CD25^+ Foxp3^+$ cells. SP-A also augments lL-2 and TGF-β levels [28].

In regard to SP-D, an anti-inflammatory effect mediated by IL-8 was detected after its binding to pollen particles, because this interleukin inhibits histamine discharge from basophils, thereby antagonizing IgE production in plasma B cells [29].

SP-D can compete with TNF-α in the proinflammatory effects on macrophages and dendritic cells by inhibiting partially this cytokine's production; thus, TNF-α contributes to an indirect increase in SP-D by inducing IL-13 [30].

It has been described that SP-D knockout mice infected with *C. neoformans* displayed less eosinophil infiltration and lower IL-5 levels in bronchoalveolar lavage fluid than wild-type mice, suggesting a direct relationship between the presence of this collectin and the response to this pathogen [31].

Interaction of collectins with respiratory fungal pathogens

The binding of SP-A and SP-D to a variety of opportunistic fungi results in the direct inhibition of fungal growth and the enhancement of phagocytosis [3]. However, the downstream immune response elicited by surfactant proteins can also contribute to the establishment of fungal infection and pathogenesis [3]. There are other medically important opportunistic fungi that cause pulmonary infections, such as *Candida* spp., which was not considered in this review because it enters the lung via hematogenous dissemination instead of the airway.

Aspergillus fumigatus

SP-A and SP-D surfactant proteins attach to *A. fumigatus* conidia via a calcium-dependent receptor, which increases phagocytosis and fungal destruction by neutrophils and alveolar macrophages [3, 32]; the fungal ligands recognized by SP-A include two N-glycosylated glycoprotein antigens (gp45 and gp55 kDa) secreted by *A. fumigatus*, which have been recovered from culture filtrates and used for immunodiagnosis of aspergillosis [32].

In a murine model of invasive pulmonary aspergillosis the intranasal administration of exogenous SP-D, either the purified human protein or a recombinant fraction containing the homotrimeric neck and the CRD domains (rSP-D), protected immunosuppressed mice from a fatal inoculum of *A. fumigatus* conidia [33]. This same experimental model was used by Singh et al. [34] and, according to their findings, the addition of native or recombinant SP-D reduced the fungal growth and increased the TNF-α and IFN-γ levels in murine lungs. SP-D gene-deficient mice are more susceptible to invasive pulmonary aspergillosis, whereas SP-A gene-deficient mice acquire resistance to this disease [35], which suggests that SP-A may facilitate the pathogenesis induced by *A. fumigatus*. Genetic studies show that mutations in the *SP-A2* gene (*G1649C*, *A1660G*, and the *AGA* allele) may increase susceptibility to *Aspergillus*-mediated allergies [10, 36].

Blastomyces dermatitidis

SP-D binds the β-glucan on the surface of *B. dermatitidis*, thereby blocking the access of β-glucan-receptors on alveolar macrophages to this molecule, which inhibits TNF-α production. *B. dermatitidis* may utilize SP-D as a strategy to reduce the host defense inflammatory reaction by diminishing TNF-α stimulation; which could favor the development of the disease [3, 37].

Coccidioides posadasii

SP-A and SP-D bind to coccidiodal antigens, but *C. posadasii* infection disrupts the expression of the pulmonary collectins, potentially enabling disease progression and promoting fungal dissemination [3, 38]. The levels of pulmonary surfactant collectins and phospholipids (measured by ELISA and Stewart method, respectively) were decreased in the lungs of mice infected intranasally with a lethal dose of *C. posadasii*; however, the collectins and phospholipid levels were normal in the lungs of *C. posadasii*-protected mice after immunization with a formalin killed spherule vaccine [38]. This study also assessed the concentration-dependent binding of SP-A and SP-D to coccidioidal antigens in vitro, and the findings sustained that pulmonary collectins were involved in the phagocytosis of *C. posadasii* by antigen presenting cells and in the downstream immune regulation of the infected host. Regarding the surfactant phospholipids, there is not sufficient information about their role of in the host defense [38].

Cryptococcus neoformans

SP-A binds to both encapsulated and non-encapsulated *C. neoformans* yeasts depending on the fungal concentration, but it does not enhance acapsular *C. neoformans* phagocytosis; this binding is calcium-dependent and can be inhibited by mannose and glucose, but not by galactose [39]. In a study on SP-A deficient and wild-type mice using an intranasal *C. neoformans* infection model, SP-A did not influence disease progression [40]. In contrast, SP-D agglutinates acapsular yeasts with a higher affinity than SP-A [41, 42], increases the phagocytosis of hypocapsular *C. neoformans* by murine macrophages under in vitro and in vivo conditions [43, 44], enhances fungal survival [44], and protects *C. neoformans* against oxidative stress in an experimental murine model, which facilitates the disease progression [43]. The ligands identified for SP-D are the capsular components glucuronoxylomannan and mannoprotein1 [42].

Histoplasma capsulatum

Only a few studies have described the particular interaction of *H. capsulatum* and surfactant proteins. McCormack et al. [19] showed that exposing *H. capsulatum* yeasts to SP-A and SP-D stimulated a dose-dependent decrease in [^{3}H]leucine incorporation, as revealed by a failure to grow on Ham's F12 supplemented medium. This exposure increased yeast permeability based on a leak of protein from the organism and enhanced the access of an impermeable substrate of the intracellular alkaline phosphatase. This mechanism is calcium-dependent, because calcium binding produces conformational shifts in the CRD region of the collectins, thus exposing charged or hydrophobic protein molecules with biological functions, such as interacting with membrane phospholipids or other surface components, and disrupting membrane function [19]. However, SP-A and SP-D did not inhibit the growth of macrophage-internalized *H. capsulatum* yeasts [19]. Besides, in this study, the authors also assayed an intranasal infection with *H. capsulatum* yeasts in SP-A null mice, demonstrating that these mice were more susceptible to the infection than age-matched wild-type control mice. This difference was associated with an abrogation in the number of pulmonary $CD8^+$ cells and a higher fungal burden in the lungs and spleen of SP-A null mice than the wild-type littermates [19]. Neither SP-A nor SP-D aggregated *H. capsulatum*, based on light microscopic inspection of the fungus incubated with these collectins for different periods of time. Besides, none of these collectins altered the phagocytosis process of this pathogen [19].

Paracoccidioides brasiliensis

After an extensive bibliographic search, we found no studies on the interaction between *P. brasiliensis* and SP-A or SP-D; the same was reported by Brummer and Stevens [16] in their review on collectins and fungal pathogens. Information on this matter remains to be investigated.

Pneumocystis sp

The CRD region of SP-A and SP-D bind the major surface glycoprotein (MSG) of *Pneumocystis* sp. [45], which is the predominant membrane protein, is rich in mannose residues, and attaches *Pneumocystis* to the alveolar epithelium; this is mediated by a time- and calcium-dependent mechanism and is competitively inhibited by mannosyl albumin [46].

Pneumocystis has a narrow relationship with its host and displays a host-specific interaction known as stenoxenism, which was first described by Gigiliotti et al. [47] in 1993, and confirmed by Demanche et al. [48] in 2001. Each *Pneumocystis* species infects a particular mammal species: *P. carinii* and *P. wakefieldiae* infect rats, *P. murina* infects mice, *P. oryctolagi* infects rabbits, and *P. jirovecii* infects humans. Thus, before 2001, *P. carinii* was the only species referred and privileged in several studies.

In rats, SP-D markedly accumulates during *P. carinii* pneumonia and binds to MSG on the surface of this fungus, which contains an N-linked carbohydrate chain that is rich in glucose, mannose, and N-acetyl-glucosamine. The binding site uses a mannose-type saccharide, which

is a calcium-dependent mechanism, and is competitively inhibited by maltose > glucose > mannose > N-acetyl-glucosamine [49]. Dodecamers and other large arrangements of SP-D bind better to *P. carinii* than oligomeric arrangements [49].

SP-D additionally binds to fungal β-glucans present on *Pneumocystis*' cystic forms, which are potent stimulators of TNF-α release [49]. *P. carinii* has other lectin binding activity, thus raising the possibility that a surface lectin on the fungus may also interact with the N-linked glycosylation of SP-D and other collectins [49].

Interestingly, none of these binding mechanisms increase the phagocytosis of this pathogen [46, 49]. However, SP-D significantly promotes *P. carinii* self-association, which results in large aggregates of organisms that may exhibit impaired uptake by macrophages; this potentially represents a mechanism for evading the microorganism's elimination by the host [49].

Pneumocystis infection results in major changes in the expression of all surfactant components [50]. The total protein content of bronchoalveolar lavage increases 10-fold during *Pneumocystis* pneumonia, which induces a 3-fold increase in the total alveolar SP-A and SP-D protein content, due to the increased expression (mRNA) and accumulation of these collectins in surfactant fluid in human [51, 52] and rat samples [53]. Moreover, *Pneumocystis* pneumonia decreases the total phospholipid levels [50]. Both collectins are known to be increased as a result of enhanced translation, constitutive release, and decreased clearance from the pulmonary fluid. Transgenic mice over-expressing IL-4, related to the Th2 cytokine response to *Pneumocystis* pneumonia, increase SP-D mRNA [50].

Aliouat et al. [53] used two corticosteroid-untreated animal models (rabbits and severe combined immunodeficiency [SCID] mice), which were intranasally inoculated with *P. carinii*, and explored the content of surfactant phospholipids and proteins in bronchoalveolar lavage. In SCID mice, the surfactant phospholipid/protein ratio remains low, whereas the parasite increases and pneumonia progresses. However, in rabbits, the surfactant phospholipid/protein ratio and parasite rates were inversely proportional, similar to the events observed in AIDS-related *Pneumocystis* pneumonia in humans; these changes were present prior to the establishment of pneumonia with *Pneumocystis*' proliferation [53].

Some studies have reported reduced surfactant phospholipid levels of bronchoalveolar lavage either in rats infected with *P. carinii* [54] or in humans with pneumocystosis [55]. The phospholipid composition of bronchoalveolar lavage was also altered in *P. carinii* pneumonia, with a slight increase in the percentage of sphingomyelin and a reduced percentage of phosphatidylcholine. SP-A inhibits phospholipids secretion by type II pneumocytes and stimulates its clearance [53]. There was no difference in the lavage phospholipase A2 activity for the *Pneumocystis*-infected and control groups [54].

The comparison among four groups of individuals: healthy volunteers, *Pneumocystis*-negative HIV-positive patients, mild *Pneumocystis* pneumonia patients, and moderate-to-severe *Pneumocystis* pneumonia patients, showed a reduction in total bronchoalveolar lavage lipids in the *Pneumocystis*-positive groups [55], particularly in the diacylglycerol lipids, whose predominant source is the surfactant's dipalmitoyl phosphatidylcholine. Furthermore, diacylglycerol is metabolized by phospholipase A2, which showed increased activity in moderate-to-severe *Pneumocystis* pneumonia (twice the level of the *Pneumocystis*-negative patients and 30-fold the normal levels) [55]. Despite the incremented activity of the phospholipase A2 enzyme, its metabolism products (monoacyl glycerols and fatty acids) were not increased; this could be explained by a dynamic cellular uptake and metabolism of lysolipids and free fatty acids or a reduced production of surfactant by alveolar type II cells. There was also a marked decrease in surfactant glycerophospholipid in patients with AIDS and *Pneumocystis* pneumonia, which suggests a potential role in the pathophysiology of this disease [55].

Conclusions

Interactions between the pulmonary collectins and different microorganisms, such as bacteria and viruses, have been extensively studied, but this is not the same for fungal pathogens. There is a lack of information on SP-A and SP-D binding to fungal carbohydrates, their relation with immune cells for the clearance of these pathogens, and the regulation of these proteins during fungal infection.

Respiratory fungal pathogens represent one of the most diverse groups of study in terms of surfactant proteins. Many of these fungi have a very narrow geographical limit, where they cause an endemic disease, such as *B. dermatitidis* and *Coccidioides* sp. Others, like *C. neoformans*, enter the organism by the respiratory route, but the lung is not their target organ; thus, they have a different association with surfactant proteins.

However, some fungi (*H. capsulatum* and *Pneumocystis* spp.) share important characteristics, including a worldwide distribution, the form of infection (inhalation of particles), and the possibility of dissemination (particularly in immunosuppressed individuals). Interestingly, they have also been found together co-infecting humans [56] and other mammals [57], which suggest a broader assembly between the fungal pathogens and the surfactant proteins than what it is known. The last feature makes them a trending study topic.

Very scarce evidence is available about SP-A, SP-D and *H. capsulatum*, contrasting the data concerning the

pulmonary collectins and *Pneumocystis* spp. However, despite the apparent understanding of the relation between the latter, there are information gaps that should be investigated, hence opening a broad research field.

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Authors' contributions
LECB and MLT conceived the study, participated in its design and coordination and helped to draft the manuscript. EMA was involved in drafting the manuscript and revising it critically for important intellectual content. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

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CAPÍTULO 9
ARTÍCULOS PUBLICADOS PREVIAMENTE, RELACIONADOS CON EL TEMA DE TESIS DE DOCTORADO

Derouiche S, Deville M, Taylor ML, Akbar H, Guillot J, **Carreto-Binaghi LE**, Pottier M, Aliouat EM, Aliouat-Denis CM, Dei-Cas E, Demanche C. *Pneumocystis* diversity as a phylogeographic tool. Mem Inst Oswaldo Cruz 2009; 104(1):112-117.

Pneumocystis diversity as a phylogeographic tool

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Parasites are increasingly used to complement the evolutionary and ecological adaptation history of their hosts. Pneumocystis *pathogenic fungi, which are transmitted from host-to-host via an airborne route, have been shown to constitute genuine host markers of evolution. These parasites can also provide valuable information about their host ecology. Here, we suggest that parasites can be used as phylogeographic markers to understand the geographical distribution of intra-specific host genetic variants. To test our hypothesis, we characterised* Pneumocystis *isolates from wild bats living in different areas. Bats comprise a wide variety of species; some of them are able to migrate. Thus, bat chorology and migration behaviour can be approached using* Pneumocystis *as phylogeographic markers. In the present work, we find that the genetic polymorphisms of bat-derived* Pneumocystis *are structured by host chorology. Therefore,* Pneumocystis *intra-specific genetic diversity may constitute a useful and relevant phylogeographic tool.*

Key words: bats - co-evolution - ethology - host specificity - phylogeography - *Pneumocystis*

Recent research suggests that parasites may help to resolve the evolutionary and ecological history of their hosts. In some cases, parasites provide an additional source of information, since parasite data can better reconstruct the common history of host and their parasite (Nieberding & Olivieri 2007). For this reason, some parasites have been recently used as phylogeographic markers (Nieberding et al. 2004, Taylor et al. 2005, Criscione et al. 2006, Nieberding & Olivieri 2007). The *Pneumocystis* species have been shown to be a powerful tool in phylogenetic studies (Demanche et al. 2001, Guillot et al. 2001, 2004, Hugot et al. 2003, Aliouat-Denis et al. 2008). These eukaryotic micro-organisms, discovered by Carlos Chagas in 1909, had been described previously as enigmatic protists but are now recognised as major fungal pathogens able to provoke severe pneumonitis in severely weakened mammals. *Pneumocystis* pneumonia (PcP) is considered one of the most serious fungal respiratory infections to occur in immunocompromised patients, especially in human immunodeficiency virus-infected individuals (Dei-Cas 2000). Numerous aspects of the biology of these fungi still need to be clarified, notably their life cycle, which remains hypothetical (Yoshida 1989, Dei-Cas 2000). Neither the life cycle stage responsible for the infection nor the eventual environmental infection sources have been identified. It has been shown however, that host-to-host *Pneumocystis* transmission via an airborne route can occur not only between immunosuppressed laboratory rodents (Hughes 1982, Soulez et al. 1991, Walzer et al. 1977) but also between non-immunocompromised hosts, which may constitute a dynamic reservoir to the *Pneumocystis* species (Dumoulin et al. 2000, Chabé et al. 2004). Indeed, healthy hosts are able to radically eliminate the pathogens from their lungs but, as long as they remain infected, they can transmit *Pneumocystis* by an airborne route to immunocompetent hosts or to weakened members of the population, which may then develop PcP (Chabé et al. 2004, Aliouat-Denis et al. 2008).

Recently, we also know that *Pneumocystis* species consist of a heterogeneous group of host specific fungal parasites that have colonised a wide range of mammalian hosts. Host species-related signatures have been reported in *Pneumocystis* populations using genomic, karyotypic, isoenzymatic and antigenic markers (Dei-Cas et al. 1998, Aliouat-Denis et al. 2008). Cross-infection experiments showed that *Pneumocystis* organisms inoculated into non-specific hosts do not induce the infection (Aliouat et al. 1993, 1994, Gigliotti et al. 1993, Durand-Joly et al. 2002) and are rapidly and radically eliminated from the lungs (Aliouat-Denis et al. 2008). Indeed, on the basis of morphological, phylogenetic and experimental data, it has been demonstrated that *Pneumocystis* constitutes a highly diversified biological group, with numerous species (Dei-Cas et al. 1998, 2006), which are strongly host-specific and well adapted to live inside the lungs of a diverse range of mammals (Demanche et al. 2001, Guillot et al. 2001, Hugot et al. 2003, Aliouat-Denis et al. 2008).

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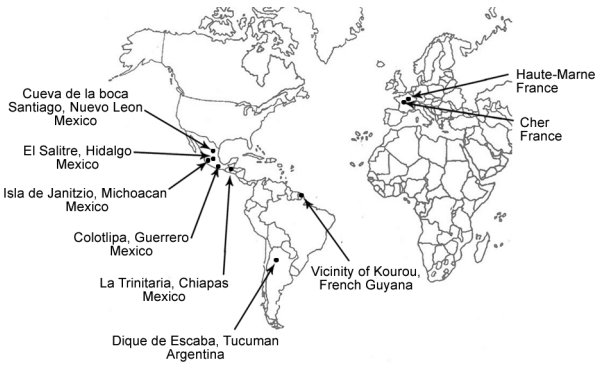
It is likely that there are as many *Pneumocystis* species as there are mammalian host species, though some mammals may be parasitised by more than one host-specific *Pneumocystis* species (Aliouat-Denis et al. 2008). The close host specificity of the numerous described or still undescribed *Pneumocystis* species led to the hypothesis of co-evolution. Consistently, several studies compared host and *Pneumocystis* phylogenies and reported a strong congruence between their phylogenetic trees (Demanche et al. 2001, Guillot et al. 2001, Hugot et al. 2003). Co-phylogeny seems to be the evolutionary pattern for *Pneumocystis* species, which have dwelt in the lungs of mammals for more than 100 million years (Demanche et al. 2001, Guillot et al. 2001, Hugot et al. 2003, Keely et al. 2003, Aliouat-Denis et al. 2008). Since *Pneumocystis* species and variants constitute interesting tools to understand the phylogeny and taxonomy of their hosts, we speculated about any related possibilities they could offer. Parasites, as proxies for host genealogy, may also be useful at the phylogeographic scale (Nieberding & Olivieri 2007).

Phylogeography is a field of research that analyses the geographical distribution of genealogical lineages and can be used to detect processes such as population subdivision, speciation events, ecological adaptation or migration routes (Emerson & Hewitt 2005). In this study, we analysed wild bat species and their corresponding *Pneumocystis*. Bats display a large biodiversity, a global distribution and a flying capacity that allow some of them to migrate over short or long distances. Bats also represent a large reservoir for emerging and re-emerging pathogens (Hance et al. 2006). Thus, the bat model seems ideally suited to assess our hypothesis of using *Pneumocystis* diversity as a potential phylogeographic tool.

MATERIALS AND METHODS

Bats - A total of 104 lung tissue samples were obtained from wild bats in Old or New World areas. Bats were captured from December 2004-July 2007. Of the samples from *Tadarida brasiliensis*, 79 were collected in Tucuman (Argentina) (16) and in four states of Mexico (63) (Table I, Figure). Nine samples of *Pipistrellus pipistrellus* from two French regions (Table I) were obtained through the Museum d'Histoire Naturelle de Bourges (France). A total of 16 samples of *Glossophaga soricina* were obtained from French Guyana (in the vicinity of Kourou) (12) and Mexico (4) (Table I). Lung tissues were frozen immediately following necropsy and stored at -20°C.



Geographical repartition of wild bat samples.

DNA extraction - DNA extraction of all the samples was carried out using the QIAamp DNA mini kit (QIAGEN) according to the manufacturer's instructions for tissue DNA extraction. Negative controls were included in the extraction procedure to monitor for contamination and extracted DNA was stored at -20°C prior to amplification.

DNA amplification and purification - Detection of *Pneumocystis* DNA was based on nested PCR, which amplified a portion of the mitochondrial large subunit (mtLSU) and small subunit (mtSSU) of the rRNA genes. Nested PCR at the mtLSU rRNA locus used the external primers published by Wakefield et al. (1990): pAZ102-H (5' -GTG-TAC-GTT-GCA-AAG-TAG-TC-3') and pAZ102-E (5' -GAT-GGC-TGT-TTC-CAA-GCC-CA-3'). The internal primers were pAZ102-X (5' -GTG-AAA-TAC-AAA-TCG-GAC-TAG-G-3') and pAZ102-Y (5' -TCA-CTT-AAT-ATTAAT-TGG-GGA-GC-3') (Wakefield 1996). Nested PCR at the mtSSU rRNA locus used the following primers: pAZ112-10F (5' -GGG-AAT-TCT-AGA-CGG-TCA-CAG-AGA-TCA-G-3') and pAZ112-10R (5' -GGG-AAT-TCG-AAC-GAT-TAC-TAG-CAA-TCC-C-3'); then pAZ112-13RI (5' -GGG-AAT-TCG-AAG-CAT-GTT-GTT-TAA-TTC-G-3') and pAZ112-14RI (5' -GGG-AAT-TCT-TCA-AAG-AAT-CGA-GTT-TCA-G-3') (Tsolaki et

TABLE I

Number of positive samples per bat species and geographical location (mitochondrial large subunit and small subunit of the rRNA loci merged)

			Mexico					Museum of Natural History, Bourges			
	Argentina	French Guyana	Chiapas	Michoacan	Guerrero	Hidalgo	Nuevo Leon	Cher	Haute-Marne	Total positive samples	Total samples
Glossophaga soricina	-	6/12	-	-	3/4	-	-	-	-	9	16
Pipistrellus pipistrellus	-	-	-	-	-	-	-	2/8	1/1	3	9
Tadarida brasiliensis	8/16	-	2/10	2/6	-	5/20	19/27	-	-	36	79

al. 1998). The Hot Master® Taq DNA Polymerase (Eppendorf) was used for amplification of both loci. For mtLSU rRNA gene amplification, the thermocycling conditions for the first PCR round were as follows: each cycle consisted of denaturation for 30 s at 94°C, annealing for 1 min at 50°C and extension for 1 min at 65°C, for 30 cycles. The second round of PCR was performed with 10% of the first-round mix and the thermocycling conditions were as follows: each cycle consisted of denaturation for 30 s at 94°C, annealing for 1 min at 55°C and extension for 1 min at 65°C, for 30 cycles. For mtSSU rRNA gene amplification, the thermocycling conditions for the first PCR round were as follows: each cycle consisted of denaturation for 30 s at 94°C, annealing for 1 min at 55°C and extension for 1 min at 65°C, for 40 cycles. The second round of PCR was performed with 10% of the first-round mix and the thermocycling conditions were as follows: the first ten cycles consisted of denaturation for 30 s at 94°C, annealing for 1 min at 52°C and extension for 1 min at 65°C, and the following 30 cycles consisted of denaturation for 30 s at 94°C, annealing for 1 min at 63°C and extension for 1 min at 65°C. Negative controls were included in each experiment to monitor for possible contamination. Amplification products were purified on a 1.5% agarose gel (Tris-borate-EDTA buffer) and extracted using the QIAEX II Gel Extraction kit (QIAGEN) when non-specific bands were detected. In the absence of smear or non-specific bands, the amplification products were directly purified using the Montage® PCR Centrifugal Filter Devices kit (Millipore). Purified DNA was stored at -20°C prior to the sequencing reaction.

Cloning of amplified and purified products - When the amplified DNA produced a band of low intensity in the agarose gel, cloning in the bacterial system was performed using the TOPO TA Cloning® kit (Invitrogen) and the One Shot® Top 10 Chemically Competent *Escherichia coli* (Invitrogen). Plasmid DNA was then extracted with the Perfectprep® Plasmid Mini kit (Eppendorf), amplified with the thermocycling conditions of the first round PCR at the locus mtLSU rRNA and purified with the Montage® PCR Centrifugal Filter Devices kit (Millipore).

Sequencing of purified DNA and sequence analysis - The sequencing reaction was carried out from both ends with the sets of internal primers (or primers included in the kit TOPO TA Cloning®, Invitrogen, for the cloned products) and the Big Dye Terminator® v3.1 kit (Applied Biosystems). The labelled products were directly sent to GenoScreen (Pasteur Institute of Lille) and analysed on an automated DNA sequencer (3730XL DNA Analyser®, Applied Biosystems). Sequences were first aligned with the software Clustal X v2.0 (Thompson et al. 1997) and then the alignment was improved manually using the software Se-Al v2.0a11 Carbon (Rambaut 2002). The aligned sequences were converted into a distance matrix (% of differences) using the Phylogenetic Analysis Using Parsimony Program (PAUP v4.0b10, Swofford 2001).

RESULTS AND DISCUSSION

Three species of bats were included in this study: *T. brasiliensis*, *P. pipistrellus* and *G. soricina*. *Pneumocystis* DNA (mtLSU and mtSSU rRNA loci merged) was detected in 48 out of 104 samples (Table I). Amplified *Pneumocystis* mtLSU and mtSSU rRNA gene fragments showed host species-specific DNA sequences. In other words, all samples from *T. brasiliensis* had a similar and unique DNA sequence of *Pneumocystis* mtLSU and mtSSU rRNA gene fragments. The same was true for *Pneumocystis* DNA samples from *G. soricina* and *P. pipistrellus*. These observations confirm the high specificity of *Pneumocystis* organisms for their hosts. Previous studies had reported high diversity in the *Pneumocystis* genus and to date at least one host-specific *Pneumocystis* sequence has been reported from each studied mammalian host species (Mazars et al. 1997, Wakefield 1998, Demanche et al. 2001, Guillot et al. 2001, Aliouat-Denis et al. 2008).

Interestingly, DNA sequences from the *Pneumocystis* organisms harboured within one host species showed some genetic polymorphism in accordance with the geographical origin of the samples. For *G. soricina* samples, the mtLSU rRNA sequences of *Pneumocystis* from French Guyana (6 identical sequences) and Mexico (2 identical sequences) were different at one base position (guanine or adenine) (Table II). Since there is low variability at the mtLSU rRNA locus from *G. soricina*-derived *Pneumocystis,* our results should be confirmed by sequencing and comparison of other loci.

For the *P. pipistrellus* samples, some polymorphism was also observed in the *Pneumocystis* mtSSU rRNA sequences which diverged in less than 1% of the sequence of the *P. pipistrellus* population (Table III) according to the geographical origin (one sequence from Cher and one sequence from Haute-Marne, France). *P. pipistrellus* bats undergo long-distance migrations (Fleming & Eby 2003); thus, mt rRNA polymorphisms could characterise two strains of *P. pipistrellus*-derived *Pneumocystis* specifically attached to two different migratory colonies of this bat species.

Comparable polymorphism was observed in the samples of *T. brasiliensis,* another long-distance migratory species. Two mtLSU and three mtSSU rRNA polymorphic sequences of *T. brasiliensis*-derived *Pneumocystis* were obtained. At the mtLSU rRNA locus, one unique sequence type was found in samples from the Argentinian colony (7 identical sequences), whereas a different sequence type was found in all Mexican samples (7 identical sequences). These two groups of sequences show only 1% of divergence from each other (Table II). At the mtSSU rRNA locus, one unique sequence type was found in samples from Argentina (7 identical sequences), another one in samples from Nuevo Leon (Mexico) (1 sequence) and another one in samples from Chiapas, Hidalgo and Michoacán (Mexico) (6 identical sequences). These sequences showed 0.9-1.6% divergence (Table III). Results in Nuevo Leon should obviously be confirmed with additional samples, since current results are based on only one *Pneumocystis* sequence. In future

TABLE II

Matrix of distance for *Pneumocystis* mitochondrial large subunit of the rRNA sequences of three bats species and two outgroup species (*P. murina* from the mouse and *P. oryctolagi* from the rabbit)

	% of divergence from					
Source of rDNA	1	2	3	4	5	6
1. *Pneumocystis* from *G. soricina* (French Guyana)	-	-	-	-	-	-
2. *Pneumocystis* from *G. soricina* (Mexico)	0.48	-	-	-	-	-
3. *Pneumocystis* from *T. brasiliensis* (Mexico)	14.49	14.03	-	-	-	-
4. *Pneumocystis* from *T. brasiliensis* (Argentina)	14.48	14.02	0.92	-	-	-
5. *Pneumocystis* from *P. pipistrellus* (France)	16.48	16.00	12.57	12.05	-	-
6. *Pneumocystis murina*	23.13	22.66	22.58	22.50	23.57	-
7. *Pneumocystis oryctolagi*	19.90	19.40	22.46	22.95	21.69	18.70

TABLE III

Matrix of distance for *Pneumocystis* mitochondrial small subunit of the rRNA sequences of three bats species and two outgroup species (hare and rabbit)

	% of divergence from						
Source of rDNA	1	2	3	4	5	6	7
1. *Pneumocystis* from *P. pipistrellus* (Haute-Marne)	-	-	-	-	-	-	-
2. *Pneumocystis* from *P. pipistrellus* (Cher)	0.28	-	-	-	-	-	-
3. *Pneumocystis* from *T. brasiliensis* (Nuevo Leon)	20.06	20.02	-	-	-	-	-
4. *Pneumocystis* from *T. brasiliensis* (Hidalgo-Chiapas-Michoacan)	20.00	19.98	0.94	-	-	-	-
5. *Pneumocystis* from *T. brasiliensis* (Argentina)	19.75	19.71	1.26	1.56	-	-	-
6. *Pneumocystis* from *G. soricina* (Mexico)	19.04	17.78	14.38	14.03	14.25	-	-
7. *Pneumocystis* f. sp. *Lepus europaeus*	26.13	25.29	26.37	26.69	26.84	21.00	-
8. *Pneumocystis oryctolagi*	20.12	19.16	21.57	21.90	21.43	18.42	8.83

studies, we should also use more polymorphic markers, like microsatellites, which offer a higher discriminating power, especially for characterising more accurately *Pneumocystis* species sub-populations linked to migratory colonies. For instance, microsatellites have already been used to characterise populations of the fungus *Histoplasma capsulatum* in the Americas (Carter et al. 2001).

Our results are in contrast with a 1994 study in which the frequently occurring one-base mutation of the mtLSU rRNA sequence could not be correlated with geography (UK, USA, Brazil and Zimbabwe) (Wakefield et al. 1994). This may be due to the fact that the portion of the mtLSU rRNA gene analysed in this study was relatively short and therefore other informative polymorphic characters may have been missed. In contrast to the Wakefield study, geography seemed to be a structuring factor to the distribution of four *Pneumocystis jirovecii* mtLSU rRNA genotypes isolated from HIV-infected patients in the USA (Beard et al. 2000). Likewise, a study in the UK showed a significant association between specific mtLSU rRNA genotypes of *P. jirovecii* and the patient's place of residence (Miller et al. 2005). Furthermore, several studies report that the relative proportions of *P. jirovecii* genotypes vary in accordance with the area from which the patients originated. The frequency pattern of DNA sequences from *P. jirovecii* that combine the association of three gene sequences (mtLSU rRNA, Dihydropteroate synthase and internal transcribed spacer) in specimens from Lisbon (Portugal) and Seville (Spain)) show inverted proportions (Esteves et al. 2008). In short, geographical location could therefore have a structuring role in the distribution of *Pneumocystis* genotypes. In the present work, the association of genotypes with migratory bat colonies suggests that there is no inter-colony transmission of *Pneumocystis* and that bats from different colonies do not share migratory routes.

On the whole, the present results based on two genetic markers (the mtLSU rRNA and mtSSU rRNA genes) widely used in *Pneumocystis* research, suggest that host species chorology could have a structuring effect on the distribution of *Pneumocystis* intra-specific variants. These could therefore be used as phylogeographic tools to approach the study of host species subpopulations. In fact, *T. brasiliensis* populations are organised into numerous colonies that could have different migratory behaviours. An attempt to clarify the migratory pathway of these different colonies was previously carried out by Russel et al. (2005). They studied bat mitochondrial DNA sequence but failed to correlate significant genetic

polymorphism to behaviourally distinct migratory colonies. *Pneumocystis* intra-specific genetic polymorphisms could perhaps help to differentiate the colonies of *T. brasiliensis* and help identify their migratory pathway.

Other studies have shown the usefulness of parasite phylogeny as a mirror to host genealogy and ecology (Nieberding & Olivieri 2007). Indeed, when parasite populations are more diversified than their host populations, gene trees of parasites can be used to infer recent migration events of infected hosts (Nieberding & Olivieri 2007). For instance, parasite genotypes identify source populations of migratory salmon more accurately than do fish genotypes (Criscione et al. 2006). Another example is the detailed study of the comparative phylogeography of the rodent *Apodemus sylvaticus* and one of its specific endoparasites, the nematode *Heligmosomoides polygyrus* (Nieberding et al. 2004, Nieberding & Olivieri 2007). Compared to the host tree, the parasite tree showed additional subgroups related to different geographic locations. Therefore, the phylogeographic pattern of the parasite suggested probable past differentiation and migration events that could not be detected through the study of the host itself (Nieberding & Olivieri 2007). A recent and similar study of the geographical distribution of *A. sylvaticus* was carried out with the same set of samples (Nieberding et al. 2004, Nieberding & Olivieri 2007), using *Pneumocystis* as the genetic marker (M Deville et al., unpublished observations). The same trend in phylogeographic history resulted: the host phylogeny showed two main geographical groups (Western Europe and Italy-Balkans) while the parasite phylogeny showed the same main groups but also many regional subgroups, suggesting past migration events of the host. Thus, these results support our hypothesis of using *Pneumocystis* as a potential phylogeographic tool. Furthermore, another pathogenic fungus, *H. capsulatum,* was also studied in *T. brasiliensis* bats (Taylor et al. 2005). Taylor et al. (2005) detected a pattern of molecular differences in accordance to the geography origin of *H. capsulatum* isolated from infected bats that could be used as a tool to monitor the distribution of the fungus and, thus, its host in the nature. These results support our data and suggest that such pathogenic fungi are a robust phylogeographic marker.

Finally, to be a good marker at a phylogenetic or phylogeographic scale, a parasite must share a common history with its host. One of the determining factors is a strong interaction between the host and the parasite. The strength of the host-parasite interaction depends on three parasitic traits: host specificity, presence or absence of intermediate hosts and presence or absence of a free-living phase (Nieberding & Olivieri 2007). The lack of a free-living phase increases the dependence of the parasite on its host, as parasite migration is then exclusively limited to host movements (Nieberding & Olivieri 2007). Thus, we can say that *Pneumocystis* species have a really intimate interaction with their hosts, since it has close host specificity and well documented co-evolution and co-speciation. In addition, *Pneumocystis* species do not have intermediate hosts and, most likely, no environmental stage. In fact, their very close host specificity and their fine adaptation to the alveolar microenvironment (Dei-Cas 2000, Dei-Cas et al. 2004, Aliouat-Denis et al. 2008) seems contradictory, or even incompatible, with any ability to multiply and survive outside their host. Furthermore, *Pneumocystis* populations seem to be more diversified than their host populations, as to be expected of parasites that can be used as proxies at the phylogeographic scale (Nieberding & Olivieri 2007). All these features provide further support to our hypothesis.

In conclusion, the genetic variability of *Pneumocystis* that we observed in bats from distinct species or from distinct geographical areas supports our hypothesis that *Pneumocystis* genetic polymorphisms provide useful and relevant phylogeographic markers of host chorology. Our results validate the use of *Pneumocystis* as a powerful tool in phylogenetic and phylogeographic studies.

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Characterizing *Pneumocystis* in the Lungs of Bats: Understanding *Pneumocystis* Evolution and the Spread of *Pneumocystis* Organisms in Mammal Populations

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Bats belong to a wide variety of species and occupy diversified habitats, from cities to the countryside. Their different diets (i.e., nectarivore, frugivore, insectivore, hematophage) lead Chiroptera to colonize a range of ecological niches. These flying mammals exert an undisputable impact on both ecosystems and circulation of pathogens that they harbor. *Pneumocystis* species are recognized as major opportunistic fungal pathogens which cause life-threatening pneumonia in severely immunocompromised or weakened mammals. *Pneumocystis* consists of a heterogeneous group of highly adapted host-specific fungal parasites that colonize a wide range of mammalian hosts. In the present study, 216 lungs of 19 bat species, sampled from diverse biotopes in the New and Old Worlds, were examined. Each bat species may be harboring a specific *Pneumocystis* species. We report 32.9% of *Pneumocystis* carriage in wild bats (41.9% in Microchiroptera). Ecological and behavioral factors (elevation, crowding, migration) seemed to influence the *Pneumocystis* carriage. This study suggests that *Pneumocystis*-host association may yield much information on *Pneumocystis* transmission, phylogeny, and biology in mammals. Moreover, the link between genetic variability of *Pneumocystis* isolated from populations of the same bat species and their geographic area could be exploited in terms of phylogeography.

Pneumocystis species are mostly airborne-transmitted, highly host-specific opportunistic microfungi responsible for severe pneumonia in a wide range of mammalian species (3). In humans, an unexpectedly high number of clustered cases of *Pneumocystis* pneumonia (PcP) occurring in 1981 revealed the AIDS pandemic (14). Nowadays, PcP is still quite frequently diagnosed in HIV-positive patients but also in patients with other causes of immunodeficiency, such as organ transplantation or anticancer therapy (5, 35, 62, 78). Still, *Pneumocystis jirovecii* remains a leading cause of high mortality in HIV patients, even after the introduction of highly active antiretroviral therapy (HAART) (11, 64). Moreover, new data have recently emerged implicating low burdens of *Pneumocystis* organisms as a cause of symptom worsening in patients with chronic obstructive pulmonary disease (42). Also *Pneumocystis* organisms may temporarily cause asymptomatic infection in immunocompetent hosts, hence constituting a potential infection source (16). Thus, immunocompetent *Pneumocystis* carriers could transmit the infection to immunocompromised individuals (16, 30, 32).

Pneumocystis organisms constitute a huge genus consisting of a large number of host-species-specific organisms found in different mammals (3, 17, 20, 21, 26, 27, 41, 49). Indeed, the narrow host specificity of *Pneumocystis* species emerged clearly from the failure of cross species infection among laboratory animals (1, 38) or between humans and SCID mice (33). Surveys in domestic, synanthropic, or wild species showed that mammals may harbor one or several host-specific *Pneumocystis* species or strains (3, 17, 27, 31, 41, 46).

Whether *Pneumocystis* organisms are able to survive or multiply in the environment remains an unanswered question. It is known that they can be transmitted by aerial route (16, 32, 45, 85) and likely by transplacental route, at least in some mammal species (15, 29, 63, 79). The unavailability of continuous *in vitro* culture systems has hindered research aiming at clarifying the role of each life cycle stage in *Pneumocystis* proliferation and transmission (44). However, ultrastructure (25, 99), short-term culture (2, 19), and cell sorting approaches (56, 57) led to quite heuristic life cycle hypotheses. Furthermore, using PcP animal models, molecular strategies and the exploring of *Pneumocystis* occurrence in wild mammals resulted in major advancements, like the notions of strong host species specificity (1, 27, 31, 33, 38, 41) and coevolution (3, 27, 31, 46).

Bats (Chiroptera) are widely distributed across various ecosystems and constitute one of the largest groups of mammals, second in number of species after the order Rodentia and first in number of individuals (69). We thus attempted to explore how *Pneumocystis* organisms can circulate and adapt within this singular clade

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of flying mammals. Indeed, about one-fourth of all the living mammalian species consists of bats, a group comprising almost 1,100 species throughout the world. Body weight of bats varies widely: from 2 g (*Craseonycteris thonglongyai*; hog-nosed or bumblebee bat) to 1,200 g (*Pteropus vampyrus*; large flying lemur) (10). The order of Chiroptera is divided into two suborders (66): megachiropters (megabats) and microchiropters (microbats). Megabats were reported to live in the Old World and microbats in the New and Old Worlds.

Chiropters occupy diversified habitats, from cities to the countryside, and exhibit the largest variety of diets among all mammalian orders (66). Most are insectivorous (about 700 species), but a significant number of bats feed almost exclusively on fruits (about 230 species), nectar or pollen (about 50 species), small vertebrates (7 species), or blood of mammals or birds (3 species) (66). Their different diets led Chiroptera to colonize diverse ecosystems, ranging from deserts to temperate forest or rainforests, of every continent of the world except Antarctica (9, 69). In some islands, for instance in New Zealand, the only native mammals are bats (22, 36, 43). Bats roost in caves, abandoned or occupied buildings, mines, tree canopies, and hollows and under leaves, bark, or rocks (9, 51). Some bat species are living solitary, whereas others form huge colonies comprising millions of individuals (9). Bats are long-lived mammals: the small 7-g Brandt's bat, *Myotis brandtii*, can live up to 38 years in the wild (98). Furthermore, some species of bats are able to migrate over long distances.

Interestingly, humans share many properties with bats: occupying diverse habitats, living in social organizations, being long-lived, able to perform long migrations, and harboring *Pneumocystis* (41). Bats may therefore represent interesting models to understand the circulation of the human-specific species *P. jirovecii* in human populations. Furthermore, bats have an unusual physiology. For instance, due to their large lungs and naked flight membranes, they are exposed to marked heat losses (66) that could influence the biology of bat-derived *Pneumocystis* organisms and reveal unknown adapting mechanisms of these singular parasitic fungi to their hosts.

The present study is based on the molecular detection of *Pneumocystis* in the lungs of bats sampled from diverse biotopes in New and Old Worlds. It explores the *Pneumocystis* infection frequency in chiropters and *Pneumocystis* genetic diversity in relation with environmental and behavioral conditions. The results suggest that wild bat populations constitute a good model for approaching the circulation of *Pneumocystis* organisms in mammal populations, host specificity, and coevolution events.

MATERIALS AND METHODS

Samples. A total of 216 bat specimens from the New World (Mexico, Guyana, Argentina) and from the Old World (France) were examined for the presence of *Pneumocystis* in their lungs. A total of 155 wild microbats belonging to 17 species were collected in different areas of Mexico (88 specimens), French Guyana (13 specimens), Argentina (16 specimens), and France (38 specimens). A total of 61 megabat specimens belonging to 2 species (*Rousettus aegyptiacus* and *Pteropus rodricensis*) were sampled from two colonies held in the same enclosure in La Palmyre Zoological Park (France). The founders of these colonies came from Northern Africa and Rodrigues Island (southwest Indian Ocean, 19°43′00′′S and 63°25′00′′E), respectively. Nineteen and 20 *Pteropus rodricensis* individuals were, respectively, transferred in 1993 and 1994 out of Jersey Zoological Park (Jersey Island) to La Palmyre Zoological Park. The colony housed in Jersey was founded by 10 individuals captured in Rodrigues Island in 1967 and 1977. The population of *Rousettus aegyptiacus* in La Palmyre was founded in 1994, with 120 individuals from various locations.

After euthanasia, the lungs were removed and immediately stored at −20°C in sterile cryotubes until used. In all cases, national rules regulating bat species protection have been respected.

Size of bat colonies. For the genus *Tadarida*, colony size was assessed by counting bat individuals. As no exact assessment of colony size was available for the other species, a semiquantitative estimation was performed on the basis of direct observational appreciation of the number of colony members. Such a method led to defined scores: big colony, >200 individuals; medium colony, 31 to 200 individuals; and small colony, 10 to 30 individuals.

DNA extraction. DNA extraction from lung tissue samples was performed using a DNeasy tissue kit (Qiagen, Courtabœuf, France) by following the manufacturer's procedure with some modifications. Part of the lung tissue (25 mg) was lysed overnight in an incubator at 56°C with permanent rotation. In order to concentrate *Pneumocystis* DNA, the column was rehydrated with 100 μl of elution AE buffer. DNA was stored at −20°C. A negative control was systematically included in each series of DNA extraction.

DNA amplification and analyses. The presence of *Pneumocystis* DNA in lungs was assessed by nested PCR at the mtLSU rRNA and mtSSU rRNA loci (91, 96), mitochondrial genes encoding rRNA. Primer sequences and PCR cycling conditions are shown in Table 1.

Negative controls were included in each experiment of PCR amplification, to monitor for eventual contamination. When nonspecific bands were detected, amplification products of the expected size (about 250 to 300 bp) were extracted from a 2% agarose gel (run in Tris-borate-EDTA buffer) using a PCR purification kit (QIAEX II gel extraction kit; Qiagen, Courtabœuf, France). When a unique band of expected size was present, amplified products were directly sent for sequencing to GenoScreen (Pasteur campus, Genopole of Lille, France). Sequencing from both ends using sets of internal primers was performed on an automated DNA sequencer (3730XL DNA Analyser; Applied Biosystems). Amplification and sequencing of each sample were repeated at least twice. The mtLSU and mtSSU sequences were aligned with already known *Pneumocystis* sequences using the computer program CLUSTAL X (version 1.63b, December 1997) (89). Then, alignments were refined by visual optimization using the software Se-Al version 2.0a11 Carbon (75). The aligned sequences were converted to distance matrix (percentage of differences). The BLASTn (http://www.ncbi.nlm.nih.gov/BLAST/) program allowed the comparison of our query sequence with the sequences available from databases in order to confirm sequence novelty and absence of contamination. To infer phenetic relationships among *Pneumocystis* isolates of our data set, an improved version of the neighbor-joining algorithm based on a simple model of sequence data (BIONJ) (37) analyses using PAUP 4.0b9 software was run (87). Evaluation of statistical confidence in nodes was based on 1,000 bootstrap replicates in BIONJ (34).

Sensitivity of the PCR. Amplification products of mtLSU and mtSSU rRNA from *Pneumocystis carinii* were cloned by using a TOPO TA cloning kit (Invitrogen). Fifteen separate colonies were selected from the transformant plates and examined for each positive sample. DNA extraction was performed with a QIAprep miniprep kit (Qiagen). To check that the insert contained in the plasmid was the expected sequence, we used the external primer set of each nested PCR mtLSU ribosomal DNA (rDNA) and mtSSU rDNA locus (Table 1). A range of dilution containing from 10^6 copies to 1 copy of the targeted gene was performed. To determine the PCR sensitivity, the presence of a *Pneumocystis carinii* gene fragment within plasmids was assessed by nested PCR at the mtLSU rRNA and mtSSU rRNA loci as described above. The amplification products were visualized on a 2% agarose gel containing ethidium bromide.

Statistical analysis. Statistical analyses were performed using SAS (version 9; SAS Institute). Variables are described as counts (proportions). A generalized linear mixed-model approach (with PROC

TABLE 1 Primer sequences and cycling conditions of *Pneumocystis* nested PCR assays at either the mtSSU rDNA or mtLSU rDNA locus (modified from references 97 and 91)

Loci or cycling step	Sequence or cycling conditions	
	mtSSU rRNA	mtLSU rRNA
Loci		
External primer pair		
Forward	pAZ112-10F/R1: 5′-GGG AAT TCT AGA CGG TCA CAG AGA TCA G-3′	pAZ102-H: 5′-GTG TAC GTT GCA AAG TAC TC-3′
Reverse	pAZ112-10R/R1: 5′-GGG AAT TCG AAC GAT TAC TAG CAA TTC C-3′	pAZ102-E: 5′-GAT GGC TGT TTC CAA GCC CA-3′
Internal primer pair		
Forward	pAZ112-13/R1: 5′-GGG AAT TCG AAG CAT GTT GTT TAA TTC G-3′	pAZ102-X/R1: 5′-GGG AAT TCG TGA AAT ACA AAT CGG ACT AGG-3′
Reverse	pAZ112-14/R1: 5′-GGG AAT TCT TCA AAG AAT CGA GTT TCA G-3′	pAZ102-Y/R1: 5′-GGG AAT TCT CAC TTA ATA TTA ATT GGG GAG C-3′
Cycling step		
1st round		
Denaturation	30 s, 94°C	30 s, 94°C
Annealing	1 min, 55°C	1 min, 50°C
Elongation	1 min, 65°C	1 min, 65°C
No. of cycles	40	30
2nd round (performed with 5% [vol/vol] of the first-round mix)		
Denaturation 1	30 s, 94°C	30 s, 94°C
Annealing 1	1 min, 52°C	1 min, 55°C
Elongation 1	1 min, 65°C	1 min, 72°C
No. of cycles	10	30
Denaturation 2	30 s, 94°C	
Annealing 2	1 min, 63°C	
Elongation 2	1 min, 65°C	
No. of cycles	30	

GLIMMIX) was used to investigate the association between *Pneumocystis* carriage and potential predictors. Models were built with a binomial error distribution and the logit link function, and capture site was included as a random factor. Covariates with a level of significance of <0.1 in the univariate analyses were entered in multivariate models, the variables being then considered significant with a P value of <0.05.

Nucleotide sequence accession numbers. *Pneumocystis* mtLSU rDNA or mtSSU rDNA sequences were deposited in GenBank under accession numbers JQ039397, JQ061293 to JQ061303, and JQ061304 to JQ061318.

RESULTS

Detection of *Pneumocystis* DNA in the lungs of bats. A sample was considered to be positive for the presence of *P. carinii* DNA when a specific band was amplified by PCR at the expected size either at the mtLSU rRNA or mtSSU rRNA locus or at both loci and then sequenced. Two hundred sixteen lung samples from 19 bat species were examined (Tables 2 and 3). None of the tested lung tissue samples gave a positive amplification after the first PCR round at either locus. At the second round, PCR amplification was positive in 71 animals (32.9%) belonging to 12 bat species: one megabat (*Rousettus aegyptiacus*) and 11 microbats (Table 3) species. Most positive lung samples were from neotropical bat specimens: 6/13 from French Guyana, 36/88 from Mexico, and 8/16 from Argentina. In metropolitan France, 14/38 lung samples from wild bats were found to be positive. In captive animals (North African bats housed in La Palmyre Zoological Park in metropolitan France), *Pneumocystis* DNA was detected in 6/61 lung samples. On the whole, a positive amplification at both mtSSU rDNA and mtLSU rDNA was obtained in 48/216 (22.2%) animals belonging to 9 bat species.

The sensitivities of the nested PCR mtLSU and the nested PCR mtSSU indicate, respectively, a limit of detection of 10 copies and 1 copy. However, our experimental results indicate that the ratios of mtLSU-positive/mtSSU-negative samples and mtLSU-negative/mtSSU-positive samples are, respectively, 33.14% and 37.2% when *P. rodricensis* flying foxes were excluded. The sensitivity of *Pneumocystis* detection is not the same at both loci. Moreover, the *Pneumocystis* sequences are variable and primers hybridize differently according to the species of bats analyzed. Consequently, the rate of the *Pneumocystis* carriage is probably underestimated in this study.

Potential influence of host suborder and geography. The frequency of *Pneumocystis* DNA detection was significantly higher in suborder Microchiroptera (41.9%) than in suborder Megachiroptera (9.8%) ($P < 0.0001$). However, only two species of megabats could be examined in the present work: Egyptian fruit bat (*R. aegyptiacus*) and Rodrigues Island flying fox (*Pteropus rodricensis*). The sampled specimens of these two species lived in the same enclosure since 1994 in La Palmyre Zoological Park. *Pneumocystis* DNA was detected in 35.3% of the 17 Egyptian fruit bats, but none of the 44 flying foxes was found to be positive.

With regard to geography, the frequencies of *Pneumocystis* carriage in bats from the New World and Old World were 43.6% and

TABLE 2 Rate of *Pneumocystis* carriage in bat species and ecological data

Origin	Bat species	Rate of *Pneumocystis* carriage[a]	Size of bat colonies[b]	Migratory	Day roost	Mating system[c]	Close physical contact	Reference(s) or source
Wild microchiropters of the New World	*Tadarida brasiliensis*	36/79 (45.6)	Big	Yes	Caves	MM/MF	Yes	10, 59
	Artibeus hirsutus	3/5 (60)	Small	No	Caves	Harem	ND	72, 88
	Mormoops megalophylla	0/3 (0)	Big	No	Caves	Harem	No	*Mormoops megalophylla*, unpublished abstract compiled and edited by the Heritage Data Management System, Arizona Game and Fish Department, and reference 88
	Myotis californicus	1/1 (100)	Small	No	Caves	ND	ND	88
	Pteronotus parnellii	1/3 (33.3)	ND[d]	No	Caves	ND	ND	88
	Natalus stramineus	1/8 (12.5)	Big	No	Caves	ND	No	88, 90
	Pteronotus davyi	0/1 (0)	ND	ND	Caves	ND	ND	
	Glossophaga soricina	9/16 (56.5)	Small	No	Caves	Harem	Yes	60, 66
	Carollia perspicillata	0/1 (0)	Small	Yes	Tree-dwelling, forest	Lek	Yes	59
Wild microchiropters of the Old World	*Nyctalus noctula*	6/20 (30)	Medium	Yes	Tree-dwelling, forest	Lek	Yes	10, 60, 59
	Pipistrellus pipistrellus	3/9 (33.3)	Medium	Yes	Anthropophilic	Harem	Yes	59
	Eptesicus serotinus	2/2 (100)	Medium	No	Anthropophilic	ND	ND	60
	Nyctalus leisleri	0/1 (0)	Small	Yes	Tree-dwelling, forest	Lek	Yes	86
	Myotis daubentoni	0/1 (0)	Medium	Yes	Tree-dwelling, forest	MM/MF	Yes	6, 60
	Myotis myotis	0/2 (0)	Big	Yes	Caves	MM/MF	Yes	10, 60, 59
	Plecotus austriacus	1/1 (100)	Medium	No	Anthropophilic	ND	Yes	80
	Plecotus auritus	2/2 (100)	Small	No	Tree-dwelling, forest	MM/MF	Yes	10, 60, 59
Captive megachiropters of the Old World	*Rousettus aegyptiacus*	6/17 (35.3)	Medium	No	Zoological park			
	Pteropus rodricensis	0/44 (0)	Medium	No	Zoological park			

[a] Number of *Pneumocystis* PCR-positive samples/total number of tested samples (%).
[b] Bat colony size was scored as follows: small, 10 to 30 individuals; medium, 31 to 200 individuals; big, >200 individuals.
[c] MM/MF, multi-male/multi-female.
[d] ND, not done.

20.2%, respectively. However, the presence of *Pneumocystis* DNA in bats from the Old World rose to 36.8% when *P. rodricensis* flying foxes were excluded (Table 4). No significant statistical association could be established between the presence of *Pneumocystis* DNA and Old or New World geographic location of the animals ($P = 0.81$) (Table 5). Consistently, no significant difference was found between microchiropters from the New (43.6%) and Old (35.6%) Worlds.

Impact of ecological and behavioral factors. Neither wild/captive state nor host phylogeny influenced *Pneumocystis* DNA detection significantly, with the obvious exception of *P. rodricensis*, in which *Pneumocystis* DNA was not found (Tables 2 and 3). In contrast, the size of the bat colony influenced the frequency of *Pneumocystis* DNA detection significantly ($P = 0.04$; Table 5). Thus, the probability of picking a *Pneumocystis*-infected bat was 3-fold higher in small colonies than in large ones, irrespective of species (Table 5). Likewise, migration and crowding influenced the frequency of *Pneumocystis* DNA detection significantly (Tables 5 and 6). The statistical multivariate analyses showed that migration and crowding are independent predictive factors of *Pneumocystis* carriage (Table 6). The probability of picking a *Pneumocystis*-infected bat was 5-fold higher in sedentary colonies than in migratory ones and 33-fold higher in crowding colonies (close physical contact between the colony members) than in non-crowding colonies (without close physical contact) (Table 6). When we limit analyses to bats in close physical contact ($n = 132$: 19 sedentary and 113 migratory bats), migration is the sole predictive factor, nonmigratory bats having an odds ratio (OR) of 5.091 (1.147 to 22.597) ($P = 0.03$) with regard to the migratory bats.

Cave temperature (°C) and relative humidity (%) were not recorded inside the main chamber. Temperature and relative humidity outside the cave were evaluated by general climate of the geographical area of the cave. The climate, roosting habits, and mating system did not seem to influence *Pneumocystis* carriage (Table 5). The elevation and food regimen did not seem to influence it either, although significance is borderline ($P = 0.07$).

TABLE 3 *Pneumocystis* carriage in bats[a]

Origin	Bat species	Targeted loci	No. of *Pneumocystis* PCR-positive samples/total number of tested samples by sampling area										
			La Trinitaria cave, Chiapas, Mexico	Isla de Janitzio cave, Michoacán, Mexico	Juxtlahuacave, Colotlipa Guerrero, Mexico	El Salitre, cave, Hidalgo, Mexico	El Salitre, cave Santa Rosa, Morelos, Mexico	La Boca cave, Nuevo León, Mexico	Dique Escaba, Tucumán, Argentina	French Guyana	Natural History Museum Bourges, France	La Palmyre zoological park, France	All areas
Wild microchiropters of the New World	*Tadarida brasiliensis*	LSU	0/10	2/6		5/20		16/27	7/16				30/79
		SSU	2/10	2/6		4/20		17/27	7/16				32/79
		Carrier	2/10	2/6		5/20		19/27	8/16				36/79
	Artibeus hirsutus	LSU					2/5						2/5
		SSU					3/5						3/55
		Carrier					3/5						3/5
	Mormoops megalophylla	LSU			0/2			0/1					0/3
		SSU			0/2			0/1					0/3
		Carrier			0/2			0/1					0/3
	Myotis californicus	LSU						1/1					1/1
		SSU						1/1					1/1
		Carrier						1/1					1/1
	Pteronotus parnellii	LSU			0/2	0/1							0/3
		SSU			0/2	1/1							1/3
		Carrier			0/2	1/1							1/3
	Natalus stramineus	LSU			1/8								1/8
		SSU			0/8								0/8
		Carrier			1/8								1/8
	Pteronotus davyi	LSU			0/1								0/1
		SSU			0/1								0/1
		Carrier			0/1								0/1
	Glossophaga soricina	LSU			3/4					6/12			9/16
		SSU			2/4					6/12			8/16
		Carrier			3/4					6/12			9/16
	Carollia perspicillata	LSU								0/1			0/1
		SSU								0/1			0/1
		Carrier								0/1			0/1
Wild microchiropters of the Old World	*Nyctalus noctula*	LSU									5/20		5/20
		SSU									6/20		6/20
		Carrier									6/20		6/20
	Pipistrellus pipistrellus	LSU									1/9		1/9
		SSU									3/9		3/9
		Carrier									3/9		3/9
	Eptesicus serotinus	LSU									2/2		2/2
		SSU									2/2		2/2
		Carrier									2/2		2/2
	Nyctalus leisleri	LSU									0/1		0/1
		SSU									0/1		0/1
		Carrier									0/1		0/1
	Myotis daubentoni	LSU									0/1		0/1
		SSU									0/1		0/1
		Carrier									0/1		0/1
	Myotis myotis	LSU									0/2		0/2
		SSU									0/2		0/2
		Carrier									0/2		0/2

	Plecotus austriacus	LSU											0/1
		SSU											1/1
		Carrier											1/1
	Plecotus auritus	LSU											2/2
		SSU											1/2
		Carrier											2/2
Captive megachiropters of the Old World	*Rousettus aegyptiacus*	LSU										4/17	4/17
		SSU										6/17	6/17
		Carrier										6/17	6/17
	Pteropus rodriensis	LSU										0/44	0/44
		SSU										0/44	0/44
		Carrier										0/44	0/44
All areas	All species	Positive *Pneumocystis* samples/total samples	2/10	2/6	4/17	6/21	3/5	20/29	8/16	6/13	14/38	6/61	71/216
		Pneumocystis carriage (%)	20.0	33.3	23.5	28.6	60.0	69.0	50.0	46.2	36.8	9.8	32.9

[a] DNA lung samples were screened by nested PCR at either the mtLSU rRNA (LSU) or mtSSU rRNA (SSU) locus or both loci for the presence of *Pneumocystis*. Results of *Pneumocystis* carriage in bats are given according to bat species, targeted locus, and sampling area. LSU, samples that are positive at the LSU locus, and some of these samples can also be positive at SSU; SSU, samples that are positive at the SSU locus, and some of these samples can also be positive at LSU; Carrier, a bat lung sample was considered positive for *Pneumocystis* DNA when nested PCR was positive at either mtLSU rRNA or mtSSU rRNA or at both loci.

The case of *Tadarida brasiliensis*, influence of elevation. Focusing on a single species of bats allowed us to remove all the behavioral factors, to evaluate only the environmental factors. The largest number of sampled bats belonged to the species *Tadarida brasiliensis*. We have collected 79 specimens of *T. brasiliensis* in five locations from North and South America (Table 3, Fig. 1): four regions in Mexico (La Trinitaria cave Chiapas [1,460 m], Isla de Janitzio cave Michoacán [2,120 m], El Salitre cave Hidalgo [1,320 m], and La Boca cave Nuevo León [445 m to 600 m]) and one province in Argentina (Dique Escaba, Tucumán Province [650 m]). The *Pneumocystis* detection rate in *T. brasiliensis* was related to elevation ($P = 0.04$): in bats coming from locations situated lower than 800 m, the *Pneumocystis* DNA carriage rate was 5-fold higher than in bats coming from locations situated at an elevation superior to 800 m (Tables 7 and 8). On the other hand, the climate (humidity and heat) did not seem to have an impact on *Pneumocystis* DNA carriage.

***Pneumocystis* genetic diversity in bats.** The genetic diversity of *Pneumocystis* from bats is examined by analyzing mtLSU rRNA and mtSSU rRNA sequences. PCR products ranged from 252 to 333 bp (nested PCR at the mtLSU rRNA gene) and from 334 to 480 bp (nested PCR at the mtSSU rRNA gene). The sequences under analysis are part of the ribosome and are coded by the mitochondrial genome, and no introns were detected in the mitochondrial genome (82). Direct sequencing of PCR products revealed that each *Pneumocystis* mtLSU rDNA or mtSSU rDNA sequence could be associated with a unique bat species (Tables 9 and 10; Fig. 2 and 3). Comparison of the mtLSU rRNA aligned sequences was carried out on 458 positions, including gaps, for a total of 14 taxa: 12 original sequences and two sequences already published, *Pneumocystis murina* (GenBank accession AF257179) and *Pneumocystis oryctolagi* (GenBank accession S42915), were chosen as outgroups. For the mtSSU rRNA locus, comparison of the aligned sequences was carried out on 530 positions, including gaps, for a total of 17 taxa: 15 original sequences and two sequences already published, *Pneumocystis* f. sp. *Lepus europaeus* (GenBank accession no. JF431106) and *P. oryctolagi* (47), were chosen as outgroups. Genetic divergence between *Pneumocystis* organisms isolated from different bat species, as assessed by *Pneumocystis* mtLSU rDNA or mtSSU rDNA sequence divergence, varied from 0.35 to 26.88% (Tables 9 and 10).

With regard to the *Pneumocystis* mtLSU rRNA locus, we found 12 sequences in the lungs of 9 bat species (Table 9, Fig. 2). In 6 bat species (*Myotis californicus*, *Nyctalus noctula*, *Artibeus hirsutus*, *Eptesicus serotinus*, *Pipistrellus pipistrellus*, *Plecotus auritus*), one specific *Pneumocystis* mtLSU rDNA sequence per species was detected (Fig. 2). In contrast, *Glossophaga soricina* and *Tadarida brasiliensis* species each harbored 2 variants of *Pneumocystis* mtLSU rDNA depending on the collection area (31). The mtLSU rDNA sequences of *Pneumocystis* detected in *G. soricina* from French Guyana and Mexico diverged by 0.35%, corresponding to one nucleotide difference (Table 9), and grouped together on a phenetic branch that is supported by a 100% bootstrap value (Fig. 2). Likewise, a relatively close order of divergence (0.78%) was found between *Pneumocystis* mtLSU rDNA sequences amplified from *T. brasiliensis* samples collected from either Argentinean or Mexican colonies (Tables 9 and 11, Fig. 1). Consistently, these sequences are included in the same group supported by a 100% bootstrap value in phenetic analysis (Fig. 2). Interestingly, mtLSU rDNA

TABLE 4 Ecological data on wild microchiropters and *Pneumocystis* carriage rates[a]

Characteristic	No. (%)		
	Whole sample ($n = 155$)	Noncarriers ($n = 90$)	Carriers ($n = 65$)
Bat colony size			
Big	92	55 (59.8)	37 (40.2)
Medium	33	21 (63.6)	12 (36.4)
Small	26	11 (42.3)	15 (57.7)
Migration			
Yes	113	68 (60.2)	45 (39.8)
No	41	21 (51.2)	20 (48.8)
World			
Old world	38	24 (63.2)	14 (36.8)
New world	117	66 (56.4)	51 (43.6)
Diet			
Insectivorous	133	80 (60.2)	53 (39.8)
Nectarivorous	16	7 (43.8)	9 (56.2)
Frugivorous	6	3 (50.0)	3 (50.0)
Day roost			
Tree-dwelling, forest	25	17 (68.0)	8 (32.0)
Cave	118	67 (56.8)	51 (43.2)
Anthropophilic	12	6 (50.0)	6 (50.0)
Mating system			
Multi-male/multi-female	84	46 (54.8)	38 (45.2)
Harem	33	18 (54.6)	15 (45.4)
Lek	22	16 (72.7)	6 (27.3)
Contact in the colony			
Crowding	132	75 (56.8)	57 (43.2)
Without contact	11	10 (90.9)	1 (9.1)
Climate			
1 = tropical	10	8 (80.0)	2 (20.0)
2 = warm temperate	49	34 (69.4)	15 (30.6)
3 = warm semi-arid	29	9 (31.0)	20 (69.0)
4 = subtropical	16	8 (50.0)	8 (50.0)
5 = equatorial	13	7 (53.9)	6 (46.2)
6 = temperate	38	24 (63.2)	14 (36.8)
Humidity			
No (climates 2, 3, 6)	116	67 (57.8)	49 (42.2)
Yes (climates 1, 4, 5)	39	23 (59.0)	16 (41.0)
Heat			
No (climates 2, 6)	87	58 (66.7)	29 (33.3)
Yes (climates 1, 3, 4, 5)	68	32 (47.1)	36 (52.9)
Elevation			
≤800 m (origins d, f, g, h)	96	48 (50.0)	48 (50.0)
>800 m (origins a, b, c, e, i)	59	42 (71.2)	17 (28.8)

[a] Bat colony size was scored as follows: small, 10 to 30 individuals; medium, 31 to 200 individuals; big, >200 individuals. Data from *P. rodricencis* were excluded from analysis. Origin: a, La Trinitaria cave, Chiapas, Mexico; b, Isla de Janitzio cave, Michoacán, Mexico; c, El Salitre cave, Hidalgo, Mexico; d, La Boca cave, Nuevo León, Mexico; e, Juxtlahuaca cave, Colotlipa Guerrero, Mexico; f, Dique Escaba, Tucumán, Argentina; g, French Guyana; h, Natural History Museum of Bourges, France; i, El Salitre cave, Santa Rosa, Morelos, Mexico.

TABLE 5 *Pneumocystis* carriage in bats: influence of ecological and behavioral factors (univariate analysis)[a]

Characteristic	OR	95% CI	*P*
Bat colony size			
Medium or big	1		
Small	3.259	1.038–10.230	0.04
Migration			
Yes	1		
No	2.959	0.997–8.779	0.05
Area			
Old World	1		
New World	1.213	0.170–8.659	0.81
Diet			
Insectivorous or frugivorous	1		
Nectarivorous	3.948	0.864–18.045	0.07
Day roost			
Tree-dwelling, forest	1		
Cavernicole	1.299	0.286–5.902	0.71
Anthropophilic	2.148	0.518–8.903	0.29
Mating system			
Lek	1		
Harem	1.905	0.512–7.080	0.33
MM-MF	1.827	0.477–6.897	0.37
Contact in the colony			
No	1		
Yes	11.387	1.154–112.354	0.038
Humidity			
Yes	1		
No	1.064	0.285–3.972	0.92
Heat			
No	1		
Yes	2.030	0.685–6.020	0.16
Elevation			
>800 m	1		
≤800 m	2.448	0.919–6.518	0.07

[a] OR, odds ratio; CI, confidence interval. Significant results at $P = 0.05$.

polymorphism was also detected in *R. aegyptiacus* (from the La Palmyre Zoo), with two *Pneumocystis* variants that diverged by a much higher percentage (19.91%; Table 9). Both Megachiroptera-derived *Pneumocystis* organism sequences were placed in a basal position according to the phenetic analysis (Fig. 2). All mtLSU rDNA sequences of *Pneumocystis* organisms from Microchiroptera were included in the same clade. In addition, *Pneumocystis* sequences derived from the New World bats and those from the Old World bats are mixed (Fig. 2).

Regarding the *Pneumocystis* mtSSU rRNA locus, we isolated 15 sequences from 11 bat species (Table 10, Fig. 3). In 8 bat species (*M. californicus*, *G. soricina*, *N. noctula*, *A. hirsutus*, *E. serotinus*, *Plecotus austriacus*, *P. auritus*, *Pteronotus parnellii*), we identified one specific *Pneumocystis* sequence per host species, while we detected two highly divergent mtSSU rRNA sequences in *R. aegyptiacus* (21.72%), two weakly divergent sequences in *P. pipistrellus*

TABLE 6 *Pneumocystis* carriage in bats: influence of ecological and behavioral factors (multivariate analysis)[a]

Characteristic	OR	95% CI	*P*
Migration			
Yes	1		
No	4.759	1.15–20.310	0.036
Contact in the colony			
No	1		
Yes	33.313	2.687–412.963	0.007

[a] OR, odds ratio; CI, confidence interval. Significant results at $P = 0.05$.

TABLE 7 *Pneumocystis* carriage in *Tadarida brasiliensis*: influence of climate and elevation

Characteristic[a]	No. (%)		
	Whole sample ($n = 155$)	Noncarriers ($n = 90$)	Carriers ($n = 65$)
Climate			
1 = tropical	10	8 (80.0)	2 (20.0)
2 = warm temperate	26	19 (73.1)	7 (26.9)
3 = warm semi-arid	27	8 (29.6)	19 (70.4)
4 = subtropical	16	8 (50.0)	8 (50.0)
Humidity			
No (climates 2, 3, 6)	53	27 (50.9)	26 (49.1)
Yes (climates 1, 4, 5)	26	16 (61.5)	10 (38.5)
Heat			
No (climates 2, 6)	26	19 (73.1)	7 (26.9)
Yes (climates 1, 3, 4, 5)	53	24 (45.3)	29 (54.7)
Elevation			
≤800 m (origins d, f)	43	16 (37.2)	27 (62.8)
>800 m (origins a, b, c)	36	27 (75.0)	9 (25.0)

[a] Origin: a, La Trinitaria cave, Chiapas, Mexico; b, Isla de Janitzio cave, Michoacán, Mexico; c, El Salitre cave, Hidalgo, Mexico; d, La Boca cave, Nuevo León, Mexico; f, Dique Escaba, Tucumán, Argentina.

(0.46%), and three lowly divergent sequences in *T. brasiliensis* (0.89% to 1.83%) (Tables 10 and 11). In the phenetic tree displaying mtSSU rDNA sequences, both sequences of macrochiroptera-derived *Pneumocystis* organisms are mixed with Microchiroptera-derived *Pneumocystis*, but the bootstrap values are not significant (Fig. 3). The *Pneumocystis* sequences derived from the same host species (*T. brasiliensis* and *P. pipistrellus*) clustered together with 100% bootstrap values (Fig. 3).

Geography seemed to have a structuring effect on *Pneumocystis* mtSSU rRNA polymorphism. Thus, the two *Pneumocystis* se-

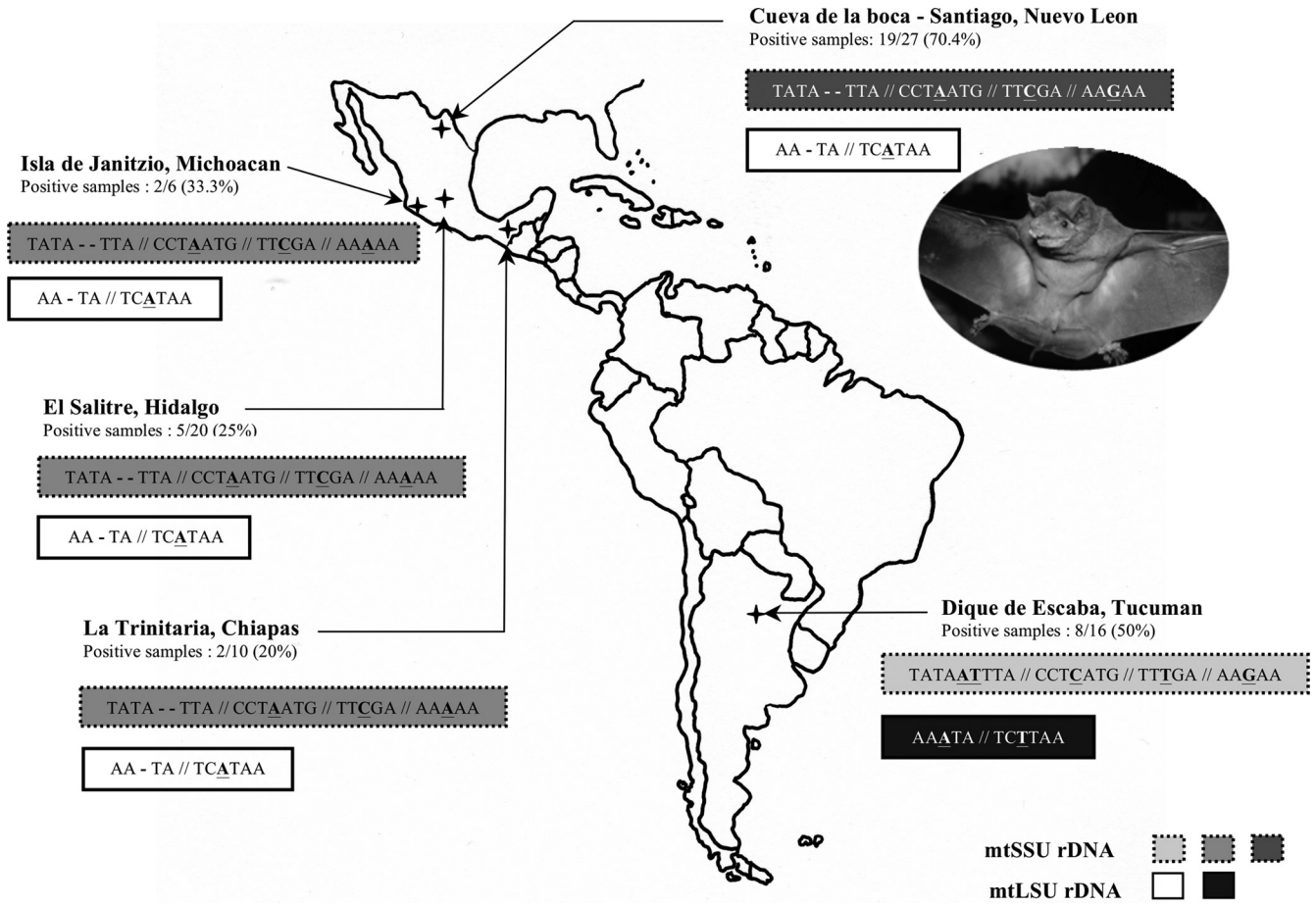

FIG 1 Genetic polymorphism of *Pneumocystis* isolates detected in the lungs of *Tadarida brasiliensis* bats sampled from North and South America. For each geographic location, mtLSU rDNA and mtSSU rDNA *Pneumocystis* polymorphic sequences are indicated and framed: two mtLSU rDNA variants and three mtSSU rDNA variants. The number of positive samples over total number of analyzed samples is also indicated below each location. The photo represents one male bat belonging to the species *Tadarida brasiliensis* and originating from Dique Escaba in Argentina.

TABLE 8 *Pneumocystis* carriage in *Tadarida brasiliensis*: influence of climate and elevation (univariate analysis)[a]

Characteristic	OR	95% CI	*P*
Humidity			
Yes	1		
No	1.450	0.074–28.587	0.73
Heat			
No	1		
Yes	2.521	0.176–36.122	0.36
Elevation			
>800 m	1		
≤800 m	5.049	1.096–23.252	0.04

[a] OR, odds ratio; CI, confidence interval. Significant results at $P = 0.05$.

quences isolated from *P. pipistrellus* (number reference P3 and P6) identified two Metropolitan French Departments (Haute Marne [51] and Cher [17], respectively) (Table 10). Likewise, the 3 *Pneumocystis* sequences isolated from *T. brasiliensis* identified 3 geographical areas: (i) Michoacán-Hidalgo-Chiapas (Mexico), (ii) Nuevo León (Mexico), and (iii) Tucumán (Argentina) (Fig. 1, Tables 10 and 11).

DISCUSSION

Detection of *Pneumocystis* DNA in the lungs of bats. The rate of the *Pneumocystis* carriage is probably underestimated in this study. Moreover, the abundance of *Pneumocystis* targets (mtLSU rRNA or mtSSU rRNA) in the bat samples studied here is generally low. Thus, chance can determine whether or not *Pneumocystis* gDNA is introduced in a given PCR. This random sampling could explain why some samples produced a positive amplification with one set of primers but not both.

Positive amplification requiring two PCR rounds is usually considered a case of *Pneumocystis* carriage (29, 30), i.e., healthy carriers harboring a low burden of *Pneumocystis* organisms. In the present work, the fact that single *Pneumocystis* PCR was negative in all cases suggests that no animal was heavily infected or developed pneumocystosis. In contrast, *Pneumocystis* carriage was a frequent event (about 33% of examined bats). Likewise, Laakkonen et al. reported high rates of *Pneumocystis* carriage (even if they used histological methods) in wild small mammals from California, but no animal showed histopathological changes typical of PcP (53). Indeed, PcP cases were only rarely reported in wild mammals (3).

Thus, mammals seem to usually develop mild though quite frequent subclinical *Pneumocystis* lung infections (17), suggesting that these microfungi develop efficient airborne circulation in natural ecosystems (3). Severe PcP would therefore be, as in humans, a rare event in the natural history of *Pneumocystis* infection (3).

This view is further consistent with the fine adaptation of *Pneumocystis* organisms to the alveolar microenvironment (8, 16, 23, 26), likely resulting from *Pneumocystis*-host species coevolution (46).

Consistently, it was experimentally shown that *Pneumocystis* organisms were able to replicate in the lungs of healthy hosts (16), which can subsequently transmit the microfungi by airborne route to susceptible or immunocompetent hosts (32, 39). These observations showed that healthy carriers could behave as a reservoir of *Pneumocystis* species, playing a critical role in the airborne circulation of *Pneumocystis* organisms in host populations (16, 17, 29, 30).

Prevalence of *Pneumocystis* colonization in bats. The reported frequency of *Pneumocystis* DNA detection varied markedly between bat species (Table 2). This finding was in accordance with previous studies, where important differences in *Pneumocystis* prevalence between mammal species have been reported (52, 53). In micromammals collected in France (58), rodent infection rates varied according to the species (e.g., 67% in *Apodemus sylvaticus*, 78% in *Eliomys quercinus*). In the same study, the *Pneumocystis* global prevalence was as high as 68%. In Thailand, 58% of the collected *Rattus norvegicus* specimens harbored *Pneumocystis* DNA (17), but infection rates in other rodents from this country

TABLE 9 Divergence matrix of *Pneumocystis* mtLSU rDNA sequences amplified from 9 bat species (GenBank accession no. JQ039397 and JQ061293 to JQ061303), one rodent (*P. murina* from *Mus musculus*, GenBank accession no. AF257179), and one lagomorph species (*P. oryctolagi* from *Oryctolagus cuniculus*, GenBank accession no. S42915)[a]

	% of divergence from:												
Host species	1	2	3	4	5	6	7	8	9	10	11	12	13
1. *G. soricina* FG													
2. *G. soricina* JG	00.35												
3. *A. hirsutus* ES	09.64	08.87											
4. *T. brasiliensis* M	11.04	10.59	17.34										
5. *T. brasiliensis* A	10.35	09.92	17.57	00.78									
6. *M. californicus* LB	11.88	12.33	14.82	08.15	09.21								
7. *P. pipistrellus* B(P6)	14.11	13.12	14.37	12.15	12.72	07.42							
8. *N. noctula* B(NO19)	10.81	10.05	13.52	09.48	09.53	05.23	02.54						
9. *E. serotinus* B(S1)	11.22	10.57	13.84	06.91	07.91	02.00	06.83	04.76					
10. *P. auritus* B(OR3)	12.75	12.03	15.09	08.93	09.30	03.53	05.44	05.52	01.94				
11. *R. aegyptiacus* P(34)	15.60	15.19	20.58	18.30	18.40	20.19	23.19	17.68	18.22	20.14			
12. *R. aegyptiacus* P(13)	16.41	16.57	19.38	16.94	16.72	15.28	19.46	15.19	15.13	16.47	19.91		
13. *P. murina*	23.48	25.19	30.24	17.95	18.10	16.06	28.32	25.98	25.34	25.16	21.89	27.01	
14. *P. oryctolagi*	19.91	21.96	28.98	18.24	17.75	16.04	27.29	24.37	26.72	26.19	18.59	28.66	17.48

[a] Numbered column headings (1 to 13) correspond to numbered sources of host species DNA in the first column. Next to each bat species Latin name, the letter and number codes indicate geographic origin and reference number of samples, respectively. FG, French Guyana; B, Bourges, France; NL, Nuevo León, Mexico; M, Michoacán, Mexico; A, Argentina; LB, La Boca; JG, Juxtlahuaca Grotto, Mexico; ES, El Salitre, Mexico; P, Zoological Park of La Palmyre, France. Letters and numbers in parentheses indicate a particular sample.

TABLE 10 Divergence matrix of *Pneumocystis* mtSSU rDNA sequences amplified from 15 bats (GenBank accession no. JQ061304 to JQ061318) and 2 lagomorph species (*P. oryctolagi* [47]; *P.* f. sp. *Lepus europaeus*, GenBank accession no. JF431106)[a]

Host species	% of divergence from:															
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1. *P. pipistrellus* B(P6)																
2. *P. pipistrellus* B(P3)	00.46															
3. *N. noctula* B(NO19)	07.21	06.52														
4. *E. serotinus* B	13.34	13.26	14.65													
5. *P. auritus* B(OR3)	12.64	12.01	14.15	02.71												
6. *M. californicus* NL	12.05	11.71	13.45	04.29	02.73											
7. *P. austriacus* B	12.34	11.57	14.23	03.24	00.49	03.06										
8. *T. brasiliensis* NL	17.25	14.63	14.58	13.31	13.43	13.06	13.53									
9. *T. brasiliensis* M	17.10	14.54	14.50	13.57	13.68	13.29	13.78	00.89								
10. *T. brasiliensis* A	18.06	15.05	15.76	13.64	14.00	13.23	13.71	01.53	01.83							
11. *G. soricina* JG	14.76	13.67	15.02	12.68	12.44	13.25	12.72	16.28	15.81	16.24						
12. *A. hirsutus* ES	13.91	13.46	14.71	12.01	11.66	11.34	11.75	15.36	14.92	15.77	08.41					
13. *P. parnellii* ES	13.83	14.04	14.64	13.71	14.33	13.36	13.74	16.93	16.45	16.62	08.81	08.45				
14. *R. aegyptiacus* P(12)	21.50	20.68	21.83	17.84	19.05	18.23	18.32	20.99	20.38	19.96	14.38	13.93	12.87			
15. *R. aegyptiacus* P(26)	23.85	23.19	24.38	23.31	23.36	22.06	26.88	21.84	21.34	21.29	15.59	12.58	16.58	21.72		
16. *P. oryctolagi*	25.12	25.25	25.79	23.97	23.34	23.60	23.48	23.32	23.50	24.15	18.50	16.33	20.22	23.02	24.77	
17. *P.* f. sp. *L. europaeus*	19.30	19.01	20.30	18.10	18.21	17.83	17.67	17.43	17.41	17.16	16.30	13.82	18.43	19.29	20.87	09.76

[a] Numbered column headings (1 to 16) correspond to numbered sources of host species DNA in the first column. Next to each bat species Latin name, the letter and number codes indicate geographic origin and reference number of samples, respectively. B, Bourges, France; NL, Nuevo León, Mexico; M, Michoacán, Mexico; A, Argentina; JG, Juxtlahuaca Grotto, Mexico; ES, El Salitre, Mexico; P, Zoological Park of La Palmyre, France. Letters and numbers in parentheses indicate a particular sample.

were quite divergent (M. Chabé and S. Morand, unpublished data). In primates, prevalence of *Pneumocystis* reached 33.6% in healthy macaques (*Macaca fascicularis*) maintained in partial release (30) and 26.5% in captive primates of 26 species taken as a whole (29).

All samples from megachiropters were collected from captive animals, and those from microchiropters were collected from wild animals. Thus, the significantly different *Pneumocystis* rates between megabats and microbats found in the present work may stem from bat taxonomy and/or from the captive/wild state of the

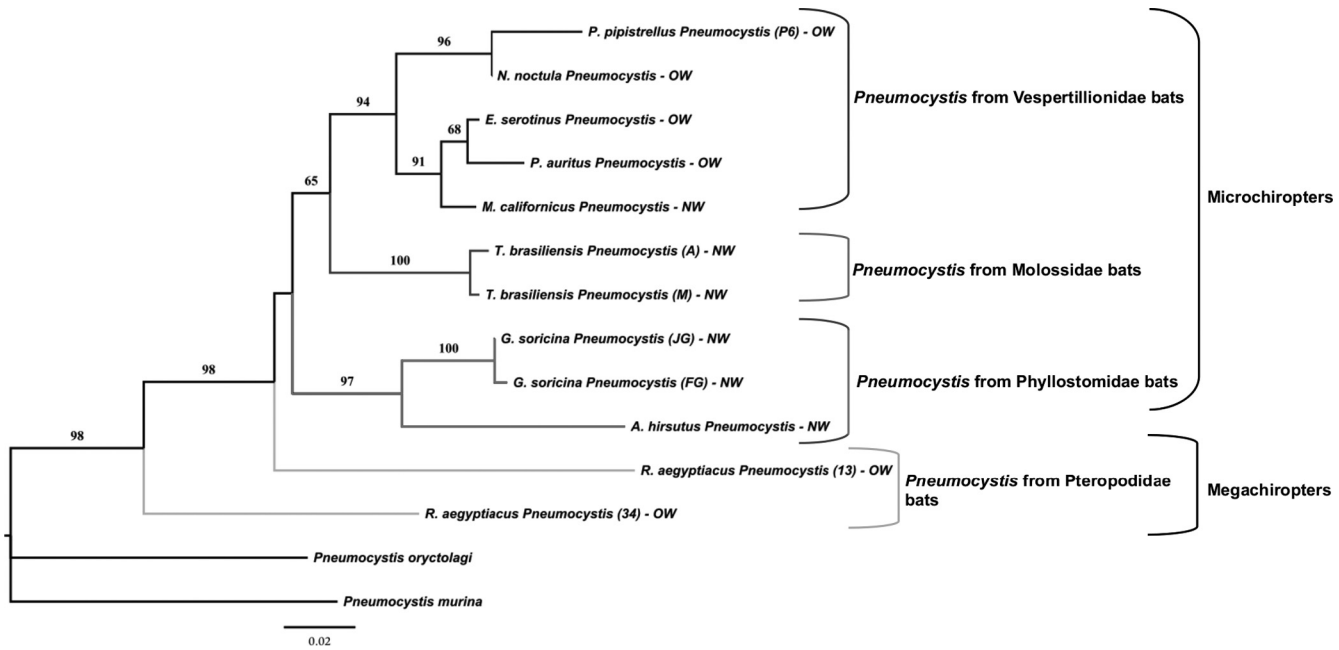

FIG 2 Phenetic relationships of *Pneumocystis* organisms from bat species inferred from mtLSU rDNA sequences. The phylogram presented resulted from bootstrapped data sets obtained by using BIONJ analysis (heuristic search option in PAUP 4.0). The percentages above the branches are the frequencies with which a given branch appeared in 1,000 bootstrap replications. Bootstrap values below 50% are not displayed. Branch lengths correspond to the total nucleotide changes assigned to each branch by PAUP 4.0. *Pneumocystis* from rabbit (*P. oryctolagi*, GenBank accession S42915) and from mouse (*P. murina*, GenBank accession AF257179) were chosen as outgroups. Letter and number codes indicate geographic origin and reference number of samples, respectively. M, Michoacán, Mexico; A, Argentina; JG, Juxtlahuaca Grotto, Mexico; OW, Old World; NW, New World. Letters and numbers in parentheses indicate a particular sample (P6, 13, 34).

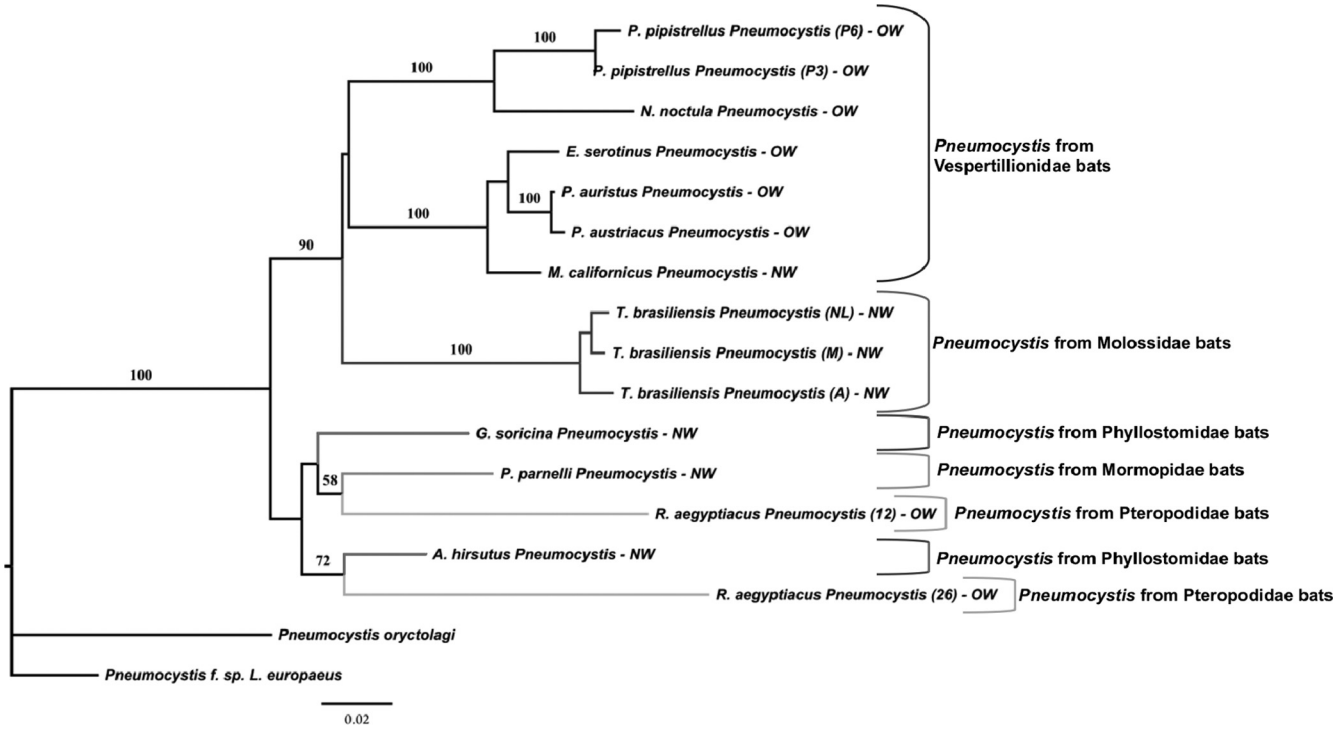

FIG 3 Phenetic relationships of *Pneumocystis* organisms from bat species inferred from mtSSU rDNA sequences. The phylogram presented resulted from bootstrapped data sets obtained by using BIONJ analysis (heuristic search option in PAUP 4.0). The percentages above the branches are the frequencies with which a given branch appeared in 1,000 bootstrap replications. Bootstrap values below 50% are not displayed. Branch lengths correspond to the total nucleotide changes assigned to each branch by PAUP 4.0. *Pneumocystis* from rabbit (*P. oryctolagi* [47]) and from hare (*P.* f. sp. *Lepus europaeus*, GenBank accession JF431106) were chosen as outgroups. Letter and number codes indicate geographic origin and reference number of samples, respectively. NL, Nuevo León, Mexico; M, Michoacán, Mexico; A, Argentina; OW, Old World; NW, New World. Letters and numbers in parentheses indicate a particular sample (P3, P6, 12, 26).

animals as well. Within megachiropters, two species, namely, Egyptian roussette (*R. aegyptiacus*) and flying fox (*P. rodricensis*), were sampled, revealing strikingly different infection rates. *Pneumocystis* DNA was detected in 35.3% of Egyptian roussettes, while no *P. rodricensis* specimen was found to be carrying *Pneumocystis*. Egyptian roussette, a fruit bat species of the Old World, can be found throughout Africa (except in the desert regions of the Sahara) and Middle East, as far as Pakistan and Northern India. In this extensive distribution area, Egyptian roussettes exist as a relatively large wild population (12). In contrast, *P. rodricensis* is restricted to Rodrigues, the smallest of the Mascarene islands, and it is considered an endangered species mainly because of deforestation and cyclones that devastate the area from time to time. Only about 4,000 individuals are thought to exist (48). Rodrigues flying foxes seem to be the sole mammal species studied so far that revealed to be entirely negative for *Pneumocystis* DNA. A first hypothesis is that *Pneumocystis* DNA sequences at the studied loci would be so divergent that they could not be detected by our techniques. Another hypothesis is that the animals used to set up the captive population were totally free of *Pneumocystis* and thus suggests that *Pneumocystis* prevalence is very low or absent in wild Rodrigues flying fox populations or that the sampling of breeders that set up the colony was too small (40 individuals from Jersey Zoological Park). A third hypothesis is that *Pneumocystis* organisms had never colonized or had been lost by this insular host species. Furthermore, Rodrigues flying foxes and Egyptian rous-

TABLE 11 *Pneumocystis* polymorphism at the mtSSU and mtLSU rRNA loci in DNA lung samples from *Tadarida brasiliensis*[a]

Sample origin	mtSSUrRNA sequence	mtLSUrRNA sequence
Mexico		
Cueva de la Boca, Nuevo León	TATA–TTA//CCT**A**ATG//TT**C**GA//AA**G**AA	AA-AT//TC**A**TAA
Isla de Janitzio, Michoacan	TATA–TTA//CCT**A**ATG//TT**C**GA//AA**A**AA	AA-AT//TC**A**TAA
El Salitre, Hidalgo	TATA–TTA//CCT**A**ATG//TT**C**GA//AA**A**AA	AA-AT//TC**A**TAA
La Trinitaria, Chiapas	TATA–TTA//CCT**A**ATG//TT**C**GA//AA**A**AA	AA-AT//TC**A**TAA
Argentina		
Dique de Escaba, Tucuman	TATA**AT**TTA//CCT**C**ATG//TT**T**GA//AA**G**AA	AA**A**AT//TC**T**TAA

[a] Results of *Pneumocystis* polymorphism in these bat specie are given according to sampling area. Bold nucleic acids indicate the mutations. *Pneumocystis* polymorphic sequences show two mtLSUrDNA variants (one from Mexico and the other from Argentina) and three mtSSUrDNA variants (two from Mexico and the third from Argentina).

settes are cohoused in the same enclosure in La Palmyre Zoological Park, and the cross-infection does not seem to occur between these 2 host species of megachiropters. This result confirms the strong host specificity in *Pneumocystis* strains (3, 27, 41).

If we exclude the absence of *Pneumocystis* DNA in Rodrigues flying foxes, the global infection rate is close between microchiropters (41.9%) and megachiropters (35.3%), suggesting a similar circulation pattern of *Pneumocystis* within both groups of either captive or wild chiropters. Likewise, *Pneumocystis* prevalence was found to be identical in bats from the New and Old Worlds. This may indicate similar intensity of circulation of *Pneumocystis* organisms in bat populations and a comparable pattern of *Pneumocystis* host-to-host transmission worldwide.

Crowding in the bat colony and the infection source issue. *Pneumocystis* rates were found to increase when the individuals of the colony are in close contact independently of colony size (Tables 5 and 6). For example, in *Natalus stramineus*, a cave-dwelling bat living in big noncrowding colonies, the *Pneumocystis* DNA carriage rate is low (12.5%) (Table 2). This could be explained by the fact that bats hang individually without contact with each other, keeping a distance of 5 to 50 cm between themselves (90). While in *Tadarida brasiliensis*, a cave-dwelling bat living in big crowding colonies, the carriage rate is high (45.6%) (Table 2). A recent study in human beings showed that the level of *Pneumocystis jirovecii* DNA in exhaled air from infected patients decreased with increased distance from the patients (18). Furthermore, it has been shown that *Pneumocystis* organisms were able to multiply transiently in the lungs of immunocompetent hosts and to transmit the infection to either susceptible or immunocompetent hosts by the airborne route (16, 32, 39). Crowding could therefore favor *Pneumocystis* host-to-host airborne transmission in bats or in other mammals. This observation raises the question of the *Pneumocystis* infection source: do *Pneumocystis* hosts contract the infection from carrier (or infected) hosts or from hypothetical environmental forms of development?

The high rate of *Pneumocystis* carriage found in *T. brasiliensis* (Tables 2 and 3) suggests a highly active interhost airborne transmission. An intensive circulation of *Pneumocystis* organisms was also reported within the members of a social organization of healthy macaques (30). The occurrence of clustered cases of PcP in hospitals (68) and the reported evidence of human-to-human *Pneumocystis* transmission in the community (76) further suggest that interhost transmission could also be highly active in humans. In addition, the fact that *Pneumocystis* organisms are able to dwell and replicate in the lungs of immunocompetent hosts points out the healthy carrier hosts as the infection source and reservoir for *Pneumocystis* species (16).

Another underlying factor which may affect transmission efficiency of *Pneumocystis* is the proportion of newly born animals in the colony. In human communities, infants could constitute a major reservoir for *Pneumocystis* organisms (67, 94), and some data collected in other mammals, like domestic or wild rabbits (26, 40), pigs (50), and macaques maintained in partial release (30), seem to strengthen this hypothesis. A survey scheduled just after the breeding period in a bat colony could provide data on the levels of infection of young bats and on their potential role in the transmission of *Pneumocystis* organisms within the colony.

Alternatively, other observations suggested that *Pneumocystis* infection could be contracted from undefined environmental sources (45). *Pneumocystis* DNA was identified in air and water samples (7, 13, 73, 96). Wakefield was able to detect DNA from rat and human-derived *Pneumocystis* in air samples from rural locations in the United Kingdom (96). Furthermore, outdoor activities, such as gardening, camping, or hiking, have been reported to be associated with PcP in HIV-infected adults (65). However, on the whole, the active airborne host-to-host circulation of *Pneumocystis* organisms and the widening of the parasite reservoir to immunocompetent hosts (16, 24, 39) render the hypothesis of *Pneumocystis* environmental forms of infection less and less plausible. The detection of *Pneumocystis* DNA in air sampled either from the room of PcP patients (7, 71) or from facilities housing laboratory animals with PcP (54, 70) could attest to *Pneumocystis* dissemination with the exhaled air of infected hosts and, therefore, potential transmission to other hosts (7, 70, 83).

In the case of *T. brasiliensis*, the existence of migratory and nonmigratory populations could render the circulation of *Pneumocystis* organisms in the population more complex (77). Thus, migratory bats could carry significantly less *Pneumocystis* organisms than sedentary bats. Nevertheless, it is possible that the increased physiological stress and immunocompromise associated with migration might increase fungal growth within bats and increase transmission (4). But new research has also shown that migration allows hosts to escape from infected habitats, reduces disease levels in successful migrants when infected animals do not migrate successfully, and may lead to the evolution of less-virulent pathogens (4). So, between intervals of habitat use, unfavorable conditions (such as a lack of hosts) could eliminate most parasites, resulting in hosts returning to these habitats after a long absence to encounter largely disease-free conditions (4). Furthermore, parasites that decline in response to host migration may include specialist pathogens, as well as those with transmission stages that can build up in the environment (4). Actually, we can assume that when migrating, bats are less exposed to high fungal loads because (i) crowding is less important, (ii) the period of reproduction is over, and (iii) changing roosts implies renewal of ambient air and, likely, exposure to lower numbers of *Pneumocystis* infective airborne forms.

Climatic factors and altitude. Temperature and humidity did not seem to impact the *Pneumocystis* DNA carriage in bats. However, the climatic factors used in our study represent global trends and did not necessarily reflect the actual conditions inside the caves. Samples from *T. brasiliensis* represent the biggest collection in the present study. In this species, *Pneumocystis* carriage rates ranged from 25% to 70% in five Latin American regions (Fig. 1). The factors accounting for such variability remain to be explored: location and sampling season but also direct exposure to weather changes versus living in a cave, where environmental conditions can be remarkably stable (66). Some studies explored the impact of climatic factors on *Pneumocystis* carriage in other host species. In Finland, an influence of seasonal changes on *Pneumocystis* carriage was reported in wild rodents and insectivores (52). In these host species, the highest prevalence of *Pneumocystis* organisms in lung samples was reported in late autumn (November), when the precipitation rate was high. The impact of environmental factors on *Pneumocystis* carriage was also evaluated in immunocompetent macaques (*Macaca fascicularis*) maintained in partial release (28). The number of macaques with detectable *Pneumocystis* DNA (assessed by nested PCR from deep nasal swab samples) was apparently correlated with mean precipitation rates (28). However, behavioral factors could also intervene. Actually, when it is rain-

ing, macaques group together, some against one another, or enter their shelters, which increases crowding and consequently the *Pneumocystis* host-to-host airborne transmission. Elsewhere, the detection rate of *Pneumocystis* DNA was higher in primates, which died (from any cause) during spring or summer (33.3%) rather than during the colder seasons (19.5%), though these differences did not reach statistical significance (29).

Regarding PcP, spontaneous pneumocystosis in domestic rabbits at weaning was usually found to be markedly less extensive in summer than in winter (74). A few studies reported seasonal variations in the occurrence of human cases of pneumocystosis (higher incidence of PcP with higher temperatures in London, Geneva, and Munich) (61, 84, 92). In contrast, surveys from Spain (93) or the United Kingdom (55) showed PcP incidence to peak in the winter months, as it occurs with other infectious respiratory diseases. In the Spanish survey, PcP incidence was negatively correlated with the mean temperature but not with rainfall activity or wind strength (93). These studies indicated that seasonal variation in PcP incidence may exist, albeit to different extents or tendencies. However, nonclimatic factors, such as human behavior or leisure activities, could also be associated with seasonal change and indirectly influence the PcP incidence (84). In bats, the present data suggest that host behavior (migration, crowding) as well as environmental factors such as climate or geographical area may influence *Pneumocystis* carriage. However, the roosting site did not seem to have a direct impact on the *Pneumocystis* DNA carriage, the elevation excepted. Altitude exposes the body to a set of constraints, the most important of which is hypoxia. The almost exponential decline of the atmospheric pressure is accompanied by a parallel decline of the oxygen pressure in the inspired air, which induces hyperventilation. Thus, altitude could directly impact on respiratory physiology and host defense mechanisms (95) and indirectly act on the development of *Pneumocystis* organisms in the alveolar microenvironment. These aspects were not explored in bats. In nonimmunocompromised laboratory rats, hypobaric hypoxia weakened host immune mechanisms and significantly impaired the surfactant composition. Such changes were not enough, however, to favor *Pneumocystis* growth or to inhibit *Pneumocystis* clearing from their lungs (95).

Genetic variability. For each bat species carrying *Pneumocystis* DNA, at least one novel sequence was amplified at both mtLSU rRNA and/or mtSSU rRNA loci, suggesting that each species of bats could be harboring specific species of *Pneumocystis* (Fig. 2 and 3). The *Pneumocystis* sequences amplified from bat lung samples were markedly different from those of other host species registered in the GenBank database. Furthermore, no cross-infection occurred between Rodrigues flying foxes and Egyptian roussettes, although they were cohoused in the same enclosure. Present data strengthened therefore the host specificity concept of *Pneumocystis* (1, 3, 38) and were consistent with the strong host specificity demonstrated in previous studies dealing with other mammals (27, 41). A first investigation of primate-derived *Pneumocystis* demonstrated that *Pneumocystis* phylogeny mirrors its host phylogeny, suggesting a long-range physiological and genetic adaptation process leading to cospeciation (46). *Pneumocystis* species may have evolved together with their hosts. Likewise, present data showed that genetic divergence in bat-derived *Pneumocystis* organisms parallels phylogenetic divergence existing among the corresponding host, also suggesting coevolution (27, 41).

Unexpectedly, in the megabat *R. aegyptiacus*, we found two sequences highly divergent from each other at both mtLSU rRNA and mtSSU rRNA loci (19.9% and 21.7%, respectively). Interestingly, this divergence was comparable to divergence existing between *Pneumocystis* organisms harbored by *M. californicus* and rabbits (16.0% at the mtLSU rRNA locus and 23.6% at the mtSSU rRNA locus), as well as between *G. soricina* and rabbits (19.9% at the mtLSU rRNA locus and 18.5%, at the mtSSU rRNA locus). The presence of two *Pneumocystis* species in *R. aegyptiacus* could therefore be hypothesized. Two species of *Pneumocystis*, i.e., *Pneumocystis carinii* and *Pneumocystis wakefieldiae* (21), have been described in the same rat species (*Rattus novergicus*), though divergence was lower (9.6% and 8.9% at mtLSU rRNA and mtSSU rRNA loci, respectively) (26). In the case of *T. brasiliensis*, *Pneumocystis* genetic polymorphism was more limited and apparently related with host infraspecific variants. We found three mtSSU rDNA sequences: two were located in Mexico (Hidalgo/Michoacán/Chiapas region and Nuevo León), and the third one was located in Tucumán (Argentina) (Fig. 1; Table 11). In contrast, one mtLSU rDNA sequence type was amplified from *T. brasiliensis* lung samples from Mexico, and another one was amplified from lung samples of *T. brasiliensis* from Argentina. Interestingly, *Pneumocystis* polymorphism seemed to be related with *T. brasiliensis* subspecies, which have been described on the basis of geographical distribution and morphology (81). As we have previously suggested (31), *Pneumocystis* strain polymorphism could be used as a phylogeographic tool to be applied to host natural populations.

Conclusions. *Pneumocystis* spp. form a group of parasitic microorganisms infecting a vast diversity of hosts in various ecosystems. A great number of mammalian species belonging to different orders of the clade Mammalia were found to be harboring *Pneumocystis* organisms. So far, all bat species (11 bat species belonging to 5 families of Chiroptera) examined in this study were found to harbor *Pneumocystis* DNA except for the flying fox species, *Pteropus rodricensis*. Interestingly, if confirmed, the absence of *Pneumocystis* organisms in 44 specimens of *P. rodricensis* is, as far as we know, the first report of a mammal population, in which no *Pneumocystis* organisms were detected. Globally, we found a high *Pneumocystis* carriage rate of 41.3% in bats. Social or behavioral factors (migration, breeding, crowding) may influence transmission of *Pneumocystis* within the colonies of bat species, while the environmental factors (such as the climate, the roosting habits, geographical place) do not seem to have of impact on carriage rate of *Pneumocystis*, with the exception of altitude.

In addition, genetic divergence existing among *Pneumocystis* DNA sequences isolated from different bat species illustrates the close host species specificity reported for *Pneumocystis* species (3, 38). A comprehensive phylogeny of *Pneumocystis* from bats is in progress, and comparison with bat phylogeny suggests coevolution (C. Demanche and C.-M. Aliouat-Denis, unpublished results) as it was reported in primates (27, 41, 46) and suggested in other mammalian groups (41). Finally, the link between genetic variability of *Pneumocystis* isolated from populations of the same bat species and their geographic localization could be exploited in terms of phylogeographical research (31).

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An *Hcp100* gene fragment reveals *Histoplasma capsulatum* presence in lungs of *Tadarida brasiliensis* migratory bats

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SUMMARY

Histoplasma capsulatum was sampled in lungs from 87 migratory *Tadarida brasiliensis* bats captured in Mexico ($n=66$) and Argentina ($n=21$). The fungus was screened by nested-PCR using a sensitive and specific *Hcp100* gene fragment. This molecular marker was detected in 81·6% [95% confidence interval (CI) 73·4–89·7] of all bats, representing 71 amplified bat lung DNA samples. Data showed a *T. brasiliensis* infection rate of 78·8% (95% CI 68·9–88·7) in bats captured in Mexico and of 90·4% (95% CI 75·2–100) in those captured in Argentina. Similarity with the *H. capsulatum* sequence of a reference strain (G-217B) was observed in 71 *Hcp100* sequences, which supports the fungal findings. Based on the neighbour-joining and maximum parsimony *Hcp100* sequence analyses, a high level of similarity was found in most Mexican and all Argentinean bat lung samples. Despite the fact that 81·6% of the infections were molecularly evidenced, only three *H. capsulatum* isolates were cultured from all samples tested, suggesting a low fungal burden in lung tissues that did not favour fungal isolation. This study also highlighted the importance of using different tools for the understanding of histoplasmosis epidemiology, since it supports the presence of *H. capsulatum* in *T. brasiliensis* migratory bats from Mexico and Argentina, thus contributing new evidence to the knowledge of the environmental distribution of this fungus in the Americas.

Key words: Bats, *Hcp100* gene, *Histoplasma capsulatum*, *Tadarida brasiliensis*.

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INTRODUCTION

Histoplasma capsulatum var. *capsulatum* is a mammal fungal pathogen distributed worldwide. The infection associated with this pathogen is relevant in those geographical areas where histoplasmosis is endemic or epidemic, such as the Ohio and Mississippi valleys in the USA [1] and some Latin American regions with a high frequency of outbreaks [2]. In Mexico, histoplasmosis is extensively distributed throughout the country [2].

Infection is caused by the inhalation of aerosolized microconidia and hyphal fragments of the *H. capsulatum* saprobe mycelial phase, which develop in bird and bat droppings accumulated in specific areas, fostered by the biotic and abiotic factors of that particular environment. *H. capsulatum* causes a systemic mycosis with a primary respiratory infection and it is not transmissible from host to host through the air or through the host's faeces. The high risk of natural bat infection with this fungus in Mexican caves has been well documented by Taylor *et al*. [3].

In recent years, *H. capsulatum* bat isolates of different geographical origin have been grouped according to their DNA polymorphism, which has revealed an important genetic diversity [2, 4, 5]. Based on sequence analyses of four protein-coding gene fragments (*Arf*, ADP-ribosylation factor; *Ole1*, delta-9 fatty acid desaturase; *H-anti*, H antigen precursor; *Tub1*, alpha-tubulin) of 137 *H. capsulatum* isolates from 25 countries, Kasuga *et al*. [6] described eight clades, seven of which were considered as phylogenetic species. The Latin American A clade shows the greatest genetic diversity and includes most of the Mexican *H. capsulatum* isolates, either from human clinical cases or naturally infected bats, whereas, the Latin American B clade consists of a clonal Argentinean *H. capsulatum* clinical isolates' population [6].

H. capsulatum infection in humans was successfully diagnosed in tissue samples using a nested-PCR assay for a fungal-specific fragment of the *Hcp100* gene. This gene encodes an *H. capsulatum* 100-kDa protein, which might be essential for fungal survival in host cells [7]. This molecular marker was validated by Maubon *et al*. [8] and by Muñoz *et al*. [9], for the diagnosis of histoplasmosis in clinical human samples. Nested-PCR of the *Hcp100* gene fragment has also been applied to detect *H. capsulatum* infection in tissue samples of captive mammals, which has been well documented in the snow leopard (*Uncia uncia*) [10]. This molecular method was also used to detect the presence of *H. capsulatum* in contaminated compost, frequently believed to be a source of fungal infection [11], and to corroborate the identification of *H. capsulatum* isolated from captive infected maras (*Dolichotis patagonum*) [12].

In general, all bat species are considered as potential reservoirs and dispersers of this pathogen in nature [4, 5, 13]. Bats can become infected irrespective of their alimentary and migratory behaviours [3, 4, 14]. However, those colonial bat species with extensive migratory routes, such as *Tadarida brasiliensis*, are probably the best candidates to spread *H. capsulatum* in the environment throughout a wide geographical area. *T. brasiliensis* form colonies comprising of thousands to millions of individuals [15], these colonies produce a large amount of droppings, thereby enhancing the fungal infection risk in their different shelters, perhaps related to high exposure to *H. capsulatum* propagules.

In the current study, we used a very sensitive and specific molecular method, nested-PCR of the *Hcp100* gene fragment, to detect the presence of *H. capsulatum* in sampled *T. brasiliensis* lungs, the primary target organ of histoplasmosis. This is the first report using nested-PCR with the *Hcp100* marker to demonstrate the presence of *H. capsulatum* in bats. The study clearly highlights the close association between bats and *H. capsulatum*, contributing to the knowledge of histoplasmosis epidemiology by recording the distribution of *H. capsulatum* infection sources related to habitats of *T. brasiliensis* migratory bats that form high-density colonies.

METHODS

Bat samples

A total of 87 *T. brasiliensis* bats were captured with mist-nets in their roosts during the day. Female and reproductive adult male bats were released, in accordance with the international rules of capture and management of this bat species, as a consequence, only young male bats of non-reproductive stage were selected for this study. Sixty-six bats came from four Mexican states: Chiapas (La Trinitaria cave, samples: M-431P, M-433P–M-437P, M-439P–M-441P), Michoacán (Isla de Janitzio cave, samples: M-444P–M-450P, M-453P), Hidalgo (El Salitre cave, samples: M-454–M-463P, M-498P–M-508P), and Nuevo León (La Boca cave, samples: M-468P–M-471P, M-473P–M-487P, M-489P–M-497P). Twenty-one bats came

from two Argentinean provinces: Tucumán (Dique Escaba grotto, samples: M-510P–M-525P) and Córdoba (Cemetery tunnel, samples: M-AR01–M-AR05). The bats from Mexico were delivered to the laboratory, alive if possible, where they were euthanized by cervical dislocation, according to the recommendations of the Animal Care and Use Committee of the Faculty of Medicine, National Autonomous University of Mexico (UNAM), and in accordance with the Mexican Official Guide (NOM 062-ZOO-1999). Each euthanized animal was placed on dry ice until necropsy. Animals captured in Argentina were euthanized in situ by cervical dislocation and their organs were preserved in 70% ethanol and sent to Mexico for DNA extraction. All captured animals were assigned a code number and they were prepared as described previously [3]. Data regarding sex, somatic measures, reproductive condition, weight, and age (determined by the nature of the hair and ossification of their phalanges) for all captured bats, from Mexico or Argentina, were recorded by bat researchers from the Instituto de Ecología, UNAM, Mexico, and from the Instituto Lillo, Tucumán, Argentina. All materials were identified and prepared as described by Anthony [16] and Handley [17]. Taxonomic determination was performed according to Hall [18] and Wilson & Reeder [19].

Bat lung samples were removed aseptically from all animals and frozen at −20 °C until DNA extraction. Systematically, each lung was processed for isolation of *H. capsulatum*, as described previously by Taylor *et al*. [3]. Briefly, the lung was homogenized in 0·1 mM phosphate-saline buffer (pH 7·2), supplemented with 50 mg/ml streptomycin and 100 U/ml penicillin, centrifuged at 300 ***g*** for 10 min, and 0·1 ml supernatant was placed on mycobiotic and brain heart infusion slants (Bioxón, México DF), containing 0·02% Bengal Rose to reduce non-pathogenic fungal contamination. Plates were incubated at 28 °C and checked daily during 6 weeks for fungal growth.

DNA samples

Each DNA sample was extracted from bat lungs using DNeasy Blood & Tissue kit (Qiagen Inc., USA) according to the manufacturer's instructions. Negative controls (Milli-Q water) were included in the extraction procedure to detect any contamination. Each extracted DNA sample was frozen at −20 °C and screened for *H. capsulatum*, using nested-PCR targeting a fragment of the *Hcp100* protein coding gene.

Nested-PCR assay of the *Hcp100* gene

This assay was performed as described by Bialek *et al*. [7] with minor modifications implemented by Taylor *et al*. [11], which did not change the specificity and sensitivity of the *Hcp100* marker. Two sets of primers, described by Bialek *et al*. [7], were used: the outer primer sets Hc I (5′-GCGTTCCGAGCCTTCCACC-TCAAC-3′) and Hc II (5′-ATGTCCCATCGGGC-GCCGTGTAGT-3′) delimit a 391-bp fragment of the gene; the inner primer sets Hc III (5′-GAGA-TCTAGTCGCGGCCAGGTTCA-3′) and Hc IV (5′-AGGAGAGAACTGTATCGGTGGCTTG-3′) delimit a 210-bp fragment unique to *H. capsulatum*. DNA amplification was conducted in a PerkinElmer Cetus DNA thermal cycler (USA) and the first PCR was set up in a 25-μl reaction mixture containing 200 μM of each dATP, dGTP, dCTP, and dTTP (Applied Biosystems Inc., USA), 2 mM $MgCl_2$, 100 pmol of each outer primer, 1 U *Taq* DNA polymerase (Applied Biosystems), and 4 μl of bat lung DNA template, which was tested at different concentrations to achieve the fungal DNA concentration required for the amplification. For the second PCR (nested reaction) the mixture consisted of 200 μM of each dNTP, 2 mM $MgCl_2$, 100 pmol of each inner primer, 1 U *Taq* DNA polymerase, and 2 μl of the first reaction product which was used as template. Cycling conditions for the first and nested reactions were performed as described previously [7]. Amplification products were electrophoresed. A 100-bp DNA ladder (Gibco Laboratories, USA) was used as molecular size marker. Heterologous DNA (20 ng) from other fungal species such as *Aspergillus fumigatus*, *Coccidioides immitis*, and *Sporothrix schenckii* (a kind gift from Dr Reyes-Montes, UNAM) together with rat and mouse lung DNA samples at different concentrations were processed as non-related DNA templates and Milli-Q water was used as a negative control. Positive amplification control was performed with DNA (20 ng) of the EH-53 *H. capsulatum* strain from a Mexican clinical case. The presence of PCR inhibitors was ruled out, since a positive control from a tissue sample of a naturally infected bat, confirmed by *H. capsulatum* culture isolation, always amplified the *Hcp100* marker generating the expected sequence.

After ethidium bromide (0·5 μg/ml) staining, the bands were visualized with a UV transilluminator and

images were captured as TIFF files. Nested-PCR products were purified using QIAquick® PCR purification kit (Qiagen) and sent to the Molecular Biology Laboratory, Institute of Cellular Physiology (UNAM, Mexico) for sequencing in an ABI-automated DNA sequencer (Applied Biosystems). Sequencing of each amplified product was the main criterion to confirm fungal presence in bat lungs.

Sequences

Sequencing reactions were performed for forward and reverse DNA strands and a consensus sequence for each amplified bat lung sample product was generated. Sequences were trimmed to ensure that all sequences had the same start and end-point. Next, they were aligned and compared using MEGA software, version 4.0 [20]. Alignment consisted of the generated sequences from bat samples together with the *Hcp100* sequence of the G-217B strain from Louisiana, USA (GenBank accession no. AJ005963) being used as reference for base positions. Pairwise and multiple parameters were established for a gap opening penalty of 15 and a gap extension penalty of 6·66; transition weight was 0·5; the use of negative matrix was off, and the delay divergent cut-off was 30%.

Neighbour-joining (NJ) [21] and maximum parsimony (MP) [22] analyses for *Hcp100* gene fragments were performed, using MEGA-4 [20] and the G-217B sequence (GenBank) as reference. The NJ tree was drawn to scale, with branch lengths in the same units as those of the genetic distances used to infer the tree. Genetic distances were computed using the Kimura [23] two-parameter model. The MP method [22] was performed, using the close-neighbour-interchange algorithm [24]. NJ or MP bootstrap values [25] for internal branches were generated by 1000 replicates.

BLASTn and BLASTx algorithms were used to search the GenBank database for homologous nucleotide sequences and putative proteins, respectively, corresponding to the nested-PCR product of the *Hcp100* gene fragment from the sequences of bat lung samples analysed.

Statistical approach

The total infection rate was estimated taking into account all studied bats from those bats that had the fungus identified by sequencing of the *Hcp100* marker. A similar estimation was conducted for bat samples from either Mexico or Argentina. The corresponding 95% confidence interval (CI) was calculated by normal distribution.

Table 1. *Number and percentage of* H. capsulatum*-infected bats from different regions of Mexico and Argentina*

Mexico				Argentina	
CS	MN	HG	NL	Tucumán	Córdoba
Infected (captured) bats, *n*					
6 (9)	6 (8)	13 (21)	27 (28)	16 (16)	3 (5)
Infected bats (%)					
66·6	75	61·9	96·4	100	60

CS, Chiapas; MN, Michoacán; HG, Hidalgo; NL, Nuevo León.

RESULTS

Based on the molecular detection of a 210-bp fragment from the *H. capsulatum Hcp100* gene of 87 *T. brasiliensis* individuals studied, 71 bats were considered as probably infected; of these, 52 bats came from four different states of Mexico (six from Chiapas, six from Michoacán, 13 from Hidalgo, 27 from Nuevo León) and 19 bats came from Argentina (16 from Tucumán, three from Córdoba) (see Table 1). Sixteen samples did not amplify the *Hcp100* marker. Three *H. capsulatum* isolates were cultured from *T. brasiliensis* lung samples (M-431P, M-436P, M-485P) of bats captured in Mexico.

A total frequency of 81·6% (95% CI 73·4–89·7) of infection was registered taking into account all studied bats; discriminating for Mexico gave a 78·8% (95% CI 68·9–88·7) infection rate from 66 bats analysed, and for Argentina a 90·4% (95% CI 75·2–100) infection rate from 21 bats studied. The numbers of infected bats according to their capture region in relation to the total number of captured bats studied, as well as the percentages of infection in each bat capture region, are presented in Table 1.

Figure 1 illustrates amplified and non-amplified lung DNA samples for the 210-bp *Hcp100* nested reaction products. In all PCR assays, non-specific bands were never observed. The DNA from EH-53 strain (positive control) amplified the expected 210-bp product in all assays. The same was observed with the non-inhibition control (data not shown), whereas Milli-Q water and/or non-related DNA templates from heterologous fungi, as well as from rat and mouse lungs, did not amplify the *Hcp100* marker.

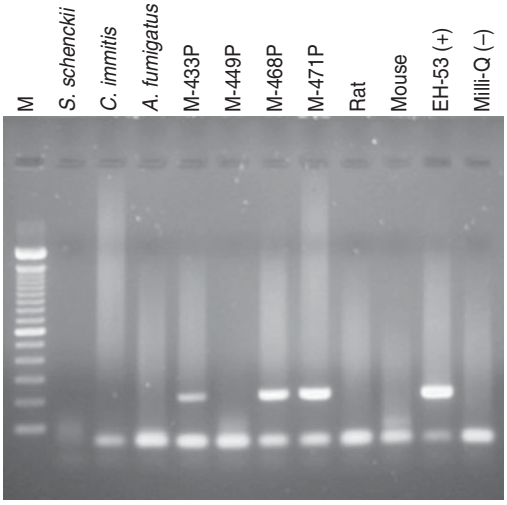

Fig. 1. Representative amplification of the *Hcp100* fragment by nested-PCR. A 100-bp DNA ladder was used as a molecular size marker (M). Heterologous DNA from other fungi and rat and mouse lung DNA samples were used as non-related DNA template. EH-53, Positive control; Milli-Q water, negative control.

Hcp100 specific DNA sequences were detected in the lungs of 71 bats sampled. These sequences were deposited in the GenBank database and bat samples with their respective accession numbers are given in Supplementary Table S1 (available online). Sequences were aligned from 2345 to 2500 (156 nt), using the *Hcp100* sequence of the G-217B strain as reference (Fig. 2), and they revealed four point mutations: thymine substituted by cytosine at position 2436 in all studied sequences; adenine substituted by guanine at position 2353 in sample M-445P (from the state of Michoacán, Mexico); cytosine substituted by thymine at position 2427 (sample M-AR03P from Argentina); and adenine substituted by guanine at position 2466 in samples from two Mexican states (M-490P–M-495P, and M-497P from Nuevo León, as well as M-503P from Hidalgo).

Based on NJ and MP analyses of 71 *Hcp100* sequences of *H. capsulatum* from bat lung samples, two clades are depicted in Figure 3. The optimal NJ tree was generated with the sum of branch length = 0·07. The MP data were: most parsimonious tree (MPT) = 170; tree length (L) = 4·0; consistency index (CI) = 1·0; retention index (RI) = 1·0, and rescaled consistency index (RC) = 1·0.

Clade I, in NJ or MP, was formed by 63 sequences of captured bats from Mexico and Argentina, which included 61 sequences with a common point mutation and two sequences, M-445P from Mexico and M-AR03P from Argentina, with one additional mutation site for each (Figs 2 and 3), achieving a 99% similarity in these sequences. Moreover, the sequence of G-217B strain, used as reference, was also included in this clade (Fig. 3). Clade II, supported by bootstraps of 64% and 99% for NJ and MP, respectively, was formed with eight sequences of captured bats from Mexico with a particular mutation (adenine substituted by guanine) (Figs 2 and 3). Seven of these sequences (M-490P–M-495P, M-497P) corresponded to La Boca cave in the state of Nuevo León and one (M-503P) from El Salitre cave in the state of Hidalgo (Fig. 3).

According to a search of the nucleotide database, using the BLASTn algorithm, all 71 sequences (156 nt each) of bat lung samples had 99% similarity with the corresponding sequence fragment of the *H. capsulatum* G-217B reference strain (GenBank), 83% similarity for the sequence of a transcription factor of *Ajellomyces dermatitidis* SLH14081 strain (GenBank accession no. XM_002628281), and 75% similarity for the fragment sequence of a hypothetic protein of *Paracoccidioides brasiliensis* Pb01 strain (GenBank accession no. XM_002793843).

A search of the protein database using the BLASTx algorithm identified a putative protein fragment of 51 amino acids (aa), corresponding to an open reading frame of 153 nt (2347–2499 nt), which is contained in the 156-nt *Hcp100* amplification product of the 71 *H. capsulatum* sequences tested. Most putative proteins resulting from the lungs of bats showed 100% identity with a fragment of 51 amino acids (731–781 aa) from a total 890 amino-acid sequence of the 100-kDa protein of the *H. capsulatum* G-217B strain (GenBank accession no. CAA06786), with only one exception (asparagine substituted by aspartic acid at position 733) for the putative protein of the M-445P sample (Michoacán, Mexico), which reached 99% identity (data not shown). The 51 amino-acid fragment had 91% identity with a transcription factor protein sequence of *A. dermatitidis* (GenBank accession no. XP_002628327) and 87% identity with a hypothetical protein of *P. brasiliensis* (GenBank accession no. EEH42596).

DISCUSSION

Molecular methods constitute a new tool to detect diverse microorganisms that infect bats and other mammals. The presence of *H. capsulatum* in bats could provide new epidemiological data concerning fungal genetic diversity in bat species and information

```
          2345     2353
G-217B    TACGGAGAAACGACCGTGAAGCGAAAAAAGCCGACGTCGTTTACATCGACTACGGCAACTCCGAAACCGTTCCGTGGACC
M-445P    ........G.....................................................................
61-seq    .............................................................................
M-490P    .............................................................................
M-491P    .............................................................................
M-492P    .............................................................................
M-493P    .............................................................................
M-494P    .............................................................................
M-495P    .............................................................................
M-497P    .............................................................................
M-503P    .............................................................................
M-AR03P   .............................................................................

            2427      2436                                2466                          2500
G-217B    CGCCTCCGGCCTCTCACGCAGCCGCAGTTTTCCGTGCAGAAAATTCGACCCCAAGCCACCGATACAGTTCTCTCCT
M-445P    ...........C.................................................................
61-seq    ...........C.................................................................
M-490P    ...........C.............................G...................................
M-491P    ...........C.............................G...................................
M-492P    ...........C.............................G...................................
M-493P    ...........C.............................G...................................
M-494P    ...........C.............................G...................................
M-495P    ...........C.............................G...................................
M-497P    ...........C.............................G...................................
M-503P    ...........C.............................G...................................
M-AR03P   ..T........C.................................................................
```

Fig. 2. Sequence alignment of 156-nt *Hcp100* fragments amplified from *T. brasiliensis* lung samples. The *Hcp100* sequence from the G-217B strain (GenBank) was used as reference. Point mutations are indicated by nucleotide abbreviations. Sixty-one identical sequences are represented by one aligned sequence, whereas sequences with different mutations are shown.

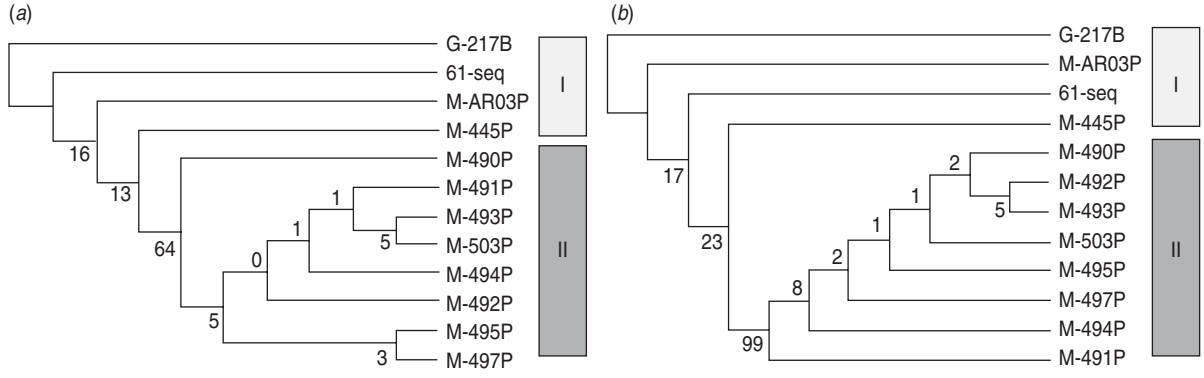

Fig. 3. Unrooted neighbour-joining and maximum parsimony trees of *Hcp100* sequences amplified from *T. brasiliensis* lung samples. (*a*) Neighbour joining; (*b*) maximum parsimony. The *Hcp100* sequence from the G-217B strain (GenBank) was used as reference. The bootstrap support values are represented below tree nodes.

regarding their behaviour related to their shelters and lifestyle, as has been suggested by Taylor *et al*. [3]. It is essential to recognize the importance of wild bats in spreading this fungus in nature and the respective repercussions for human disease. The association between bats and *H. capsulatum* had even been observed before the 1960s [26–28], and throughout the 1980s, other studies have also been published on this subject [14, 29–33]. However, this paper is the first to report on the use of molecular screening to detect the presence of *H. capsulatum* in a large sample (87 individuals) of the insectivorous migratory bat *T. brasiliensis* (Chiroptera: Molossidae), which is one of the most abundant bat species in the Western Hemisphere [15, 34–36], and therefore of importance for the study of bats from different geographical origins from Latin America. The findings described indicate the utility of the molecular marker studied, a unique *Hcp100* gene fragment that successfully identifies the presence of *H. capsulatum* in tissues of captured wild animals, supporting the role of this marker as an available epidemiological tool, as has been demonstrated in histoplasmosis diagnosis in human tissues [7–9].

The present results emphasize an important percentage of *T. brasiliensis* infection with *H. capsulatum*, in either Mexican or Argentinean bats, representing a substantial rate of $\geqslant 60\%$ infection (Table 1). Establishing the presence of *H. capsulatum* in a wide number of lung samples of *T. brasiliensis* was our main aim. Young male bats in non-reproductive stage were selected for this purpose, according to the criterion mentioned in the Materials and Methods section. Further, based on our experience with other animal models, young male mice are one of the most susceptible hosts for experimental histoplasmosis [37, 38], which allow us to infer that young male bats may also be susceptible hosts for *H. capsulatum* infection compared to adults. Although the young male bat cohort is not representative of bat populations, our findings highlight the high risk of infection of *T. brasiliensis* bats and emphasize the role of bats as reservoirs and dispersers of this fungus. In addition, the high percentages of infected bats could be associated with the high density of *T. brasiliensis* colonies [15], enhancing their infection risk. Moreover, physical conditions of bat shelters could influence the infection risk factor for humans and bats, as has been documented by Taylor *et al*. [3].

Despite the fact that fungal isolation is the gold standard for demonstrating a state of fungal infection, this method has a major limitation in its low sensitivity (a high fungal burden is needed in tissue samples to obtain a successful fungal isolation). Moreover, in environmental samples, contamination with other fast-growing microorganisms interferes with a favourable *H. capsulatum* isolation process. *H. capsulatum* isolation in nature is an unusual finding and, in general, the rate of fungal isolation from bat droppings and/or other contaminated soil, as well as from infected bats, is low [14, 30–33, 39, 40]. However, a high infection rate of 66% in bats captured in Mexico was reported by Taylor *et al*. [3], through *H. capsulatum* cultures from different bat organs.

Although fungal isolation in organs of captured bats is a fortuitous event, three *H. capsulatum* isolates were recovered from cultured bat lungs. Assays were also performed to isolate *H. capsulatum* from other bat organs such as spleen, liver, and intestine, of which we also obtained three isolates (two from spleen and one from liver) of all *T. brasiliensis* bats captured in Mexico (data not shown), confirming the low fungal burden in the tissue of the bats studied. Correlation between mycological and molecular findings was very low, since only a small number of positive fungal cultures were attained. Molecular methods have been implemented in recent years to detect the presence of fungal pathogens in tissue samples of infected hosts, especially for the molecular diagnosis of histoplasmosis. For this reason, for the detection of *H. capsulatum* in bat tissues we selected a unique molecular marker (*Hcp100* gene fragment) which has been shown to be an excellent tool for revealing the presence of *H. capsulatum* in infected clinical samples [8, 9]. The *Hcp100* marker has a higher sensitivity than fungal isolation in culture media, irrespective of fungal burden in the host, supporting the use of this molecular marker for detection of the pathogen's presence in bat tissues. The high specificity of the *Hcp100* marker was corroborated by absence of non-specific bands, as well as by non-amplification of heterologous DNA from either other fungi or mammalian hosts and negative controls, as can be seen in Figure 1.

A 156-nt sequence included in the 210-bp fragment of the *Hcp100* marker was sufficient to reveal similarity in the sequences obtained from the lungs of bats. Hence, 61 bat lung samples presented identical sequences, supporting a high similarity in these samples. Moreover, 10 sequences, mostly from bats captured on the same date in the La Boca cave, and having an additional mutation site, also remained closely related to all sequences studied. Detected mutations are not due to sequencing errors, since they appeared in several *Hcp100* fragments and were confirmed in both DNA strands. The sequencing of 71 products of the 156 nt sequence revealed high similarity with the corresponding fragment of the *H. capsulatum Hcp100* gene from the G-217B strain (GenBank), confirming that all *Hcp100* sequences from the bat samples belong to *H. capsulatum*.

Despite the *Hcp100* marker showing low polymorphism, two clades associated with infected bats were identified based on the topologies of the NJ and MP trees. Analyses of NJ and MP topologies revealed differences in the studied sequences, irrespective of the geographical origin of *T. brasiliensis* bats sampled in Mexico. It is important to emphasize that some bat lung samples from Nuevo León (M-490P–M-495P, M-497P) and Hidalgo (M-503P), Mexico, diverge from other studied lung samples (clade II). These findings suggest that the clade II *Hcp100* genotype, from the Nuevo León and Hidalgo samples, is associated with distinct shelters for bat infection from these regions, and it may be related to *T. brasiliensis*

migration routes, which are shared throughout the Mexican territory [35, 36].

BLASTn analysis of the 156-nt sequences from 71 bat lung samples revealed the highest similarity for the *H. capsulatum* G-217B strain, followed by the *A. dermatitidis* SLH14081 strain and *P. brasiliensis* Pb01 strain (now classified as *P. lutzii* according to Teixeira *et al.* [41]), which supports the specificity of the 156-nt fragment to detect *H. capsulatum*.

Similarly, a BLASTx analysis also revealed identity in most protein fragments resulting from the sequences of the *Hcp100* marker amplified from the lungs of infected bats and the 100-kDa protein of the *H. capsulatum* G-217B strain, despite three silent mutations being found in most of the sequences analysed. A non-silent mutation (asparagine substituted by acid aspartic at position 2353) of sample M-445P (Michoacán, Mexico) has no known biological implication. BLASTx analysis also supports the specificity of the 156-nt fragment to detect *H. capsulatum*.

Wild bats have a high risk of infection with airborne propagules from this pathogenic fungus that are found in their shelters. Although bats are easily infected, they do not normally develop a severe course of the disease [3]. This study has a critical relevance that should be noted by specialized researchers who work with *H. capsulatum* and bats, which are considered potential reservoirs of this fungus and play an important role in the spread of *H. capsulatum* in the environment [3–5, 13].

NOTE

Supplementary material accompanies this paper on the Journal's website (http://journals.cambridge.org/hyg).

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DECLARATION OF INTEREST

None.

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Histoplasma capsulatum and *Pneumocystis* spp. co-infection in wild bats from Argentina, French Guyana, and Mexico

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Abstract

Background: *Histoplasma capsulatum* and *Pneumocystis* organisms cause host infections primarily affecting the lung tissue. *H. capsulatum* is endemic in the United States of America and Latin American countries. In special environments, *H. capsulatum* is commonly associated with bat and bird droppings. *Pneumocystis*-host specificity has been primarily studied in laboratory animals, and its ability to be harboured by wild animals remains as an important issue for understanding the spread of this pathogen in nature. Bats infected with *H. capsulatum* or *Pneumocystis* spp. have been found, with this mammal serving as a probable reservoir and disperser; however, the co-infection of bats with both of these microorganisms has never been explored. To evaluate the impact of *H. capsulatum* and *Pneumocystis* spp. infections in this flying mammal, 21 bat lungs from Argentina (AR), 13 from French Guyana (FG), and 88 from Mexico (MX) were screened using nested-PCR of the fragments, employing the *Hcp100* locus for *H. capsulatum* and the *mtLSUrRNA* and *mtSSUrRNA* loci for *Pneumocystis* organisms.

Results: Of the 122 bats studied, 98 revealed *H. capsulatum* infections in which 55 of these bats exhibited this infection alone. In addition, 51 bats revealed *Pneumocystis* spp. infection of which eight bats exhibited a *Pneumocystis* infection alone. A total of 43 bats (eight from AR, one from FG, and 34 from MX) were found co-infected with both fungi, representing a co-infection rate of 35.2% (95% CI = 26.8-43.6%).

Conclusion: The data highlights the *H. capsulatum* and *Pneumocystis* spp.co-infection in bat population's suggesting interplay with this wild host.

Keywords: *Histoplasma*, *Pneumocystis*, Co-infection, Bats, PCR

Background

Histoplasmosis due to *Histoplasma capsulatum* and pneumonia caused by *Pneumocystis* spp. are fungal diseases that can generate serious and even life-threatening pneumonia in immunosuppressed hosts. *H. capsulatum* is a fungal pathogen that affects a wide range of mammal species, including the human. Autochthonous clinical cases have been reported between the latitudes 54° 05′ North (Alberta, Canada) and 38° South (Neuquén, Argentina) [1,2]. The disease associated with this fungus is relevant in the geographical areas where histoplasmosis is endemic or epidemic, such as the Missouri, Ohio, and Mississippi river valleys, in the United States of America (USA), and some Latin American countries with a high frequency of outbreaks [3,4]. In Mexico, histoplasmosis is widely distributed and case reports are rather variable [4]. Infection is caused by the inhalation of fungal saprobe mycelial-phase propagules (infective form) that develop in special environments and are mainly found in bat guano accumulated in confined spaces such as caves and abandoned mines and buildings. The potential role of bats in spreading *H. capsulatum* in nature remains unclear. The high risk of natural bat infection with this fungus in Mexican caves has been well-documented [5-8].

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According to their genetic diversities, *H. capsulatum* isolates from different geographical origins have been grouped into eight clades; seven of which are considered phylogenetic species. Among these, highlight the LAm A clade that harbours significant genetic variability [9].

The genus *Pneumocystis* contains highly diversified fungal pathogens that are harboured by a wide range of mammal hosts [10-16]. *Pneumocystis* organisms, which are transmitted via host-to-host airborne route, have a marked host-species-related diversity that is associated with close host specificity. The high divergence among *Pneumocystis* species most likely resulted from a prolonged process of co-evolution with each mammal host, mostly associated with co-speciation, as suggested by Demanche et al. [12] and Hugot et al. [13]. Although most phenotypic and genotypic data supporting *Pneumocystis* stenoxenism derives from laboratory animal models or captive animals, reports about *Pneumocystis* prevalence and circulation in wild fauna are scarce [12-16].

Unpublished preliminary data by our team revealed *H. capsulatum* and *Pneumocystis* co-infection in two randomly captured bats, identifying these mammals as probable reservoirs and dispersers of both parasites in nature (Dei-Cas E and Taylor ML, comm. pers.). The study of co-infection systems, where the host (i.e. a wild host) usually harbours two or multiple parasites, requires an in-depth investigation to determine a comprehensive understanding of this multi-infectious process in regards to its dynamics and consequences.

H. capsulatum and *Pneumocystis* share a number of features that justify their concomitant study, including: a low pathogenicity in healthy hosts and severe disease in immunocompromised hosts; an induced immune response; a respiratory portal of entry; and the ability to disseminate from the lungs to other organs. Therefore, it is possible that co-infection between both parasites is highly common in nature.

The aim of the present research was to detect the frequency of the *H. capsulatum* and *Pneumocystis* organisms' infection and co-infection in the lung samples of a number of wild bat species from three countries from Latin America. For this purpose, we used a highly sensitive PCR with specific molecular markers for each pathogen that have been used successfully in clinical patients.

Methods

Bat samples

A total of 122 bats from different species and families were randomly captured as reported by Taylor et al. [7]: 21 came from Argentina; 13 came from French Guyana; and 88 came from Mexico. In all cases national rules regulating bat species protection, capture, and processing have adhered to strict ethical recommendations and to the guidelines published by Gannon, Sikes and the Animal Care and Use Committee of the American Society of Mammalogists [17].

The bats were euthanized by cervical dislocation and processed according to recommendations and approval of the Faculty of Medicine Ethics Committee, in accordance with the Animal Care and Use Committee of the UNAM and the Mexican Official Guide (NOM 062-ZOO-1999). The lungs from each bat captured in Mexico were separated and immediately frozen at −20°C. Animals captured in Argentina and French Guyana were also euthanized by cervical dislocation and processed *in situ* and their lungs were separated and preserved in 70% ethanol until DNA extraction.

DNA samples

DNA was extracted from the bat lungs using a DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA, USA). After extraction, the DNA samples were frozen at −20°C. The DNA samples were screened for *H. capsulatum* infection using nested-PCR for a fragment of the gene encoding a 100-kDa protein (*Hcp100*) [18], a molecular marker considered to be highly specific for this pathogen. Molecular screening for *Pneumocystis* spp. infection was conducted in parallel, using nested-PCR for fragments of the rRNA mitochondrial large [19,20] and small [21] subunit loci, *mtLSUrRNA* and *mtSSUrRNA*, respectively.

Nested PCR assay of the *Hcp100* locus for the detection of *H. capsulatum*

The assay was performed as described by Bialek et al. [18] with minor modifications by Taylor et al. [22] that did not change the specificity and sensitivity of the *Hcp100* marker. Two sets of primers, described by Bialek et al. [18], were used: the outer primer set included HcI (5′-GCG-TTC-CGA-GCC-TTC-CAC-CTC-AAC-3′) and HcII (5′-ATG-TCC-CAT-CGG-GCG-CCG-TGT-AGT-3′); the inner primers were HcIII (5′-GAG-ATC-TAG-TCG-CGG-CCA-GGT-TCA-3′) and HcIV (5′-AGG-AGA-GAA-CTG-TAT-CGG-TGG-CTT-G-3′) and delimit a 210 base pair (bp) fragment unique to *H. capsulatum*. The primers were supplied by Operon Technologies Inc. (Alameda, CA, USA).

The first and second PCR reactions of the *Hcp100* locus were standardised elsewhere [6]. For the first round of amplification, the thermocycling conditions were as follows: one cycle at 94°C for 5 min; 35 cycles at 94°C for 30 s, 50°C for 30 s and 72°C for 1 min; and a final cycle at 72°C for 5 min. Thermocycling conditions for the second PCR (nested reaction) were: one cycle at 94°C for 5 min; 30 cycles at 94°C for 30 s, 65°C for 30 s and 72°C for 1 min; and a final extension cycle at 72°C for 5 min. The DNA (20 ng) of the EH-53 *H. capsulatum* strain from a Mexican clinical case was used as a positive amplification

control, and Milli-Q water was processed as a negative control.

Nested PCR assays of the *mtLSUrRNA* and *mtSSUrRNA* loci for the detection of *Pneumocystis* spp.

The assays were based on amplifying fragments of the mitochondrial large (*mtLSU*) and small (*mtSSU*) subunits of the rRNA gene.

Nested PCR at the *mtLSUrRNA* locus employed the outer primer set published by Wakefield et al. [19], pAZ102-H (5′-GTG-TAC-GTT-GCA-AAG-TAG-TC-3′) and pAZ102-E (5′-GAT-GGC-TGT-TTC-CAA-GCC-CA-3′). The inner primers pAZ102-X (5′-GTG-AAA-TAC-AAA-TCG-GAC-TAG-G-3′) and pAZ102-Y (5′-TCA-CTT-AAT-ATT-AAT-TGG-GGA-GC-3′) and delimit a 267 bp fragment for *Pneumocystis* [20]. The first and nested PCR reactions of the *mtLSUrRNA* locus were standardised as described elsewhere [14,19,20]. For the first round of amplification, the thermocycling conditions were as follows: 30 cycles at 94°C for 30 s, 50°C for 1 min, and 65°C for 1 min. The nested reaction was performed with 10% of the first-round amplification product and the thermocycling conditions were: 30 cycles at 94°C for 30 s, 55°C for 1 min, and 65°C for 1 min.

Nested PCR at the *mtSSUrRNA* locus was performed with the outer primers, pAZ112-10 F (5′-GGG-AAT-TCT-AGA-CGG-TCA-CAG-AGA-TCA-G-3′) and pAZ112-10R (5′-GGG-AAT-TCG-AAC-GAT-TAC-TAG-CAA-CCC-3′). The inner primers pAZ112-13RI (5′-GGG-AAT-TCG-AAG-CAT-GTT-GTT-TAA-TTC-G-3′) and pAZ112-14RI (5′-GGG-AAT-TCT-TCA-AAG-AAT-CGA-GTT-TCA-G-3′) and delimit a 300 bp fragment for *Pneumocystis* species, as reported by Tsolaki et al. [21]. PCR for the *mtSSUrRNA* locus was previously described by Tsolaki et al. [21] and Akbar et al. [14]. For the first round of amplification, the thermocycling conditions were as follows: 40 cycles at 94°C for 30 s, 55°C for 1 min, and 65°C for 1 min. The nested round was performed with 10% of the first-round amplification product and the thermocycling conditions were: 10 cycles at 94°C for 30 s, 52°C for 1 min, and 65°C for 1 min, followed by 30 cycles at 94°C for 30 s, 63°C for 1 min, and 65°C for 1 min.

Primers for the *mtLSUrRNA* and *mtSSUrRNA* loci were supplied by Operon Technologies. The DNA (20 ng) of rabbit *Pneumocystis* (*Pneumocystis oryctolagi*) was processed as a positive amplification control, and Milli-Q water was used as a negative control for both *Pneumocystis* molecular markers.

Amplified products

Amplicons from each PCR assay were electrophoresed through 1.5% agarose in 0.5X Tris-borate-EDTA buffer. Electrophoresis was conducted at 120 V for 50 min. The 100 bp DNA Ladder (Gibco Laboratories, Grand Island, NY, USA) was used as a molecular size marker. The bands were visualised using a UV transilluminator after ethidium bromide staining (0.5 μg/mL). The amplicons were purified using the QIAquick® PCR and the QIAEX II kits (Qiagen) for the *H. capsulatum* and *Pneumocystis* organisms, respectively. Afterwards, the amplicons were sent to the Molecular Biology Laboratory, Institute of Cellular Physiology (UNAM, Mexico) for sequencing in an ABI-automated DNA sequencer (Applied Biosystems Inc., Foster City, CA, USA). Sequencing reactions were performed for forward and reverse DNA strands, and a consensus sequence for each amplified bat lung sample product was generated. The sequences were edited and aligned using the MEGA software, version 5 (http://www.megasoftware.net).

Most of the *Hcp100* sequences of *H. capsulatum* were previously reported in González-González et al. [6], and the other sequences were deposited in a database [GenBank: from JX091346 to JX091370 accession numbers]. All sequences generated by both molecular markers for *Pneumocystis* spp. were reported by Derouiche et al. [16] and Akbar et al. [14].

The sequences of the specific markers for each pathogen (i.e., *Hcp100* for *H. capsulatum* and *mtLSUrRNA* or *mtSSUrRNA* for *Pneumocystis* spp.) that were obtained in the same animal were the main inclusion criterion for considering bat co-infection.

Statistics

The infection and co-infection rates for each pathogen were estimated by considering all of the bats studied from the three countries and from each country separately (Argentina, French Guyana, and Mexico), in relation to those bats with *H. capsulatum* and *Pneumocystis* spp. infections as identified by sequencing their respective molecular markers. The corresponding 95% confidence interval (CI) was calculated using a normal distribution.

Results

Data from nine bat species studied belonging to five different families, highlighting their particular behaviours, such as migration, nourishment, distribution in the American continent and colony size, are referred to in Table 1, according to Ceballos and Oliva [23]. These behaviours varied considerably among the bat species studied (Table 1). The different species captured, their numbers, and their geographical origins are registered in Table 2. Although most of the bat species studied were non-migratory, the number of migratory bats from three processed species was greater than that of the non-migratory species (Tables 1 and 2). It is noteworthy that among the 122 bats studied, 84 (68.80%) belonged to the migratory species *Tadarida brasiliensis*, from which 63 individuals were captured in Mexico and 21 in Argentina (Table 2).

Table 1 Families, species, and behaviours of the bats studied

(Family) Species	Migration	Nourishment	Distribution	Colony size
(Phyllostomidae)				
Artibeus hirsutus	Non-migratory	Frugivorous	From south of Sonora to south of Guerrero, Mexico	Not defined
Carollia perspicillata	Non-migratory	Frugivorous	From Tamaulipas to Oaxaca, Mexico, and to south of Bolivia, Brazil and Paraguay	Small groups from 10 to 100 individuals
Glossophaga soricina	Non-migratory	Nectarivorous, polinivorous, frugivorous and insectivorous	From Mexico to South America	From a few to 2,000 individuals
(Natalidae)				
Natalus stramineus	Non-migratory	Insectivorous	From north of Mexico to Brazil	Approximately 10,000 individuals
(Mormoopidae)				
Pteronotus davyi	Non-migratory	Insectivorous	From north of Mexico to Brazil	From hundreds to thousands of individuals
Pteronotus parnellii	Non-migratory	Insectivorous	From north of Mexico to Brazil	From hundreds to thousands of individuals
Pteronotus parnellii	Non-migratory	Insectivorous	From north of Mexico to the north of Argentina and Paraguay	Thousands of individuals
Mormoops megalophylla	Migratory	Insectivorous	From south USA to Venezuela and Peru	From a few to thousands of individuals
(Molossidae)				
Tadarida brasiliensis	Migratory	Insectivorous	From central USA to Chile and Argentina	Generally, thousands to millions of individuals
(Vespertilionidae)				
Myotis californicus	Occasionally migratory	Insectivorous	From western Canada and USA to Guatemala	Small groups or gregarious

Data from the bat species were reported by Ceballos and Oliva [23].

Table 2 Species, numbers, and geographical origins of the bats infected with *H. capsulatum* or *Pneumocystis* spp.

Species	Geographical origins/ localities						Number of bats infected with *H. capsulatum*/ *Pneumocystis*			
	Argentina		French Guyana	Mexico						(Total samples per species)
	TUC	CBA	Kourou	CS	MN	GR	HG	MS	NL	
	***H. capsulatum*/*Pneumocystis* (total samples)**									
A. hirsutus								5/3 (5)		5/3 (5)
C. perspicillata			1/0 (1)							1/0 (1)
G. soricina			3/6 (12)			4/3 (4)				7/9 (16)
N. stramineus						5/1 (8)				5/1 (8)
P. davyi						1/0 (1)				1/0 (1)
P. parnellii						2/0 (2)	0/1 (1)			2/1 (3)
M. megalophylla						2/0 (2)			1/0 (1)	3/0 (3)
T. brasiliensis	16/8 (16)	3/ND (5)		8/2 (8)	7/2 (8)		13/5 (20)		26/19 (27)	73/36 (84)
M. californicus									1/1 (1)	1/1 (1)
Number of bats infected with *H. capsulatum*/*Pneumocystis* (Total samples per locality)	16/8 (16)	3/ND (5)	4/6 (13)	8/2 (8)	7/2 (8)	14/4 (17)	13/6 (21)	5/3 (5)	28/20 (29)	98/51 (122)

Abbreviations: TUC = Tucumán; CBA = Córdoba; CS = Chiapas; MN = Michoacán; GR = Guerrero; HG = Hidalgo; MS = Morelos; NL = Nuevo León.
ND = Not determined.

Detection of *H. capsulatum* infection in the bat lung samples

Of the 122 bat lungs that were molecularly screened for *H. capsulatum* infection, 98 bats generated sequences for the *Hcp100* marker, of which 55 bats were found to be infected with this pathogen alone, corresponding to 45.1% (95% CI = 36.4-53.6%) of the 122 bats from the three geographical regions studied (Figure 1).

Table 2 displays the number of infected bats with *H. capsulatum* in relation to the total number of each bat species sampled at different localities from the monitored Latin American countries.

Detection of *Pneumocystis* spp. infection in the bat lung samples

Of the 122 lungs that were molecularly screened for *Pneumocystis* spp., 51 bats generated sequences for one or both of the *Pneumocystis* molecular markers assayed. From these sequences, seven matched the *mtLSUrRNA* locus and another seven matched the *mtSSUrRNA* locus, while 37 sequences were generated at both loci. *Pneumocystis* spp. infection alone was found only in eight bats, corresponding to 6.6% (95% CI = 2.25-10.85%) of the total bats studied (Figure 1).

Table 2 displays the number of infected bats with *Pneumocystis* spp. in relation to the total number of each bat species sampled at different localities from the monitored Latin American countries.

H. capsulatum and *Pneumocystis* spp. co-infection in the bat lung samples

Of the lung samples from the 122 bats captured in Argentina, French Guyana, and Mexico that were molecularly screened for *H. capsulatum* and *Pneumocystis* infections, 43 samples revealed the specific sequences of each pathogen, corresponding to 35.2% (95% CI = 26.8-43.6%) of the samples being co-infected with both pathogens in bats from the three geographical regions studied (Figure 1).

Table 3 displays the number of co-infected bats with both pathogens in relation to the total number of each bat species sampled at different localities from the monitored Latin American countries.

Finally, of the total number of bat lungs sampled, 106 (86.8%, 95% CI = 80.92-92.68%) were found to be infected with one or both pathogens, whereas 16 (13.1%, 95% CI = 7.22-18.98%) did not show evidence of infection with any of the pathogens studied (Figure 1).

Discussion

The co-infection relationship between the host and different parasite species could occur in natural conditions, although it has been scarcely studied due to its complexity and poor understanding [24]. The presence of more than one parasite species in a single host can lead to positive or negative interactions. In the positive interaction, the parasite could favour the entry and survival of another parasite, whereas in the negative interaction the establishment of a parasite prevents the entry of other parasites and abolishes their survival [24]. It is well accepted in medical research that the infection concept implies the presence of the pathogen in the infected host's tissues, which does not necessarily indicate a disease status that is supported by characteristic signs and symptoms. Although bats, in general, have a high infection rate with *H. capsulatum* in their shelters, they most likely do not develop a severe course of the disease [7], and the impact of this infection on the survival of their population is unknown. With regard to *Pneumocystis* bat infection, this wild host could present a latent infection

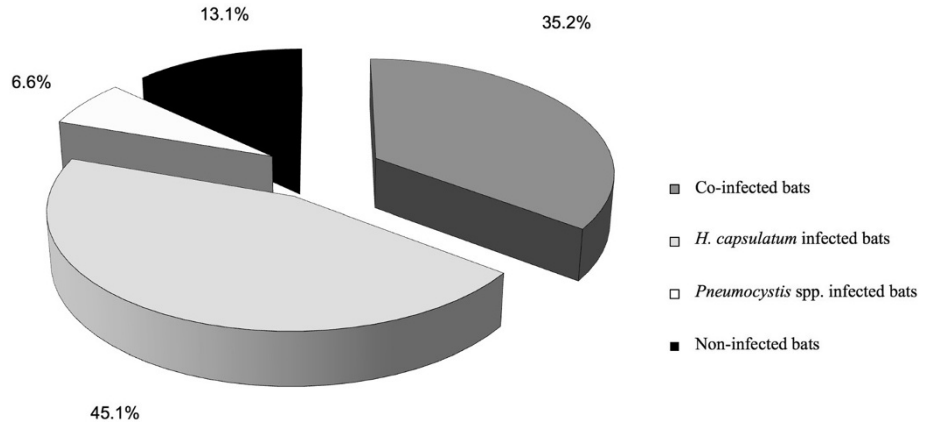

Figure 1 Percentages of *H. capsulatum* and *Pneumocystis* infection and their respective co-infection in bats randomly sampled in Argentina, French Guyana, and Mexico. Each percentage value was calculated based on the 122 bats captured. Bat infection was screened with specific molecular markers for each pathogen, as described in the Methods section.

Table 3 Species, numbers, and geographical origins of the bats co-infected with *H. capsulatum* and *Pneumocystis* spp.

Species	Geographical origins/localities									
	Argentina (n = 21)		French Guyana (n = 13)	Mexico (n = 88)						Number of co-infected bats
	TUC	CBA	Kourou	CS	MN	GR	HG	MS	NL	(Total samples per species)
	Co-infection (total samples)									
A. hirsutus								3 (5)		3 (5)
C. perspicillata			0 (1)							0 (1)
G. soricina			1 (12)			3 (4)				4 (16)
N. stramineus						1 (8)				1 (8)
P. davyi						0 (1)				0 (1)
P. parnellii						0 (2)	0 (1)			0 (3)
M. megalophylla						0 (2)			0 (1)	0 (3)
T. brasiliensis	8 (16)	0 (5)		2 (8)	2 (8)		3 (20)		19 (27)	34 (84)
M. californicus									1 (1)	1 (1)
Number of co-infected bats (Total samples per locality)	8 (16)	0 (5)	1 (13)	2 (8)	2 (8)	4 (17)	3 (21)	3 (5)	20 (29)	43 (122)

Abbreviations: TUC = Tucumán; CBA = Córdoba; CS = Chiapas; MN = Michoacán; GR = Guerrero; HG = Hidalgo; MS = Morelos; NL = Nuevo León.

without evidence of any disease signs and symptoms [12,14]. Consequently, bats could be potential carriers of both parasites in the environment.

H. capsulatum and *Pneumocystis* spp. cause a host infection through the respiratory airway, mainly affecting the pulmonary tissue. After infecting the lungs, each parasite develops on distinct host environments and exploits different host resources. The *H. capsulatum* parasitic yeast-phase is an intracellular pathogen of the lung phagocytic cells. In contrast, *Pneumocystis* organisms are extracellular pathogens that frequently attach to type I pneumocytes [10].

Histoplasma-Pneumocystis co-infection has been reported in immunosuppressed human patients [25], whereas reports of co-infection in wild mammals have not been published. This fact should be re-examined because both parasites are able to share the same wild hosts in a particular manner, likely associated with the host immune status related to stress, sickness, and nutrient starvation.

PCR assays that utilize specific molecular markers are very sensitive tools for detecting a low fungal burden in clinical samples from asymptomatic patients. Currently, *H. capsulatum* and *Pneumocystis* spp. infections are detected by different PCR methods, either in human clinical cases or in experimental models [14,26-29]. The present study is the first report for detecting a natural co-infection in wild bats from three distant geographical Latin American regions, using specific PCR assay for each parasite.

The numbers of wild bats infected with *H. capsulatum* or *Pneumocystis* organisms varied, with the number of *H. capsulatum* infected bats surpassing the number of *Pneumocystis* infected bats (Figure 1). No association was found between a bat species' susceptibility and nourishment and the rate of infection with both pathogens. However, it is possible that some bat behaviours promote different infection rates for either *H. capsulatum* or *Pneumocystis* spp. According to published findings, the rates of each pathogen infection could be associated with the bat colony size and their movements, in the case of *H. capsulatum* [7], or with behavioural factors such as bats crowding and migration in the case of *Pneumocystis* spp. [14]. The biggest colonies, mainly of *T. brasiliensis*, had the highest rate of infection with *H. capsulatum*, most likely due to bat colony movements within enclosed spaces, especially in shelters where short ceiling-to-floor distances prevails, which facilitate the development of a great number of airborne infective propagules on the abundant guano accumulated underneath bat colonies [7]. Hence, each of these factors allows the co-infection state with both pathogens.

Based on the following evidence, it is likely that either *H. capsulatum* or *Pneumocystis* displayed an interaction with different bat species since million of years ago (Ma): 1.- Bat fossils (*Tadarida* sp.) were reported approximately 3.6 – 1.8 Ma in the Late Pliocene [30]; 2.- the *H. capsulatum* complex most likely started its radiation at 13–3 Ma in the Miocene [9]; and 3.- the *Pneumocystis* species have had interaction with mammal hosts for more than 100 Ma [10-13,31]. Under this assumption, the co-infection of both pathogens most likely generated a co-evolution process between each pathogen and the wild host.

Data pertaining to *Histoplasma-Pneumocystis* co-infection reveal a rate of 35.2%; this finding could be useful for understanding the persistence of both infections in susceptible hosts. The absence of *Histoplasma* or *Pneumocystis* infections in 13.1% of the bats studied could suggest that most of the analysed bat populations

were exposed to a high risk of infection with these pathogens in their shelters. Co-infection interactions could cause ecological and immunological implications for the host. For the ecological implications, space and alimentary competitions are involved. For the immunological implications, the host immune response against *H. capsulatum* at the pulmonary level involves cells and molecules that could also participate in the host immune response against *Pneumocystis,* or vice versa.

Conclusion

The impact of the present findings highlights the *H. capsulatum* and *Pneumocystis* spp. co-infection in bat population's suggesting interplay with this wild host. In addition, this co-infection state could also interfere with the outcome of the disease associated with each pathogen.

Competing interests

The authors declare that they have no conflicts of interest.

Authors' contributions

MLT and EDC contributed equally to the design of this study. AEGG coordinated and performed the molecular assays for *H. capsulatum* detection. MLT and AEGG contributed equally to draft the manuscript. JARB and LECB processed the bat samples from Argentina and Mexico and collaborated in the molecular assays for *H. capsulatum*. EDC, ELMA, CMAD, CD, and MC coordinated the molecular assays of *Pneumocystis* and revised the manuscript draft. MP, HA, and SD performed molecular assays for *Pneumocystis* detection. All authors have read and approved the manuscript.

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CAPÍTULO 10
PRESENTACIONES EN CONGRESOS NACIONALES E INTERNACIONALES DURANTE EL DOCTORADO

- **Carreto-Binaghi LE**, Morales-Villareal FR, Martínez-Orozco JA, Taylor ML. Utilidad del diagnóstico molecular en histoplasmosis. XXXIV Congreso Interamericano de Infectología Pediátrica. León, Guanajuato, México. 14-17 octubre 2015, Resumen IB-12. Presentación oral.
- **Carreto-Binaghi LE**, Martínez-Orozco JA, Morales-Villarreal FR, Cabello C, Palma G, Taylor ML. "Histoplasmosis in Mexico". Round Table "Sharing local experiences on histoplasmosis". First Histoplasmosis in the Americas and the Caribbean meeting. Paramaribo, Suriname. 4-6 diciembre 2015. Presentación oral.
- **Carreto-Binaghi LE**, Morales-Villarreal FR, Martínez Orozco JA, Taylor ML. Papel de las proteínas surfactantes en la co-infección de *H. capsulatum* y *P. jirovecii* en pacientes con virus de la inmunodeficiencia humana. 7mas jornadas Académicas de Químicos del INER. Ciudad de México, México. 1-3 marzo 2017. Presentación oral.
- Taylor ML, **Carreto-Binaghi LE**, Morales-Villarreal FR, Martínez Orozco JA. Co-infección de *H. capsulatum* y *P. jirovecii* en pacientes con SIDA. X Congreso Nacional de Micología Médica "Dr. Amado González Mendoza y Dr. Jesús Mayorga Loera". Guadalajara, Jalisco, México. 19-21 octubre 2017. Presentación de cartel.
- **Carreto-Binaghi LE**, Morales-Villarreal FR, Aliouat EM, García-de la Torre G, González-González AE, Martínez Orozco JA, Taylor ML. Co-infección de *Histoplasma capsulatum* y *Pneumocystis jirovecii* en pacientes del Instituto Nacional de Enfermedades Respiratorias. XLIII Congreso Nacional de Infectología y Microbiología Clínica. Mérida, Yucatán, México. 23-26 mayo 2018.
- **Carreto-Binaghi LE**, Tenorio EP, Morales-Villarreal FR, Aliouat EM, Zenteno E, Martínez-Orozco JA, Taylor ML. Lung inflammatory mediators in *Histoplasma capsulatum* and *Pneumocystis jirovecii* infection. LAMIG-SMI Mucosal Immunology Simposium. Estado de México, México. 4-6 septiembre 2019.

EL COMITÉ ORGANIZADOR DEL
XXXIV CONGRESO INTERAMERICANO DE INFECTOLOGÍA PEDIÁTRICA
"Dra. Mercedes Macías Parra"

Otorga la presente

CONSTANCIA A:

LAURA ELENA CARRETO BINAGHI, FERNANDO R. MORALES VILLAREAL, JOSÉ ARTURO MARTÍNEZ OROZCO, MARIA LUCIA TAYLOR.

POR SU PARTICIPACIÓN EN LA SESIÓN DE TRABAJOS LIBRES CON EL TRABAJO:

UTILIDAD DEL DIAGNÓSTICO MOLECULAR EN HISTOPLASMOSIS

ESTAMOS AGRADECIDOS POR LA ALTA CALIDAD DE SU PRESENTACIÓN LA CUAL CONTRIBUYO AL ÉXITO DEL CONGRESO

LEÓN, GUANAJUATO A 17 DE OCTUBRE DE 2015

DRA. HILDA G. HERNÁNDEZ OROZCO
Coordinadora de Trabajos Libres

Certificate of participation

Laura Carreto - Binaghi

is hereby recognized for the participation in

The First Meeting of Histoplasmosis in the Americas and the Caribbean

December 4 - 6, 2015 Paramaribo - Suriname

Organized by:
Foundation for Scientific Research Suriname (SWOS)

Prof. Dr. Mathieu Nacher
French Guiana

Dr. Stephen.G.Vreden
Suriname

Dr. Tom Chiller
USA

SALUD
SECRETARÍA DE SALUD

INSTITUTO NACIONAL
DE ENFERMEDADES
RESPIRATORIAS
ISMAEL COSÍO VILLEGAS
INER

El Instituto Nacional de Enfermedades Respiratorias
Ismael Cosío Villegas

Otorga la Presente

CONSTANCIA

A:

DRA. LAURA ELENA CARRETO BINAGHI

Por su excelente participación como Ponente dentro del *SIMPOSIUM 3.*
MICROBIOLOGIA 1. "Tópicos de Micología"
con el Tema:
"Papel de las Proteínas surfactantesen la Co-infección de Histoplasma capsulatum y Pneumocystis jiroveci en pacientes con virus de la inmunodeficiencia humana."
en las ***7as. Jornadas Académicas de Químicos del INER "En Torno de la Esencia está la Morada de la Ciencia"***
que se llevaron a cabo del 1 al 3 de marzo del presente año en este Instituto.

Ciudad de México, a 2 de marzo de 2017.

Dra. Rosaura Esperanza Benítez Pérez
Jefa del Departamento de
Educación Continua

Q. C. René Guevara Gutiérrez
Jefe del Departamento de Laboratorio
Clínico y Profesor Titular

7mas. Jornadas Académicas de Químicos del INER
"En Torno de la Esencia está la Morada de la Ciencia" Platón
1° al 3 de marzo del 2017

Dirección de Enseñanza
Departamento de Educación Continua

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LA ASOCIACIÓN MEXICANA DE MICOLOGÍA MÉDICA A.C.

OTORGA LA PRESENTE:

CONSTANCIA

9 CONGRESO NACIONAL DE MICOLOGIA MEDICA

A

Dra. Taylor María L, Carreto-Binaghi LE, Morales-Villareal FR, Martínez-Orozco JA.

POR SU PARTICIPACIÓN EN:

CARTELES

CO-INFECCIÓN DE *Histoplasma capsulatum* Y *Pneumocystis jirovecii* EN PACIENTES CON SIDA.

EN EL IX CONGRESO NACIONAL DE MICOLOGÍA MÉDICA

"DR. AMADO GONZÁLEZ MENDOZA Y DR. JESÚS MAYORGA LOERA"

Guadalajara, Jalisco, 19 al 21 de Octubre de 2017

Dra. Francisca Hernández Hernández
Vice-Presidente

M. en C. Jorge A. Mayorga Rodríguez
Presidente (2015-17)

Dr. Víctor Manuel Tarango Martínez
Secretario

MUCOSAL IMMUNOLOGY SYMPUOSIUM

LAMIG-SMI 2019

CERTIFICATE

to

Carreto-Binaghi LE, Tenorio EP, Morales-Villarreal FR, Aliouat EM,
Zenteno E, Martínez-Orozco JA, Taylor ML.

By its relevant participation at the poster session
With the presentation of the work

to

"Lung inflammatory mediators in Histoplasma capsulatum and Pneumocystis jirovecil infection"

THE MUCOSAL IMMUNOLOGY SYMPOSIUM

Iztacala Cultural Center, FES Iztacala, UNAM, Tlalnepantla, Mexico State,
Mexico, 4-6 September 2019

Organizers

Leticia Moreno Fierros PhD

Michael Schnoor PhD

Asociación Mexicana de Infectología y Microbiología Clínica, A.C.

Otorga la presente

CONSTANCIA

por su presentación de Trabajo Libre

Cartel **B19**

Título CO-INFECCIÓN DE HISTOPLASMA CAPSULATUM Y PNEUMOCYSTIS JIROVECII EN PACIENTES DEL INSTITUTO NACIONAL DE ENFERMEDADES RESPIRATORIAS

Autores

**Carreto-Binaghi L1,Morales-Villarreal F1,Aliouat-. E2,García-De la Torre G3,González-González A3,Martínez-Orozco J1,Taylor-Da Cunha e Mello M3, (1) Instituto Nacional de Enfermedades Respiratorias, México; (2) Institut Pasteur de Lille, Francia Metropolitana; (3) Universidad Nacional Autónoma de México, México.

Durante el **XLIII Congreso Nacional de Infectología y Microbiología Clínica** del 23 al 27 de Mayo del 2018 en el Centro Internacional de Congresos de Yucatán, Mérida, Yucatán, México.

Dra. Noris Marlene Pavia Ruz
Presidente AMIMC A.C.

Dra. Patricia Cornejo Juárez
Secretario Académico

PREMIOS OBTENIDOS EN CONGRESOS NACIONALES E INTERNACIONALES DURANTE EL DOCTORADO

- Tercer lugar en la categoría de Investigación Básica dentro de los trabajos libres en modalidad de presentación oral. **Carreto-Binaghi LE**, Morales-Villareal FR, Martínez-Orozco JA, Taylor ML. Utilidad del diagnóstico molecular en histoplasmosis. XXXIV Congreso Interamericano de Infectología Pediátrica. León, Guanajuato, México. 14-17 octubre 2015. Resumen IB-12.
- Young Investigator Award. Otorgado por el Instituto Mérieux y la Asociación Mexicana de Infectología Pediátrica en el marco del XXXVII Congreso Interamericano de Infectología Pediátrica. Chihuahua, Chihuahua, México. 1º diciembre 2018.
- SLB Trainee Award. **Carreto-Binaghi LE**, Tenorio EP, Morales-Villarreal FR, Aliouat EM, Zenteno E, Martínez-Orozco JA, Taylor ML. Lung inflammatory mediators in *Histoplasma capsulatum* and *Pneumocystis jirovecii* infection. LAMIG-SMI Mucosal Immunology Simposium. Estado de México, México. 4-6 septiembre 2019.

Asociación Mexicana de Infectología Pediátrica, A.C.

EL COMITÉ ORGANIZADOR DEL
XXXIV CONGRESO INTERAMERICANO DE INFECTOLOGÍA PEDIÁTRICA
"Dra. Mercedes Macías Parra"

Otorga la presente

CONSTANCIA A:

LAURA ELENA CARRETO BINAGHI, FERNANDO R. MORALES VILLAREAL, JOSÉ ARTURO MARTÍNEZ OROZCO, MARIA LUCIA TAYLOR.

POR HABER OBTENIDO EL **3ER LUGAR EN EL AREA DE INVESTIGACION BÁSICA** CON EL CON EL TRABAJO:

UTILIDAD DEL DIAGNÓSTICO MOLECULAR EN HISTOPLASMOSIS

ESTAMOS AGRADECIDOS POR LA ALTA CALIDAD DE SU PRESENTACIÓN LA CUAL CONTRIBUYO AL ÉXITO DEL CONGRESO

LEÓN, GUANAJUATO A 17 DE OCTUBRE DE 2015

DRA. HILDA G. HERNÁNDEZ OROZCO
Coordinadora de Trabajos Libres

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Institut Mérieux and the Asociación Mexicana de Infectología Pediátrica (AMIP) are pleased to provide their joint research Award 2018 to:

Dr. Laura CARRETO BINAGHI

Instituto Nacional de Enfermedades Respiratorias "Ismael Cosío Villegas"

Institut Mérieux – AMIP Award 2018

Chihuahua, December 1st, 2018

Marc BONNEVILLE
Vice President, Medical and Scientific Affairs
Institut Mérieux

Dr. Antonio LUÉVANOS
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Laura Elena Carreto Binaghi

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Non-member awardees may utilize the certificate for a complimentary one-year student or post-doc membership. Current society members may utilize the certificate for a $25 discount off the cost of annual meeting registration. Reference certificate code SLB2019SPDA. Email questions to jholland@leukocytebiology.org.