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INSTITUTO DE INVESTIGACIONES BIOMÉDICAS

DEGRADACIÓN Y APROVECHAMIENTO DE LAS PROTEÍNAS DEL HUÉSPED INTERNALIZADAS POR CISTICERCOS DE *Taenia solium* Y *Taenia crassiceps*

TESIS

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Contenido

Resumen

La presencia de proteínas del huésped al interior de los tejidos de cisticercos de *Taenia solium* y *Taenia crassiceps* ha sido reportado ampliamente en las últimas décadas, sin embargo, el destino final de estas proteínas o su papel fisiológico al interior del cisticerco apenas comienza a dilucidarse. Cabe mencionar que el cisticerco es capaz de internalizar casi cualquier proteína que se le proporciona en el medio de cultivo. En el presente trabajo se estudió si el cisticerco degrada las proteínas internalizadas del huésped y si aprovecha los aminoácidos provenientes de ellas para sintetizar proteínas propias. Para evaluar la degradación, se marcaron metabólicamente dos proteínas con Leu-^{3}H, que se adicionaron al medio en el que se cultivaron cisticercos de *T. crassiceps*, a fin de rastrear el destino estas proteínas, así como el de sus productos de degradación. Los resultados obtenidos mostraron que el uso de las proteínas como fuente se aminoácidos fue muy escaso. En vista de esos resultados se investigaron posibles usos alternos de las proteínas del huésped que internaliza el cisticerco. Los nuevos resultados mostraron que en parte de esas proteínas son almacenadas en depósitos de sales de calcio en los llamados corpúsculos calcáreos. Lo anterior sugiere que las proteínas del huésped son re-direccionadas para convertirse en el componente principal de la matriz orgánica de los corpúsculos calcáreos, sobre la cual se depositan las sales de calcio que forman los corpúsculos.

Abstract

The presence of host's proteins inside the tissues of *Taenia solium* and *Taenia crassiceps* cysticerci has been widely reported in the last decades, however, the final destination of these proteins and their physiological role inside the cysticercus only recently has been investigated. It is worth mentioning that the cysticercus is capable of internalizing almost any protein provided in the culture medium. In this work we studied if cysticerci degrade internalized host proteins and take advantage of the resulting amino acids to synthesize their own proteins. To evaluate host protein degradation, a strategy of producing Leu-^{3}H metabolically labeled proteins was developed, so that when cysticerci were cultivated in presence of radiolabeled proteins, we could track incorporation of the free amino acids in newly synthesized proteins. Results showed that the use of host proteins as a source of amino acids was very scarce. In view of these findings, we decided to investigate alternative uses of the host proteins internalized by cysticerci. New results showed that part of these proteins are stored in deposits of calcium salts in the so-called calcareous corpuscles. This suggests that the host proteins are re-routed to become a main component of the organic matrix in calcareous corpuscles.

I. **Introducción**

La *T. solium* es un céstodo causante de la teniasis en humanos en su estadio adulto, y de la cisticercosis porcina y humana en su estadio larvario. En regiones endémicas de México y otros países de Sudamérica, África y del Sureste Asiático, caracterizadas por condiciones de pobre higiene, ocurre la cisticercosis humana. El cisticerco está formado por una vesícula llena de líquido con un escólex, que se aloja en diversos tejidos de su huésped intermediario, tales como el músculo esquelético y el cardiaco, así como en el sistema nervioso central y en el tejido subcutáneo (Sciutto *et al*., 2000).

I.1 Ciclo de vida de *Taenia solium*

El ciclo de vida de este parásito involucra dos huéspedes, el cerdo como huésped intermediario en el cual se desarrolla la fase larvaria también llamada metacéstodo o cisticerco, y el humano, que es el único huésped definitivo para este parásito, en el que se desarrolla el gusano adulto (Fig. 1). Cuando el humano ingiere carne de cerdo cruda o mal cocida, infectada con cisticercos de *T. solium,* los cisticercos se activan por la acción de las enzimas intestinales y se fijan en la pared de la primera porción del intestino delgado (duodeno) en donde se desarrollan hasta convertirse en el parásito adulto, conocido coloquialmente como “solitaria”. El gusano adulto está conformado por un escólex que posee una corona de ganchos llamada rostelo y cuatro ventosas como órganos de adhesión; el cuello es una región de proliferación celular, a partir del cual se forma una cadena de segmentos o proglótidos, que poseen tanto genitales masculinos como femeninos. La *T. solium* es un organismo hermafrodita capaz de auto-fecundarse. Los proglótidos más distales al escólex se encuentran grávidos o llenos de huevos y son liberados al ambiente junto con las heces fecales del huésped. El cerdo puede consumir heces humanas o alimentos contaminados con huevos y desarrolla la cisticercosis, el ciclo se completa cuando el humano ingiere carne de un cerdo cisticercoso (Fig. 1). Las enzimas gástricas e intestinales inducen la evaginación del escólex, que se fija a la pared intestinal a través del rostelo y las ventosas, e inicia su desarrollo hasta alcanzar el estadio adulto. Como se mencionó antes, el humano también puede desarrollar cisticercosis al ingerir alimentos contaminados con huevos de *T. solium*

(Sciutto *et al*., 2000). De hecho, el problema de salud de mayor interés sanitario es la neurocisticercosis que se produce cuando los cisticercos se alojan en el sistema nervioso central del humano (SNC). La neurocisticercosis es una enfermedad pleomórfica con signos y síntomas que dependen del número de parásitos y de su localización en el SNC del paciente, así como del estado de la relación huésped-parásito. En general se puede afirmar que cuando los cisticercos aparecen viables, la sintomatología es moderada o inexistente, mientras que cuando se encuentran cisticercos hialinos o calcificados, la sintomatología puede involucrar cuadros graves de hipertensión craneal, eventos convulsivos y muchos otros síntomas, debido al proceso inflamatorio que se establece (Fleury *et al*., 2010).

Figura 1. Ciclo de vida de *Taenia solium*. El parásito tiene como huésped intermediario al cerdo y como huésped definitivo al ser humano. Tomado de Cistimex.

I.2 Distribución mundial de cisticercosis por *T. solium*

De acuerdo con la Organización Mundial de la Salud (Report of the WHO Expert Consultation on Foodborne Trematode Infections and Taeniasis/Cysticercosis), la distribución de la taeniasis/cisticercosis, incluye diferentes países de Latinoamérica, África en la región Sub-Sahara y en Asia, especialmente en países de la región sureste (Fig. 2). En México, la teniasis/cisticercosis ya no se considera un problema

de salud pública como había sido hace más de tres décadas, sin embargo, en zonas rurales relativamente aisladas, todavía se reportan altas prevalencia de cisticercosis porcina y aún se reportan casos de neurocisticercosis humana (Fleury *et al.,* 2010 y 2012).

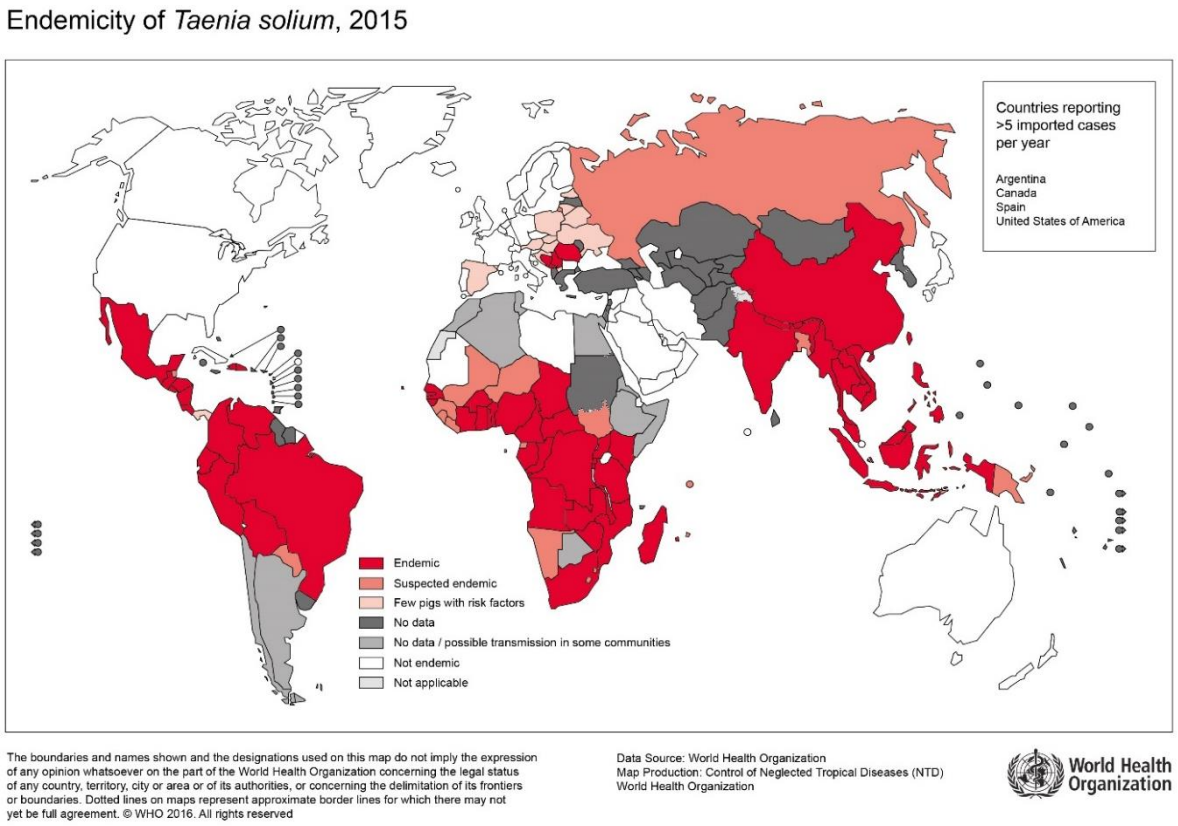


Figura 2. Distribución mundial de la Taeniasis/cisticercosis. Tomado de: Working to overcome the global impact of neglected tropical diseases: first WHO report on neglected tropical diseases (2015).

I.3 El modelo de cisticercosis murina: *Taenia crassiceps*

Taenia crassiceps es un parásito cercanamente relacionado a *T. solium* que presenta un ciclo de vida similar (Fig. 3). T. crassiceps también requiere de dos huéspedes: el intermediario que pueden ser roedores, ratones o ardillas, y el definitivo que suelen sus predadores, perros y zorros (Freeman, 1962; Willms y Zurabian, 2010). El cisticerco de *T. crassiceps* presenta similitudes morfológicas, fisiológicas y moleculares que lo han convertido, desde hace varias décadas, en un

modelo de estudio de la cisticercosis en ratones (Sciutto *et al*., 2011). Esta especie de parásito tiene la capacidad de reproducirse asexualmente mediante gemación, lo cual facilita su mantenimiento en laboratorio a través de transferencia de los cisticercos del peritoneo de ratones infectados peritonealmente hacia ratones sanos (Fig. 3) (Dorais y Esch, 1969). Después de varias semanas de infección, los cisticercos se obtienen de la cavidad peritoneal para su estudio, incluso, se pueden mantener *in vitro* por semanas. Una cepa de *T. crassiceps*, ORF, que ha sido propagada en condiciones de laboratorio durante décadas, ha perdido la capacidad de formar el escólex, lo que la hace incapaz de propagarse en condiciones naturales, pero cuya facilidad de manejo y disponibilidad en el laboratorio, le ha permitido convertirse en un modelo muy utilizado para el estudio de la cisticercosis, tanto *in vitro* como *in vivo*. Otra cepa mantenida en laboratorio es la WFU, que sí forma un escólex y, por lo tanto, puede alcanzar la fase adulta del parásito, también ha sido ampliamente utilizada en el laboratorio (Willms y Robert, 2007).

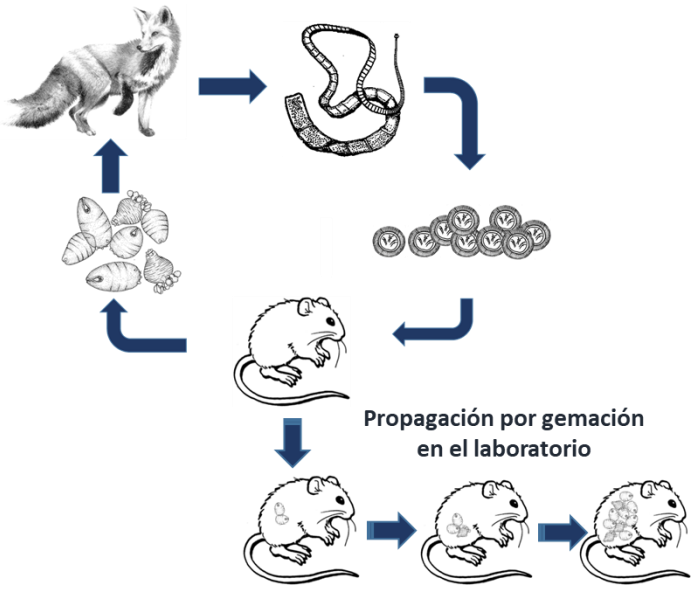


Figura 3. Ciclo de vida de *Taenia crassiceps*. El huésped definitivo pueden ser zorros o perros y el huésped intermediario roedores como ratones y ardillas. Los cisticercos pueden ser mantenidos en condiciones del laboratorio mediante inoculaciones intraperitoneales en ratones sanos. Ilustración original preparada por JFB.

El cisticerco de *T. crassiceps* y el uso del modelo murino de cisticercosis ha permitido entender diversos aspectos de relación huésped-parásito, como la influencia genética en la susceptibilidad a esta parasitosis en ratones (Fragoso *et al*., 1996; 1998; 2008); los mecanismos que utiliza el parásito para evadir la respuesta inmune del huésped a través de la inducción de un tipo de respuesta Th2 que favorece la proliferación del cisticerco (Terrazas *et al*.,1998; Gómez-García *et al*., 2006; Peón *et al*.,2013); la respuesta a hormonas del huésped como la progesterona e insulina (Vargas-Villavicencio *et al*., 2005,2008; Jiménez *et al*., 2006, Escobedo *et al*., 2004, 2005, 2009; Ostoa-Saloma *et al*.,2010), ensayos de vacunación que condujeron al desarrollo de varios antígenos candidatos para desarrollar una vacuna contra la cisticercosis (Sciutto *et al*., 2011), así como estudios morfológicos fisiológicos y moleculares, tales como la caracterización de proteasas (White AC, *et al*., 1997, Baig *et al*.,2005), procesos de endocitosis y absorción de nutrientes (Haynes *et al*., 1968; Threadgold y Dunn, 1984; Ambrosio *et al*., 1994), entre muchos otros.

I.4 El genoma de *Taenia solium*

Cabe mencionar que los céstodos se han adaptado evolutivamente al modo de vida parasitario a través de una serie de modificaciones, morfológicas, fisiológicas y moleculares. Por ejemplo, entre las modificaciones morfológicas se puede mencionar la ausencia de un aparato digestivo y en su lugar posee una superficie tegumental sincicial, a través de la cual absorbe ávidamente nutrientes de su huésped (Burton *et al*., 2012). Con la secuenciación y análisis del genoma de *Taenia solium* y otros parásitos céstodos se hizo evidente que estos organismos son altamente dependientes de su huésped (Tsai *et al*., 2013). El análisis reveló que este parásito al igual que los otros céstodos, presentan una extensa reducción de sus capacidades metabólicas y un incremento de su habilidad para absorber nutrientes comparada con otros animales. Algunos ejemplos son, su reducida o casi nula habilidad para sintetizar ácidos grasos y colesterol *de novo*, en su lugar poseen familias de genes para proteínas capaces de unir y transportar ácidos grasos esenciales (Fatty acid binding proteins, FABP) y posee las enzimas para elongar

lípidos (Illescas *et al*., 2012; Alvite *et al*., 2012, Tsai *et al*., 2013). Otro ejemplo es la reducida capacidad de síntesis de aminoácidos y de nucleótidos, que están completamente ausentes en el genoma de *T. solium* (Tsai *et al*., 2013) (Fig.4).

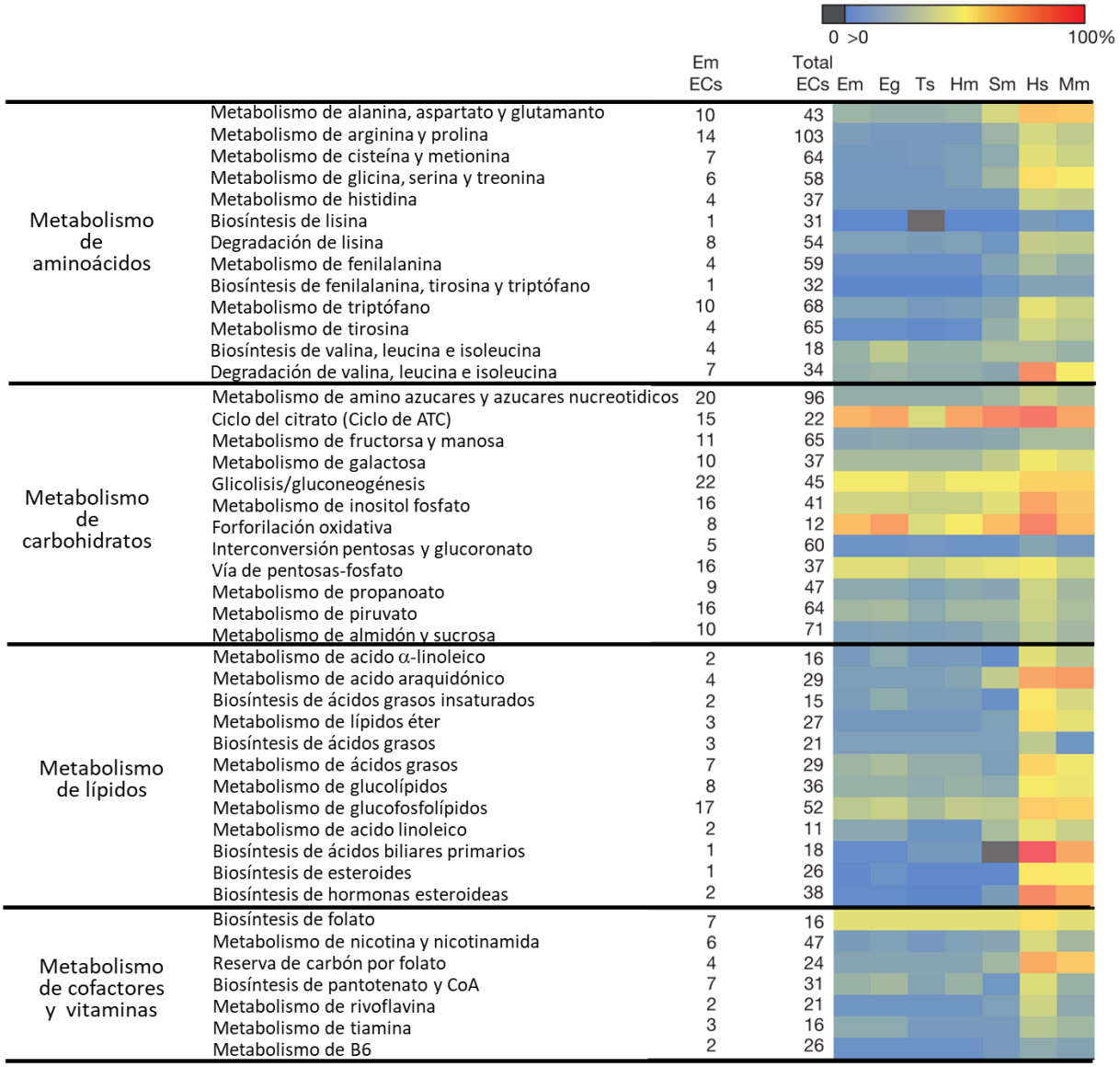


Figura 4. Conservación de rutas metabólicas. Mapa de intensidad que muestra la conservación de rutas metabólicas individuales para *Echinococcus multilocularis* (Em), *E. granulosus* (Eg), *T. solium* (Ts), *Hymenolepis microstoma* (Hm) y *Schistosoma mansoni* (Sm) comparados con los de humano (Hs) y del ratón (Mm). Cada fila indica una ruta metabólica individual agrupada por su pertenencia a una superclase (definida de acuerdo con KEGG (Kyoto Encyclopedia of Genes and

Genomes)). Los mosaicos coloreados indican el nivel de conservación (porcentaje de enzimas detectadas) de cada ruta dentro de cada especie. CoA: coenzima A; EC: número de enzima; TCA: ciclo de ácidos tricarboxílicos. Tomado y traducido de Tsai *et al*., 2013.

II. Antecedentes

II.1 Internalización de proteínas del huésped

Desde hace varias décadas se ha reportado la presencia de proteínas del huésped (desde un inicio se identificaron albúmina e inmunoglobulinas) en el fluido vesicular (FV) del cisticerco de diferentes especies de *Taenia*, incluidas *solium y crassiceps* (Kagan *et al.,* 1963, Chordi *et al*., 1965, 1975; Hustead *et al.,* 1977; Machnicka *et al.,* 1986; Hayunga *et al*., 1989; Shapiro Z, 1992) así como de *Echinococcus (*Kagan *et al.,* 1963, Chordi *et al*., 1965; Coltorti *et al*., 1975; Hustead *et al*., 1977b; Shapiro Z, 1992; Zeghir-Bouteldja *et al*., 2017);. Sin embargo, poco se sabe de la función y abundancia de estas proteínas al interior del cisticerco. Por ejemplo, se reportó que la albúmina actúa como un regulador de la presión oncótica entre el cisticerco de *T. crassiceps* y su ambiente (tejido del huésped) (Aldrige *et al*., 2006). Respecto a la incorporación de proteínas del huésped, en específico albúmina e inmunoglobulinas, nuestro grupo de trabajo describió que el proceso de internalización se lleva a cabo a través de un mecanismos inespecífico y una alta tasa de incorporación; se propuso como mecanismo a la pinocitosis fluida y se describieron dos posibles rutas de incorporación de macromoléculas través de los citones usando como marcador la Peroxidasa de rábano y otra directamente por debajo de la membrana basal con Rojo de rutenio (Ambrosio *et al*., 1994) (Fig. 5). Estudios recientes también realizados en nuestro grupo, describieron la abundancia y la función de las proteínas del huésped; ensayos de electroforesis en dos dimensiones permitieron determinar que las proteínas de huésped representaban 11-13% de la proteína total del FV, se logró identificar 15 proteínas adicionales a las anteriormente descritas, tales como hemoglobina, serpina A3-8, haptoglobina, proteína activadora de rho GTPasa, fosfoglicerato cinasa 1, entre otras (Navarrete-Perea *et al*., 2014). En este estudio también se demostró que las proteínas del

huésped en el FV presentaban las mismas abundancias y proporciones que en el huésped, lo cual corroboraba que su incorporación al interior de los cisticercos ocurre de forma inespecífica, mediante pinocitosis fluida (Navarrete-Perea *et al*., 2014). Estudios más recientes y aplicando técnicas de proteómica más sensibles permitieron identificar y cuantificar 4,259 proteínas en un extracto total de cisticercos, de las cuales un 20% corresponde a proteínas del huésped. Además, se logró identificar alrededor de 900 especies de proteínas del huésped presentes en los tejidos del cisticerco (Navarrete-Perea *et al*., 2017). Estos hallazgos, nos indican que el intercambio molecular en la cisticercosis porcina alcanza niveles no descritos para otras relaciones huésped-parásito. Se trata de una “intimidad molecular” sin precedente en la literatura.

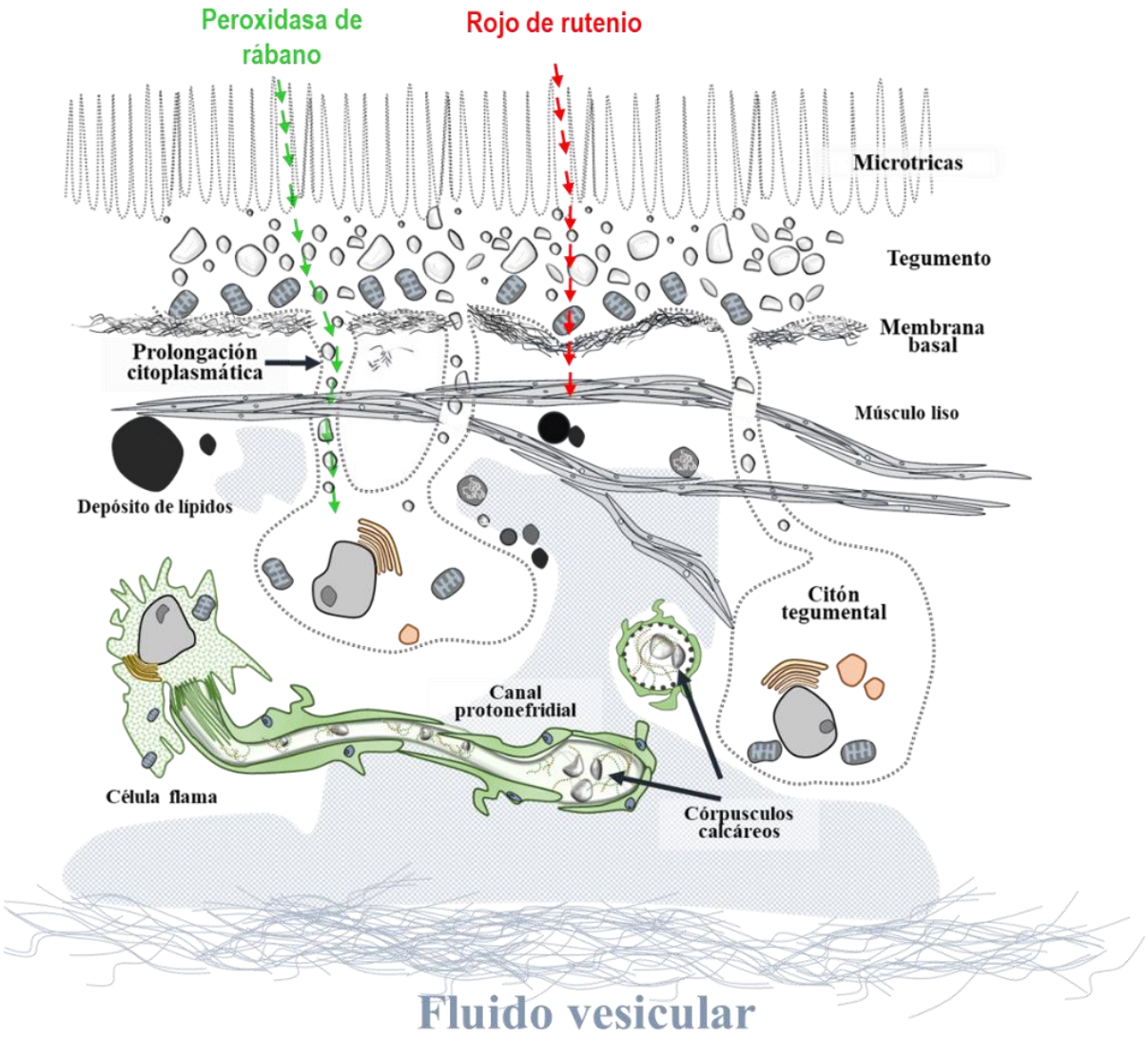


Figura 5. Rutas de incorporación y esquema del tegumento. Nuestros resultados apuntaron a que existen dos rutas para la internalización de macromoléculas hacia los tejidos y fluido vesicular del cisticerco; la primera ruta es la que siguen proteínas solubles, tales como la peroxidasa de rábano, que parece ser incorporada al tegumento y posteriormente es direccionada hacia el citoplasma de los citones subtegumentales vía prolongaciones citoplasmáticas. Por otro lado, reactivos como el rojo de rutenio se une a glicoproteínas que forman el glicocáliz tegumental. Estas proteínas parecen ser liberadas hacia el espacio intersticial por a través de la membrana basal que se localiza debajo del tegumento. Ilustración original preparada por JFB.

Respecto a la función de las proteínas del huésped que incorporan los cisticercos al fluido vesicular y hacia los tejidos, se ha propuesto que algunas de ellas pueden aportar al parásito, funciones similares a las que juegan para el huésped. Tal es el caso de la haptoglobina, que es una proteína a cargo de unir la hemoglobina, liberada al plasma por daño de los eritrocitos, con el objeto de recuperar el hierro. Se ha propuesto que el cisticerco toma ventaja de la función del par haptoglobina-hemoglobina, para manejar sus necesidades de hierro, que a su vez influye en la regulación de la respuesta inflamatoria alrededor del cisticerco (Navarrete-Perea *et al*., 2016). En la presente tesis se explora la posible función que juegan las inmunoglobulinas del huésped incorporadas hacia el FV y los tejidos del cisticerco. A pesar de lo sugerente de nuestros resultados, es mucho lo que falta por explorar para entender a cabalidad la complementación molecular que el cisticerco logra incorporando las proteínas del huésped.

II.2 Proteasas del cisticerco

Se dispone de muy poca información acerca de si las proteínas del huésped que internalizan los cisticercos son degradadas como fuente de aminoácidos, o si tienen algún otro destino en los tejidos del cisticerco. Se han caracterizado diversas proteasas presentes en los tejidos de varias especies de cisticercos taénidos; incluyendo cisteín-proteasas, aspartil-proteasas y metalo-proteasas (White *et al*., 1992). Las cisteín-proteasas han sido las más estudiadas y se ha descrito que son tipo catepsina, siendo activas a un pH ácido y capaces de degradar *in vitro* algunas proteínas del huésped como hemoglobina, inmunoglobulinas o albúmina (White *et al*., 1992,1997; Baig *et al*., 2005; Li *et al.,* 2006). La localización de estas proteasas se ha descrito al interior de los citones y en vesículas tipo-lisosoma del tegumento (Khalil *et al*., 1998). Al igual que las cisteín-proteasas, las aspartil-proteasas son activas a pH ácido y solo se ha demostrado *in vitro* su capacidad de degradar hemoglobina (White *et al*., 1992). De las metalo-proteasas se ha reportado que tienen actividad a pH neutro y son liberadas al medio, posiblemente como un mecanismo para modular la respuesta del huésped, de este tipo de enzimas se ha descrito su presencia en el sobrenadante de cisticercos en cultivo y su capacidad

para degradar albúmina, IgG's e IL-2 (White *et al*., 1992, Baig *et al*., 2005). Sobre esta línea de investigación resta averiguar si estas proteasas juegan un papel equivalente *in vivo.*

II.3 Corpúsculos calcáreos

Los corpúsculos calcáreos (CC) son estructuras presentes al interior de los canales protonefridiales tanto en el parásito adulto como en el metacéstodo (cisticerco) de *T. solium* y llegan a representar hasta un 40% del peso seco del parásito (Baron ,1968; Vargas-Parada *et al*., 1999a, 1999b). El estudio de los CC en diferentes céstodos, *in vivo* e *in vitro*, ha llevado a plantear diversas hipótesis respecto a su origen, formación y estructura. Los CC consisten de una matriz orgánica compuesta de proteínas, carbohidratos, lípidos, RNA y DNA dispuestos en anillos concéntricos, y un depósito inorgánico principalmente de sales de calcio, magnesio, fósforo y carbonato (apatita e hidroxiapatita) (Smith *et al*.,1993). La cantidad, la forma, el tamaño y la composición química varía entre individuos y organismos (von Brand *et al*., 1965; Smith *et al*., 1993). La formación de CC en el cisticerco de *T. solium* se ha descrito que ocurre, a diferencia de otros céstodos, en el medio extracelular en la luz de los canales protonefridiales y el material orgánico es provisto por microproyecciones provenientes de la pared del canal (Vargas-Parada *et al*., 1999b).

Respecto a la función de los CC en la fisiología de los céstodos, incluyendo *Taenia* spp se ha propuesto su papel como reservorios de carbonatos que protegen los tejidos del parásito contra condiciones ácidas como su paso por el estómago del huésped definitivo o como reserva de fosfatos que varía en respuestas al estado nutricional del organismo (von Brand *et al.,* 1969; Zurabian *et al*., 2005). En el caso específico de *Taenia* spp la presencia de estos corpúsculos al interior de los canales protonefridiales podría estar implicada con la regulación de los fluidos tisulares, así como la eliminación de desechos metabólicos y de antígenos potenciales (Vargas-Parada *et al*., 1999b). También se ha propuesto que los CC podrían participar como depósitos de los excedentes de calcio, lo cual podría proteger el cisticerco contra la

calcificación, ya que se ha reportado la presencia de proteínas de unión a calcio en la matriz orgánica en su interior (von Brand et al., 1975; Zurabian *et al*., 2005).

III. **Justificación del proyecto**

La cisticercosis humana y porcina es causada por el metacéstodo de *T. solium* y se adquiere por ingerir alimentos contaminados con huevos de *T. solium*. El cisticerco se puede localizar en diversos tejidos de su huésped como el músculo esquelético y sistema nervioso central (Willms K, *et al*., 2008). Para que el cisticerco pueda sobrevivir en los tejidos del huésped, requiere de una serie de mecanismos que le permitan evadir la respuesta inmune y obtener nutrientes. Diversos esfuerzos se han llevado a cabo para dilucidar los mecanismos implicados en la supervivencia de los cisticercos en los tejidos del huésped, por ejemplo, se ha reportado la excreción de paramiosina como un mecanismo para evitar la activación del complemento (Laclette *et al*., 1989,1992) además de que el cisticerco incorpora moléculas como la glucosa y aminoácidos (Pappas *et al*., 1973; Rodríguez-Contreras *et al*., 1998).

Otro aspecto estudiado en la interacción cisticerco-huésped es la internalización de proteínas del huésped. Nuestro grupo ha reportado que las proteínas del huésped pueden llegar a representar hasta un 13% del contenido proteico del fluido vesicular (FV) y que la internalización de estas sigue un mecanismo inespecífico (Ambrosio J, *et al*., 1994; Navarrete J, *et al*., 2014, 2016, 2017). Algunos análisis recientes del proteoma del cisticerco por espectrometría de masas, permitieron identificar y cuantificar 4259 proteínas en un extracto total de cisticercos, de las cuales el 20% corresponden a proteínas del huésped. Los resultados incluidos en ambos reportes sugieren que la internalización de las proteínas del huésped se lleva a cabo a través de un mecanismo inespecífico; posiblemente pinocitosis fluida. En concordancia con lo anterior, el porcentaje de proteínas del huésped en los tejidos del cisticerco refleja cercanamente la abundancia de esas proteínas en los fluidos del huésped, vgr., suero o líquido cefalorraquídeo. Entre las proteínas del huésped presentes en el FV las de mayor abundancia son la albúmina y las inmunoglobulinas. En el caso de la internalización de la albúmina se le ha atribuido un rol en la regulación de la presión oncótica del cisticerco, y en el caso de las inmunoglobulinas se ha propuesto su participación en diversos mecanismos de evasión inmune, o incluso de

explotación inmune, es decir, usarlas como una fuente de nutrientes (Aldridge *et al*., 2006).

Sin embargo, se desconoce el destino de las proteínas del huésped internalizadas por el cisticerco. Al respecto, se han reportado estudios *in vitro* que muestran que los extractos crudos del cisticerco poseen proteasas que pueden degradar proteínas del huésped, incluyendo a las inmunoglobulinas, la albúmina y la hemoglobina (White *et al*., 1992,1997), aunque no se ha determinado si estas enzimas cumplen este papel *in vivo*. La evidencia indica que el cisticerco de la *T. solium* utiliza las chaperonas del huésped para secuestrar el hierro de éste y para disponer de él en sus tejidos (Navarrete-Perea *et al*., 2016).

Con la publicación del genoma completo de *T. solium* quedó claro que estos organismos poseen una capacidad biosintética muy limitada (Tsai *et al*., 2013). Ahora sabemos que el cisticerco es incapaz de sintetizar la mayor parte de los aminoácidos, por lo que debe obtenerlos de su entorno, es decir, de los tejidos del huésped, para sobrevivir. Considerando lo antes mencionado, parecía probable que el cisticerco usara las proteínas internalizadas como una fuente de aminoácidos para llevar a cabo la síntesis de sus propias proteínas.

En el presente trabajo decidimos abordar el estudio del destino las proteínas del huésped al interior del cisticerco ya sea como fuente de aminoácidos o rastrear qué otro destino podría tener. Cabe mencionar que el cisticerco es capaz de internalizar casi cualquier proteína que se le provea en el medio de cultivo. Nuestra estrategia consistió en utilizar proteínas marcadas metabólicamente, una que participa en la relación huésped-cisticerco, así como otra proteína marcada que no tiene relación alguna con el parásito o la enfermedad. Esta estrategia pretende generar datos que permitan un mejor entendimiento de la fisiología del parasito y de la relación con su huésped.

IV. **Hipótesis y objetivo**

IV.1 Hipótesis

Los cisticercos utilizan las proteínas del huésped como una fuente de aminoácidos para sintetizar proteínas propias.

IV.2 Objetivo general

Identificar las proteínas que son sintetizadas por cisticercos de *T. crassiceps* a partir de las proteínas del huésped internalizadas.

IV.3 Objetivos específicos

- Determinar si el cisticerco es capaz de sintetizar sus proteínas a partir de la IgG y de la proteína recombinante marcada metabólicamente con Leu-^{3}H.
- Identificar las proteínas sintetizadas *de novo* por el cisticerco a partir de los productos de degradación de las proteínas marcadas con Leu-^{3}H.
- Explorar destinos alternativos de las proteínas del huésped que son internalizadas por el cisticerco de Taenia spp.

V. **Materiales y Métodos**

Una parte de los métodos se encuentra descrita en las secciones correspondientes de los dos artículos ya publicados. Sin embargo, en este capítulo se añade mayor detalle sobre los procedimientos empleados e información no contenida en los artículos publicados por razones de limitación del espacio.

V.1 Parásitos

Los experimentos *in vitro* se llevaron a cabo usando cisticercos de *Taenia crassiceps* cepa ORF, los cuales son mantenidos en ratones hembras BALB/c (Sciutto *et al*., 2011), alojados en la Unidad de Modelos Biológicos del Instituto de Investigaciones Biomédicas de la UNAM. A los tres meses posteriores a la infección, se sacrificaron los ratones de acuerdo con los Lineamientos para el Manejo y Uso de Animales de laboratorio. Cabe mencionar que el protocolo utilizado fue aprobado por la Comisión de Bioética del Instituto de Investigaciones Biomédicas (Número de aprobación ID 199). Los cisticercos se colectaron de la cavidad peritoneal y se lavaron varias veces con solución salina de fosfatos estéril, pH7.4 (PBS). Posteriormente se pusieron en cultivo en el medio correspondiente a 37°C y en una atmosfera 5% CO2. Los cisticercos de *T. crassiceps* de la cepa WFU también se mantuvieron por inoculaciones intraperitoneales en ratones. Similarmente, estos cisticercos se obtuvieron y lavaron varias veces con PBS, para ser almacenados a -70°C hasta su uso.

Los cisticercos de *T. solium* fueron obtenidos por disección de músculo esquelético de cerdos infectados en condiciones naturales, que fueron sacrificados de forma humanitaria en las instalaciones de la Facultad de Veterinaria y Zootecnia de la UNAM, siguiendo los lineamientos del Código Ético del Instituto de Investigaciones Biomédicas. Al igual que los cisticercos de *T. crassiceps*, los de *T. solium* fueron lavados varias veces con solución salina de fosfatos y almacenados a - 70°C hasta ser usados.

V.2 Extractos de cisticercos

V.2.1 Extracto total en urea

A 20 cisticercos de *T. crassiceps* se les añadió una solución de lisis (7 M de urea, 2 M de tiourea, 4 % de CHAPS, 10 mM de Tris e inhibidores Complete-Roche) antes de ser homogenizados con un Benchmark D1000 y sometidos a 3 ciclos de congelamiento-descongelamiento, para luego centrifugar la suspensión a 14,000 x g durante 15 min y recolectar el sobrenadante. La concentración de proteína fue determinada usando el NI-protein assay kit (GBiosciences) siguiendo las instrucciones del fabricante.

V.2.2 Extractos de tejido, fluido vesicular y medio de cultivo

Posterior al cultivo de los cisticercos durante nueve días (ver sección V.11) se colectó el medio y se le añadió una mezcla de inhibidores (Complete Roche) siguiendo las recomendaciones del fabricante. Los cisticercos fueron lavados varias veces con PBS. Una vez lavados los cisticercos las vesículas fueron seccionadas sobre una superficie de vidrio estéril, usando un bisturí y se recolectó el FV, al que se añadió solución de lisis (ver sección IV.2.1) en relación 1:1 (v/v). Para la obtención del extracto de tejido, los cisticercos ya sin fluido, fueron lavados 3 veces con PBS-inhibidores. Después se les añadió la solución de lisis y se homogenizaron con un Benchmark D1000 *y sometidos a 3 ciclos de congelamiento-descongelamiento con nitrógeno líquido,* para luego centrifugar la suspensión a 14,000 x g durante 15 min y recolectar el sobrenadante. La concentración de proteína en los extractos y en los fluidos fue determinada también con el kit mencionado arriba.

V.2.3 Extracto salino

En este caso, un volumen aproximado de 30-40 mL de cisticercos completos (incluido el FV) de *T. crassiceps* fueron suspendidos en PBS-inhibidores y homogenizados con un Benchmark D1000 y sometidos a 3 ciclos de congelamiento-descongelamiento con nitrógeno líquido, para luego centrifugar la suspensión a

14,000 x g y colectar el sobrenadante. El extracto se almacenó a -70°C hasta su uso.

V.3 Marcaje metabólico de IgG murina con tritio (^{3}H)

Se cultivaron varios hibridomas murinos para evaluar su productividad durante el cultivo *in vitro*. Entre ellos se seleccionó un hibridoma productor de IgG, dirigido hacia una proteína no relacionada con las cisticercosis (donado por S. López); el hibridoma (anti-NR) fue cultivado en medio RPMI-1640 (Biowest) suplementado con suero bovino fetal al 10% y ampicilina-estreptomicina y adicionado con 5 µCi/mL of Leucina-^{3}H (Leu-^{3}H) (Perkin-Elmer), partiendo de una densidad de $1x10^5$ células/mL. El cultivo se mantuvo a 37° C en una atmósfera de 5% CO_2 durante 14 días. Posteriormente se separaron las células por centrifugación y se recolectó el sobrenadante para mezclarse en una relación 1:1 con una solución de fosfatos, 20mM a pH 7.0. Esta solución se mantuvo a -70° C hasta que se utilizó.

V.4 Marcaje metabólico de proteína verde fluorescente (GFP) recombinante con tritio (^{3}H)

Una cepa recombinante de *Escherichia coli* que expresa la GFP, fue amablemente donada por P. Gaytán. La bacteria fue cultivada en medio Luria-Bertani (LB) adicionado con Kanamicina y 5 µCi/mL of Leu-^{3}H (Perkin-Elmer) durante 24 h a 37°C. Las células bacterianas se cosecharon y suspendieron en solución de lisis (Guanidina 5M, Tris 10mM, inhibidores Complete-Roche, pH 7.4). Posteriormente se lisaron mediante sonicación durante 3 minutos (6 ciclos de 30 segundos), el homogenizado se centrifugó 30 min a 4000 x g y se colectó el sobrenadante. Esta solución se mantuvo a -70° C hasta su uso.

V.5 Pre-cultivo y cultivo de los cisticercos de *T. crassiceps*

Después de múltiples ensayos, se determinó que la eliminación de macromoléculas presentes en el tejido y en el FV de los cisticercos de *T. crassiceps*, ocurría de manera óptima en un medio que contenía las sales correspondientes al RPMI-1640, pero carente de aminoácidos. A este medio de cultivo, que se preparaba en nuestro

laboratorio, se le denominó como Medio Mínimo para Cisticercos (MMC). En breve, varios grupos de 20 cisticercos se cultivaron previamente en 10 volúmenes de MMC durante 3 días llevando a cabo cambios del medio cada 24 h para favorecer la excreción/secreción de las proteínas del huésped y las del cisticerco, contenidas en el FV.

Para evaluar la incorporación y el uso de las proteínas marcadas metabólicamente (IgG-^{3}H o GFP-^{3}H), después del pre-cultivo, los cisticercos fueron mantenidos durante 9 días en 1 mL de MMC suplementado con 2% de albúmina, al que se añadió alrededor de 500 µg de proteínas marcada (IgG-^{3}H o GFP-^{3}H) a 37°C en una atmosfera de 5% de CO_2. A diferentes tiempos de cultivo se evaluaba la cantidad de marca incorporada por los cisticercos, hasta que se determinó que el tiempo de mayor incorporación de las proteínas marcadas en el que los cisticercos se mantenían viables era de 9 días. Con el objetivo de evaluar la actividad biosintética basal de los parásitos bajo estas condiciones de cultivo, un grupo de cisticercos tratados de manera idéntica fueron cultivados en presencia de 30 µCi de Leu-^{3}H. Al final del cultivo, se obtuvieron los medios de cultivo y los tejidos de los tres grupos de cisticercos (IgG-^{3}H, GFP-^{3}H y Leu-^{3}H). Estos materiales fueron almacenados a -70°C para su posterior procesamiento para obtener extracto de tejido y FV.

V.6 Geles de poliacrilamida

La preparación de geles con diferentes porcentajes (10% y 15%) de poliacrilamida se realizó de forma estándar (Maniatis *et al.,* 1989). Usualmente se cargaron hasta 100 µg de extractos de proteína en una proporción de 5:1 con solución amortiguadora de carga 6x (Glicerol 30%, SDS 10%, DTT 0.5 M y trazas de azul de bromofenol). La mezcla fue desnaturalizada durante 5 min a 96°C. Al terminar la electroforesis, los geles fueron sumergidos e incubados en una solución de Coomassie.

IV.7 Western-blot

Los geles fueron transferidos a membranas de nitrocelulosa en una cámara húmeda (BioRad), durante 70min a 120V. Las membranas fueron bloqueadas con leche descremada al 10% en PBS pH 7.4. El anticuerpo primario fue diluido en la misma solución e incubado 2 h a temperatura ambiente. Se lavó la membrana 3 veces durante 10 min con PBS-Tween 20 al 0.1%. El anticuerpo secundario se diluyó también en PBS-Tween 20 y se incubó durante 2 h a temperatura ambiente y posteriormente se lavó 3 veces. El reconocimiento por los anticuerpos se reveló con el kit de quimioluminiscencia West-Femto (Thermo Scientific) siguiendo las instrucciones del fabricante.

V.8 Cromatografía

V.8.1 Purificación de la IgG murina

El sobrenadante del cultivo del hibridoma recolectado como se describió antes, fue mezclado en una relación 1:1 con solución de fosfatos 20 mM pH 7.0, ajustando el pH a 7.0. La mezcla se pasó por la columna de Sepharosa-Proteína G (Sigma) previamente equilibrada con la solución de fosfatos. Posteriormente la columna fue lavada con 5 volúmenes de la solución de fosfatos y la fracción unida fue eluida usando glicina 0.1 M, pH 2.7.

IV.8.2 Purificación de GFP

Aprovechando que la proteína recombinante posee una etiqueta de poli-histidinas, la GFP recombinante se purificó a través de cromatografía de afinidad a metales; para ello, el sobrenadante obtenido de la lisis de las bacterias se pasó por una columna His Trap FF (Sigma) acoplada a sulfato de níquel y previamente equilibrada con la misma solución de lisis descrita arriba. Se lavó la columna con 5 volúmenes de solución amortiguadora de lisis y la proteína unida se eluyó con la misma solución, pero con imidazol 10mM.

V.8.3 Actividad específica

La actividad específica de cada proteína marcada, IgG-^{3}H o GFP-^{3}H, fue determinada disolviendo10µg de proteína en Tritosol (Fricke, 1975) y midiendo la radiactividad en un contador de centelleo (Beckman Coulter LS6500).

V.9 Fluorografía

La detección de la marca radiactiva en el gel de poliacrilamida fue realizada por fluorografía (Bonner and Laskey, 1974). El gel fue sumergido dos veces durante 30 min en dimetil-sulfóxido (DMSO) y posteriormente en una solución al 20% (w/w) de PPO (2,5-Difeniloxazol) durante 3 horas. Finalmente, el DMSO fue removido sumergiendo el gel en agua. Se secó el gel y se expuso a una placa radiográfica a -70°C durante 10 o 30 días.

V.10 Ensayo por inmuno-absorción ligado a enzimas (ELISA)

Los ELISA se realizaron para evaluar la productividad de los hibridomas de los que se disponía para elegir el que mayor cantidad de inmunoglobulina generaba. Brevemente, se sensibilizó una placa toda la noche con el sobrenadante de cada uno de los hibridomas en una relación 1:5 (sobrenadante: PBS-Tween 20 0.05%). Posteriormente se realizaron 3 lavados de 5 min con PBS-Tween 20 0.05% y después se incubó con el anticuerpo secundario (anti-IgG de ratón) durante 2 h. La placa se lavó una vez más y se procedió a revelar el reconocimiento con orto-fenilendiamina (OPD) y se leyó la reacción colorimétrica a 495 nm.

V.11 Obtención de corpúsculos calcáreos

Los CC se obtuvieron a partir de un extracto salino de cisticercos de *T. solium*, descrito previamente. Se usaron varios grupos de cisticercos obtenidos de cerdos infectados de forma natural. Los extractos se centrifugaron a 14,000X g durante 30 min a 4°C para que los CC sedimentaran en el fondo del tubo. Puesto que los corpúsculos calcáreos son depósitos de sales de calcio sobre una matriz orgánica, su alta densidad permite obtenerlos al fondo del tubo después de la centrifugación del extracto de cisticercos. Los restos celulares y sobrenadante fueron eliminados,

se suspendió el sedimento con CC en agua bidestilada (Milli-Q, Millipore Co) y se centrifugó. Este proceso de lavado del sedimento fue repetido de 8-10 veces hasta que se observaba completamente limpio. Se hizo un lavado rápido del sedimento con 0.5 mL de una solución de HCl 0.1 N con el fin de eliminar proteínas que no son parte de la matriz y que pudieran haber sido adsorbidas en la superficie de los CC durante la obtención del extracto. Finalmente, los CC fueron centrifugados y disueltos en 2mL de HCl 0.1 N. El material disuelto de los CC fue concentrado usando viales de ultrafiltración Amicon (3 kDa de punto de corte) y suspendido en 150 μL una solución de 7 M urea, 2 M tiourea, 4% CHAPS y 10 mM Tris.

V. 12 Espectrometría de masas del extracto proteico de los CC

Previo al análisis, las proteínas fueron reducidas con ditiotreitol (DTT) (BioRad) 10mM durante 1 h y alquiladas con iodoacetamida (BioRad) 15 mM durante 30 min. La reacción de alquilación fue detenida usando DTT 15mM. Cada muestra fue digerida con tripsina porcina modificada y preparada para Cromatografía Liquida acoplada a Espectrometría de Masas en tándem (LC/MS/MS). Las muestras fueron inyectadas en una columna Acquity UPLC-BEH-C18 (Waters, Milford, MA), la cual está acoplada en-línea con un LTQ Orbitrap XL (Thermo Fisher Scientific, Waltham, MA) equipado con una fuente de ionización por electro-pulverización ("electrospray"). Las muestras fueron equilibradas en el solvente A que contiene 100% H_2O y 0.1% de ácido fórmico y eluída a través de un gradiente con el solvente B el cual contiene 100% de acetonitrilo y 0.1% de ácido fórmico. Una muestra de 3 μL de proteínas digeridas fue retenida usando una pre-columna (Symmetry® C18, 5 μm, 180 μm × 20 mm, Waters) la cual fue cambiada en-línea a una columna capilar UPLC de 10-cm (100 μm ID BEH-C18 1.7 μm de tamaño de partícula). La columna fue enmarcada en un calentador de columna operando a 35°C. Después de cargar, los péptidos se separaron en un gradiente de 60-min a una tasa de flujo de 400nL/min. EL gradiente fue el siguiente: 3-50% Solvente B; 30 min, 50-85%; 1 min, 85%; 7 min y 3%; 22 min. Los péptidos eluidos fueron directamente electro-pulverizados dentro del espectrómetro de masas a través de una punta cubierta de sílice estándar (NewObjective, Woburn, MA).

EL espectrómetro LTQ fue operado en modo de adquisición independiente de datos para alternar automáticamente entre un escaneo completo en un rango de m/z 400 a 2000, y los 3 iones más intensos se aislaron secuencialmente y fragmentados usando una disociación inducida por colisión (Collision-induced dissociation, CID). La disociación se llevó a cabo con helio como gas de colisión a una energía normalizada de 40% y un tiempo de activación de 10 ms. La adquisición de datos se controló mediante el programa Xcalibur 2.0.7 (Thermo Fisher Scientific). Los espectros obtenidos del LTQ Orbitrap XL fueron extraídos mediante Proteome Discoverer versión 1.3 (Thermo Scientific) y buscada mediante el uso de Sequest contra la base de datos de *T. solium.*

Todos los archivos generados por Sequest se buscaron con los siguientes parámetros: la tolerancia para péptidos fue de 1.6 Da, la tolerancia de masa de los fragmentos fue de ±0.8 Da y un máximo de dos cortes faltantes. La carbamidometilación de las cisteínas fue establecida como una modificación estática y la oxidación de metioninas fijada como una modificación variable.

V.13 Análisis de datos de la espectrometría de masas

La abundancia relativa de proteínas, representada por el factor de abundancia espectral normalizado (Normalized spectral abundance factors, NSAF) se calculó por el número de conteos espectrales (SpC) identificando una proteína, dividido entre la longitud de la proteína y dividido por la suma de conteo espectral (SpC) para todas las proteínas en el experimento (Zybailov *et al*., 2006). El análisis de ontología para el huésped se llevó a cabo usando el algoritmo de PantherGO (Thomas, *et al*., 2003). En el caso de las proteínas de *Taenia* se anotaron y analizaron para ontología usando Blast2GO (Götz, *et al*., 2008).

VI. **Resultados**

VI.1 Producción de las proteínas marcadas

VI.1.1 Estandarización de la producción y marcaje de IgG

Se cultivaron varios hibridomas murinos productores de IgG. Algunos de ellos habían sido parcialmente caracterizados; se sabía únicamente que reconocían proteínas no-identificadas de *T. solium*; otro hibridoma se sabía que no reconocía proteínas del cisticerco (Tabla 1).

Tabla 1.- Lista de hibridomas murinos productores de IgG. Información de los hibridomas disponible al inicio del presente estudio.

Nombre	Características
ET-150*	Hibridoma anti-extracto crudo de cisticercos de *T. solium*
C10*	Hibridoma anti-productos de excreción-secreción del cisticerco de *T. solium* (se desconoce su reconocimiento por western-blot)
ASC*	Hibridoma anti-productos de excreción-secreción del cisticerco de *T. solium* (reconoce una glicoproteína de ~10 kDa).
F6*	Hibridoma anti-extracto crudo *T. solium*, ensayos de inmunofluorescencia en cortes de cisticercos, mostraron reconocimiento del canal espiral (reconoce una banda de 200 kDa); muestra reacción cruzada con *T. saginata*
NR **	Hibridoma dirigido a un producto no relacionado con la cisticercosis

Donados por los Dres. *Yolanda Medina y **Sullivan López.

Para evaluar el reconocimiento de cada uno de los hibridomas se realizó western blot contra extractos totales de cisticerco de *T. solium* y *T. crassiceps.* Asimismo, se cuantificó la producción de IgG de cada uno, con el fin de determinar cuál permitía obtener una mayor cantidad. Por medio de un ELISA se observó que el hibridoma no relacionado (α-NR) era el que producía mayor cantidad (Fig.6); además, proliferaba *in vitro* más rápido que el resto.

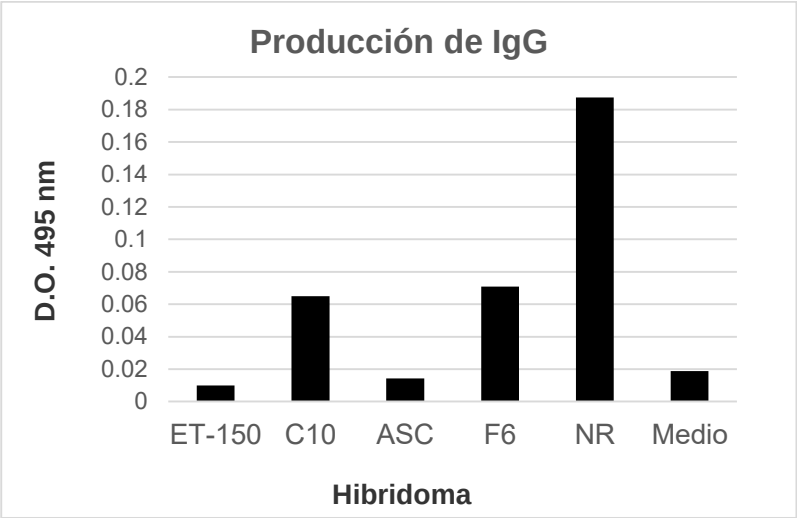


Figura 6.- ELISA directo para la evaluación de la producción de IgG por parte de los hibridomas. La placa fue sensibilizada con el sobrenadante de cada hibridoma, después fue incubada con anti-IgG de ratón-peroxidasa; el reconocimiento fue revelado con O-fenilendiamina como sustrato y se determinó la absorbancia a 495 nm.

Para confirmar la información acerca de la especificidad, provista para cada uno de los hibridomas se realizó un western blot contra extractos totales de cisticercos de *T. solium* y *T. crassiceps*, usando como anticuerpo primario el sobrenadante de cada uno de los hibridomas y como secundario un anti-IgG murina. Como se observa en la figura 7, el hibridoma NR, no reconoció algún antígeno en el extracto de cisticercos de *T. solium* ni de *T. crassiceps*, tal como se esperaba. En el caso del hibridoma ET-150 se reconocieron bandas de alto peso molecular (>150 kDa) en ambos extractos de cisticercos. No se observó reconocimiento alguno por los otros monoclonales; además, su crecimiento resultó lento en cultivo. Tomando en cuenta los resultados obtenidos, se decidió usar el hibridoma NR por su alta capacidad proliferativa y mayor producción de IgG comparado con los demás hibridomas. El hecho de que el hibridoma no reconociera los extracto no afectaba nuestros propósitos, pues ya estaba reportado que la incorporación de IgG durante el cultivo de los cisticercos no es influida por la especificidad de la inmunoglobulina (Ambrosio *et al*., 1994). Se determinó que el crecimiento y la producción de IgG del hibridoma NR alcanzaban condiciones óptimas en 14 días de cultivo (Fig. 8).

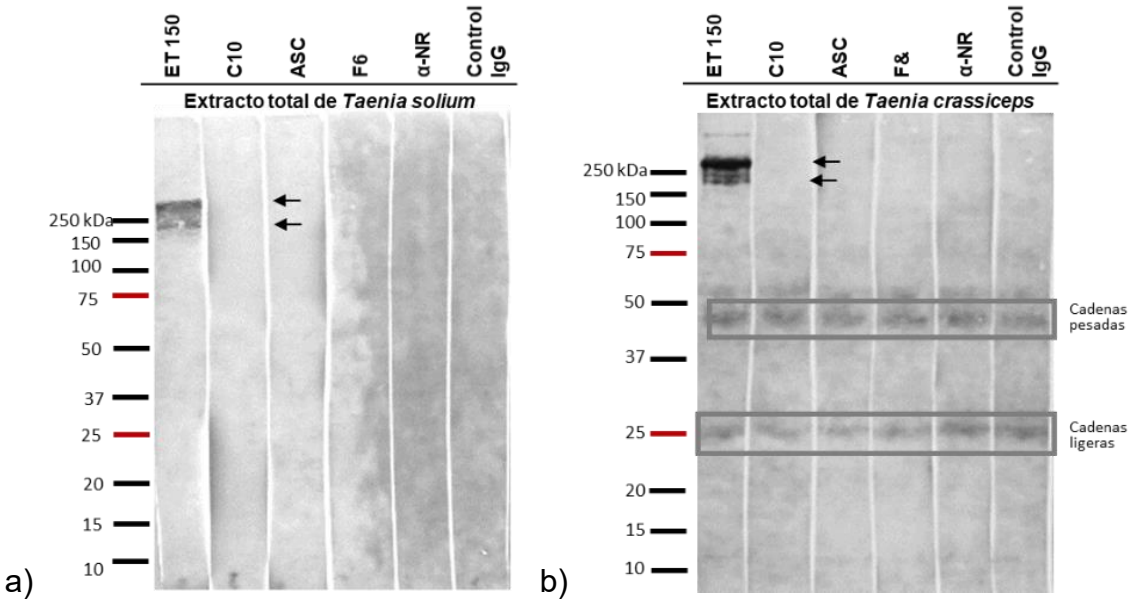


Figura 7. Western blot de los sobrenadantes de hibridomas reaccionados contra extractos totales de cisticercos de a) *T. solium* o b) *T. crassiceps*. Las flechas señalan las bandas reconocidas por el hibridoma ET-150. También se indica el reconocimiento de las cadenas ligeras y pesadas de las IgG debidas al segundo anticuerpo.

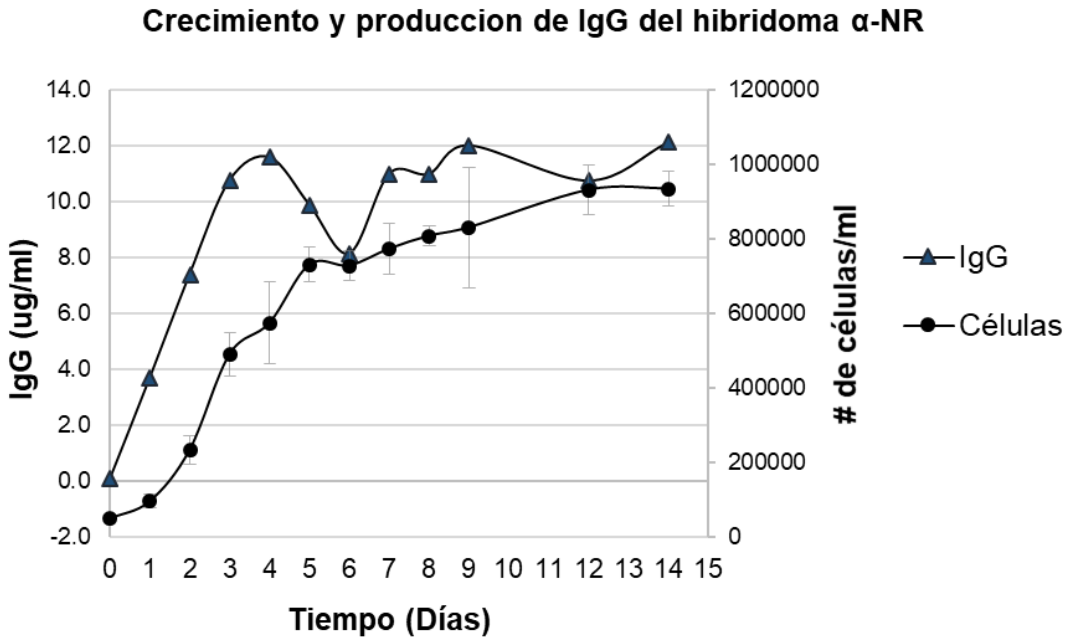
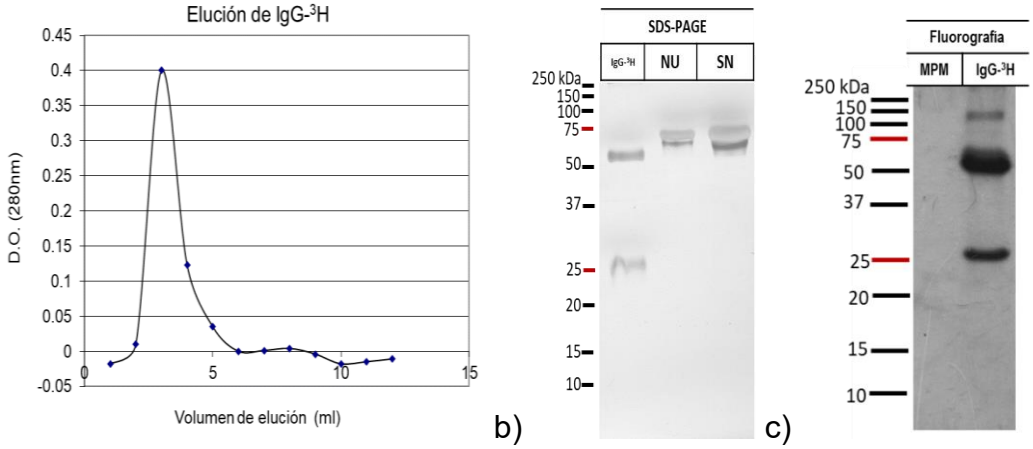


Figura 8. Crecimiento y producción de IgG del hibridoma NR.

Se procedió a realizar el cultivo del hibridoma NR adicionando marca radiactiva (Leu-^{3}H); después de 14 días de cultivo se separó el sobrenadante de las células y se purificó la fracción de IgG, a través de una columna acoplada a proteína G; obteniéndose un solo pico de elución. Se confirmó la pureza de la fracción IgG, unida a la proteína G, a través de SDS-PAGE (Fig. 9 a, b). Asimismo, para evaluar la incorporación de Leu-^{3}H en la inmunoglobulina, se sometió el gel de poliacrilamida a tratamiento para fluorografía para detectar el ^{3}H incorporado por auto-radiografía. Se logró detectar las bandas correspondientes a las cadenas pesadas (50 kDa) y ligeras (25 kDa) de las IgG, además de una banda de 150 kDa con menor intensidad, que posiblemente correspondía a la IgG completa (Fig. 9 c).



a) b) c)

Figura 9. Purificación y determinación del marcaje isotópico de la IgG producida por el hibridoma NR. a) Perfil de elución del sobrenadante del hibridoma NR a través de una columna de proteína G; b) Análisis de las fracciones obtenidas de la cromatografía en SDS-PAGE; c) Detección de la radiactividad en las IgG-^{3}H por medio de fluorografía. NU= fracción no unida a la columna de proteína G; SN= sobrenadante del cultivo del hibridoma NR; MPM= Marcador de peso molecular.

VI.1.2 Producción y purificación de proteína GFP marcada metabólicamente

Se cultivó *in vitro* una cepa de células BL21 D3 (*E. coli*), recombinante para GFP, que posee un promotor constitutivo que alcanza su máxima expresión a las 24 h (Rodríguez-Mejía *et al*., 2017). Al final del cultivo, se purificó la proteína por afinidad a metales, a partir de un extracto total de bacterias, utilizando una columna acoplada

a níquel. Como se muestra en la figura 10, se observó el mayor enriquecimiento de la GFP a las 24 h. Se confirmó la pureza de la GFP, unida a la columna de sefarosa acoplada a níquel, a través de SDS-PAGE (Fig. 10b). Asimismo, para evaluar la incorporación de Leu-^{3}H en la GFP, se sometió el gel de poliacrilamida a tratamiento para fluorografía para detectar el ^{3}H incorporado por auto-radiografía. Se logró detectar una banda marcada de 27 kDa, correspondiente a la GFP (Fig. 10c).

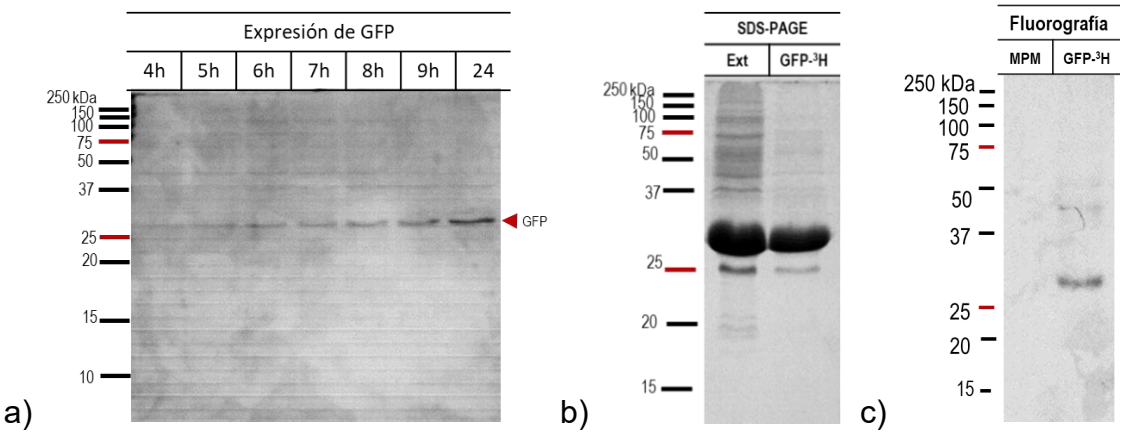
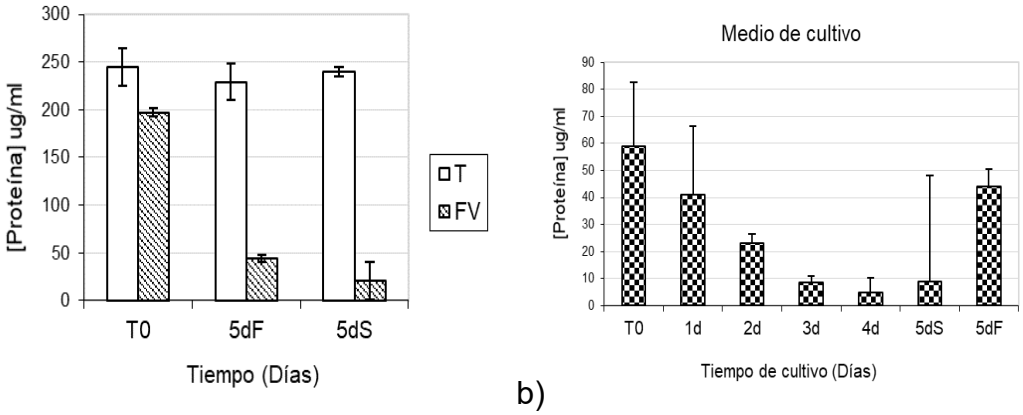


Figura 10. Expresión *in vitro* de una cepa de células recombinantes y purificación de GFP-^{3}H. a) Se colectaron muestras del cultivo *in vitro* de una cepa de células BL21 D3 (*E. coli*), recombinante para GFP a 4, 5, 6, 7, 8, 9 y 24 h. Cada muestra se ajustó a una D.O de 0.1 evaluada a 600 nm y se corrieron en un SDS-PAGE. b) Análisis de las fracciones obtenidas de la cromatografía de afinidad a níquel en SDS-PAGE; c) Detección de la radiactividad en la GFP-^{3}H por medio de fluorografía. Ext = Extracto bacteriano total no unido a la columna de níquel; GFP-^{3}H = fracción unida a la columna de níquel; MPM= Marcador de peso molecular.

V.2 Estandarización del cultivo de cisticercos en presencia de IgG-^{3}H o GFP-^{3}H

Tomando en consideración los estudios *in vitro* reportados en la literatura (Ambrosio *et al*., 1994, Aldridge *et al*., 2006), se decidió utilizar 20 cisticercos de *T. crassiceps* en cada pozo de cultivo. Los cisticercos fueron seleccionados por su tamaño homogéneo, diámetro (5-8 mm) y por no mostrar gemas en la vesícula principal. A partir de varios ensayos se estableció la necesidad de realizar un pre-cultivo durante 3 días, con cambios de medio (MMC) cada 24 h, para disminuir la cantidad de

proteínas del huésped en el tejido y el FV de los cisticercos (Fig. 11). Durante el pre-cultivo, los cisticercos mantuvieron su motilidad normal. También a través de múltiples ensayos, se determinó que el tiempo óptimo de cultivo de los grupos de cisticercos, en presencia de 500 µg de IgG-^{3}H, era de 9 días en medio MMC suplementado con albúmina al 2%. En este tiempo de cultivo se alcanzó una incorporación suficiente de marca para realizar los análisis subsecuentes; además, los cisticercos mantenían su viabilidad. Se colectaron muestras de tejido, FV y medio de cultivo para evaluar la presencia de IgG-^{3}H.



a) b)

Figura 11. Análisis de la liberación de IgG por parte de los cisticercos de *T. crassiceps* en durante el cultivo en MMC. El cultivo se realizó durante 5 días con cambios de medio cada 24 h. a) Densitometrías de geles de SDS-PAGE para determinar la cantidad de proteína presente en extractos de Tejido (T) y FV. b) Densitometría de geles de SDS-PAGE para determinar la cantidad de proteína liberada al medio de cultivo. T0 = tiempo cero; 5dF = grupo sin cambios de medio; 5dS = grupo con cambios de medio.

Una vez establecidas las condiciones del pre-cultivo para disminuir la cantidad de proteínas del huésped en el tejido y el FV de los cisticercos, se determinó usar el mismo MMC suplementado sólo con albúmina para establecer condiciones que favorecieran la incorporación y uso de la proteína marcada (IgG-^{3}H o GFP-^{3}H), disminuyendo el estrés osmótico de los cisticercos durante el cultivo *in vitro*.

VI.3 Cultivo de cisticercos de *T. crassiceps* con IgG-^{3}H o GFP-^{3}H

Los cultivos de los grupos de cisticercos en presencia de cada una de las proteínas marcadas (IgG-^{3}H o GFP-^{3}H), se llevó a cabo durante 9 días y se obtuvieron extractos de Tejido (T), Fluido Vesicular (FV) y Medio de Cultivo (MC), que se analizaron mediante SDS-PAGE, incluyendo la determinación de radioactividad por fluorografía, para detectar la marca radiactiva de las proteínas. En el caso de los cisticercos cultivados en presencia de IgG-^{3}H, los geles mostraron claramente las bandas correspondientes a las cadenas pesada (50 kDa) y ligera (25 kDa) de las IgG en el extracto de tejido. En contraste, ninguna de las dos bandas se observó en el FV y MC (Fig. 12). También se observaron bandas tenues adicionales, menores a 50 kDa, lo que sugiere una degradación modesta de la IgG-^{3}H. En el caso de los cisticercos cultivados con GFP-^{3}H, se observó la banda correspondiente a la GFP marcada (27 kDa) y varias bandas extra que están presentes también en el carril de la GFP purificada. Estos resultados son consistentes con una actividad proteolítica muy limitada en los tejidos del cisticerco. Es importante hacer notar que los extractos proteicos del grupo de cisticercos cultivados solo con Leu-^{3}H mostraron numerosas bandas radiactivas entre 20 kDa y más de 250 kDa, lo que indica que los cisticercos mantienen una alta actividad biosintética bajo las condiciones de cultivo usadas en los experimentos (Fig. 12). Es decir que, si bien los cisticercos incorporan ambas proteínas durante el cultivo, los resultados indican que no las aprovechan como fuentes de aminoácidos libres. En contraste con la ausencia de IgG y GFP en los extractos de FV y MC, el SDS-PAGE de los mismos extractos mostraron una abundante presencia de albumina sérica bovina en el FV (Fig. 12), indicando que esta proteína fue incorporada durante el cultivo, lo cual apoya la hipótesis el concepto de que la BSA juega un papel de regulador osmótico para el cisticerco (Aldridge *et al*., 2006).

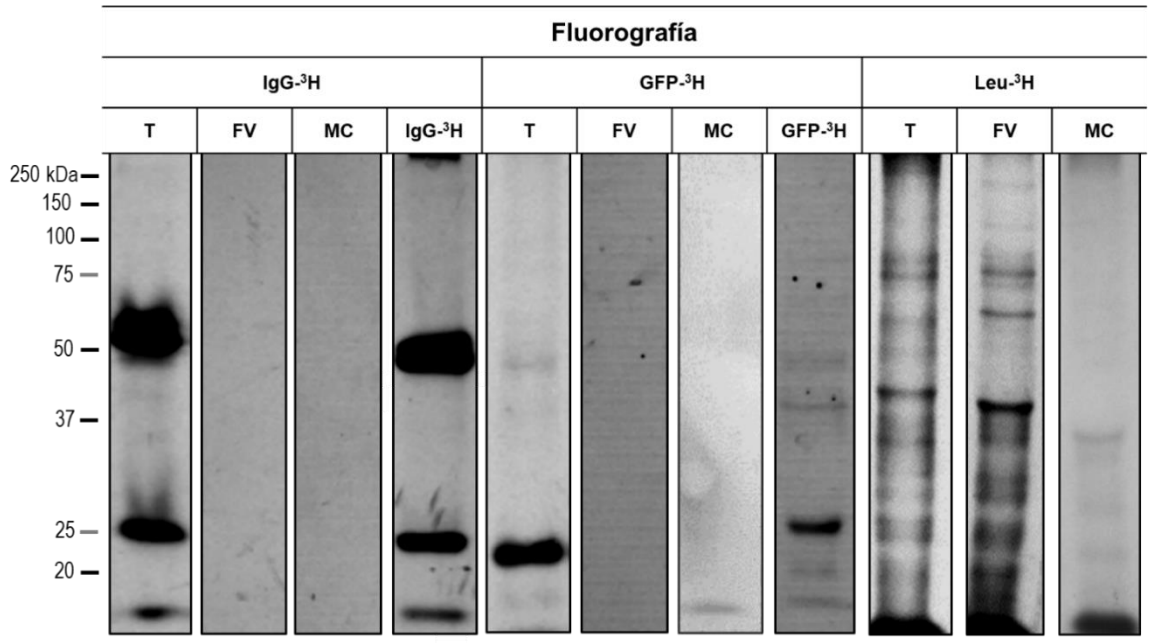


Figura 12. Detección de radiactividad en los extractos proteicos de cisticercos de *T. crassiceps* cultivados *in vitro* en presencia de IgG-^{3}H o GFP-^{3}H. Un grupo de cisticercos fue cultivado con 30 µCi of Leu-^{3}H en MMC suplementado con 2% de BSA, durante 9 días. Al final del cultivo, se obtuvieron los extractos de tejido (T), fluido vesicular (FV) y el medio de cultivo (MC) y se resolvieron por SDS-PAGE; los geles al 10% fueron tratados para fluorografía y expuestos ante una placa radiográfica.

VI.4 Ensayo funcional de las IgG del huésped purificadas de un extracto crudo de *T. crassiceps*

En vista de la escasa degradación de la IgG del huésped incorporada por los cisticercos en los ensayos anteriores y con el fin de evaluar si las IgG presentes en los tejidos del cisticerco mantenían su actividad de anticuerpo, como se había reportado para *T. solium* (Navarrete-Perea, *et al*., 2017), se purificaron las IgG de ratón a partir de un extracto salino de cisticercos de *T. crassiceps* por cromatografía de afinidad con proteína G. La elución de la columna mostró un pico único de IgG murina, cuya pureza fue evaluada por SDS-PAGE y western-blot, usando un anticuerpo anti-IgG de ratón. Los resultados de la SDS-PAGE mostraron dos bandas de 50 y 25 kDa, que fueron identificadas como cadenas pesadas y ligeras

de IgG mediante western-blot con los anticuerpos específicos dirigidos a IgG de ratón (Fig. 13). Cabe mencionar que la fracción eluída de la columna de proteína G mostraba bandas adicionales a la IgG, algunas reconocidas por anticuerpo policlonal contra IgG y otras no.

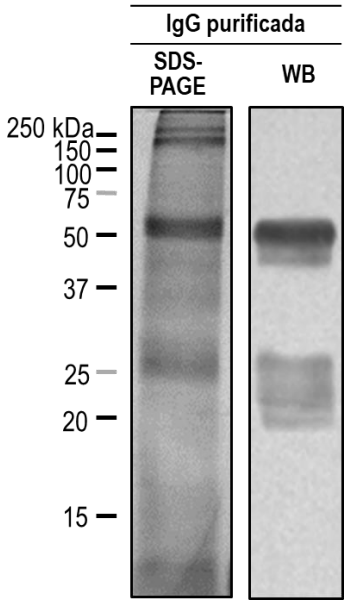


Figura 13. Aislamiento e identificación de las IgG del huésped incorporadas por los cisticercos de *T. crassiceps*. La IgG del huésped presente en los extractos proteicos de cisticercos de *T. crassiceps,* fue aislada por medio de cromatografía de afinidad usando una columna de proteína G. El carril izquierdo muestra el SDS-PAGE de la fracción eluída, mientras que el carril derecho muestra el reconocimiento de los anticuerpos anti- IgG murina mediante un ensayo de western-blot.

Para evaluar la actividad de anticuerpo de la IgG murina purificada de los extractos crudos de cisticercos de *T. crassiceps*, se realizó un western-blot de T y FV que fueron puestas a reaccionar con la fracción de IgG murina purificada. Los resultados mostraron que la IgG murina purificada mantiene actividad de anticuerpo en contra de una variedad de antígenos parasitarios dentro de un rango de 25-100 kDa para T y de 12- >250 kDa para el FV (Fig.14).

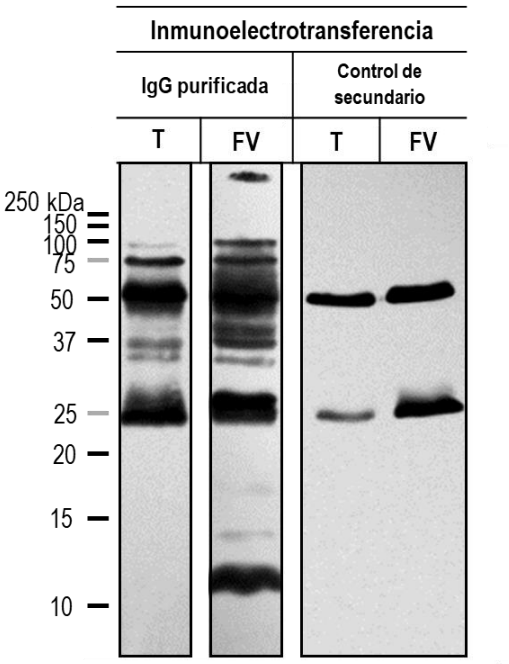


Figura 14. Reconocimiento antigénico de la IgG murina purificada de los extractos crudos de cisticercos de *T. crassiceps*. Muestras de T y FV fueron separadas por SDS-PAGE y transferidas a una membrana de nitrocelulosa; ambas tiras fueron puestas a reaccionar con la fracción de IgG murina purificada. Como anticuerpo secundario se utilizó un anticuerpo anti-IgG de ratón preparado en conejo, acoplado a peroxidasa. En las dos tiras del lado derecho, se muestran los controles reaccionados únicamente con el anticuerpo secundario.

VI.5 Análisis proteico de los corpúsculos calcáreos de *T. solium* por espectrometría de masas

La gran abundancia de inmunoglobulinas en los tejidos del cisticerco, contrasta con su escasa degradación como fuente de aminoácidos, demostrada arriba. En consecuencia, se llevó a cabo un análisis de algunos destinos alternativos para las proteínas del huésped, incluidas las inmunoglobulinas. En este sentido, es bien sabido que los CC de los taénidos son concreciones minerales de sales de fósforo y calcio, depositadas sobre una matriz orgánica, compuesta de proteínas, polisacáridos y lípidos. La formación de los CC en estos organismos parásitos se ha propuesto como un mecanismo de aislamiento y acumulación de metabolitos

tóxicos y otros materiales de desecho (Vargas-Parada*, et al*., 1999). Es decir, que los CC podrían constituir un destino para las proteínas del huésped internalizadas por el cisticerco. Los CC se localizan principalmente en el escólex de los cisticercos. En consecuencia, fue necesaria una adaptación del proyecto. Puesto que la primera parte de este trabajo se realizó usando la cepa ORF de *T. crassiceps* que carece de escólex, y, por lo tanto, la disponibilidad de CC en estos parásitos es escasa, se decidió utilizar cisticercos de *T. solium*, por tener escólex, y para conseguir suficiente material de CC para el análisis proteómico.

Lo anterior implica el supuesto de que el rol fisiológico de los CC es similar en céstodos estrechamente relacionados (*T. solium* vs. *T. crassiceps*). Como antecedente de este análisis proteómico, vale la pena mencionar que se sabía muy poco acerca la composición proteica de la matriz de los CC, incluyendo un reporte de nuestro propio grupo hace más de una década (Zurabian, *et al.*, 2005; Chun-Seob, *et al.,* 2017). El análisis de la matriz proteica de los CC que se realizó en el presente proyecto doctoral, constituía un acercamiento mucho más amplio a través de las modernas herramientas proteómicas. Además, algunos aspectos funcionales de las proteínas del huésped fueron explorados a través de ensayos específicos, por ejemplo, se realizaron ensayos de western blot usando anticuerpos específicos dirigidos contra proteínas del huésped (IgG y albúmina), para complementar este análisis.

Los extractos de CC se obtuvieron a partir de dos grupos de cisticercos de *T. solium* y se procesaron para espectrometría de masas. Se identificaron y cuantificaron de 636 y 760 proteínas en las dos muestras y se procedió a la identificación de las proteínas obtenidas. Un hallazgo notable fue que el 70% de las proteínas identificadas en ambas muestras de CC (412 y 506) correspondían a proteínas de huésped (Fig. 5), mientras que sólo 224 y 251 de las proteínas pertenecían a *T. solium*. La variabilidad de las proteínas identificadas en ambas muestras fue considerablemente alta, solo se compartían 111 proteínas (73 del huésped y 38 del parásito), esto indica que 75-80 % de las proteínas identificadas variaron entre muestras. Ello sugiere que la regulación de las proteínas que se incorporan a los

CC es pobre. Sin embargo, al hacer un análisis ontológico de las proteínas tanto del huésped como de *Taenia*, estas resultaron en patrones de función similares (Fig. 15, Fig. 16).

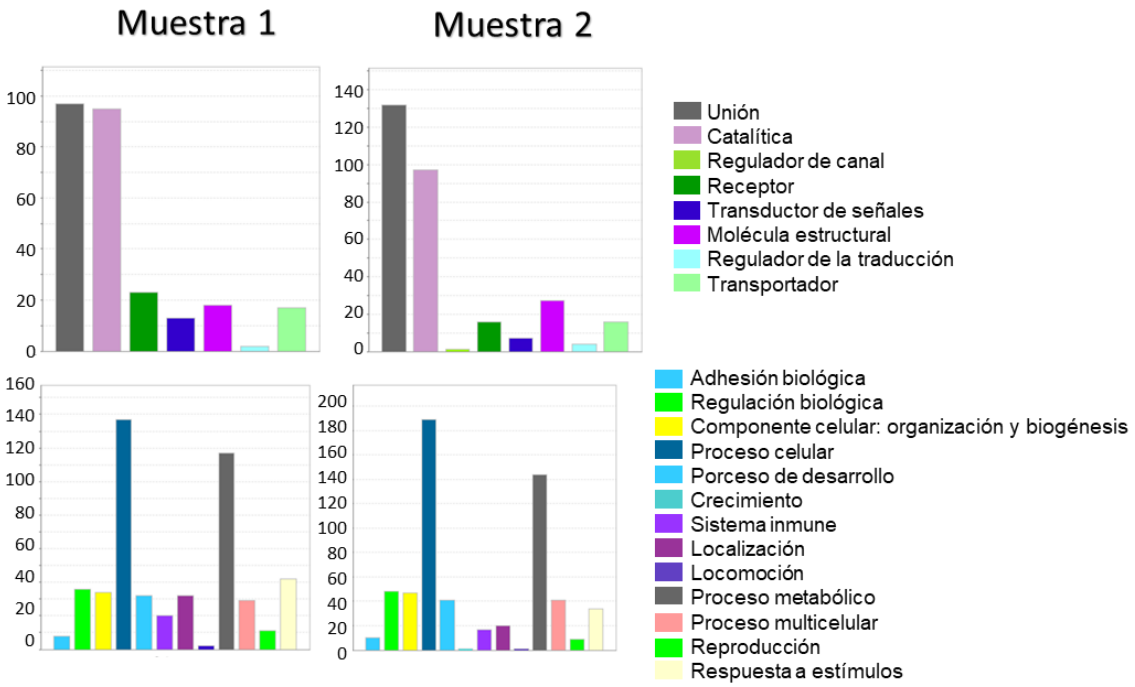


Figura 15.- Perfil funcional de las proteínas de *T. solium* identificadas por espectrometría de masas en la matriz proteica de los corpúsculos calcáreos. El análisis de ontología génica de las proteínas de huésped se realizó considerando la función molecular (fila superior) y proceso biológico (fila inferior) usando la base de datos de PANTHERGO.

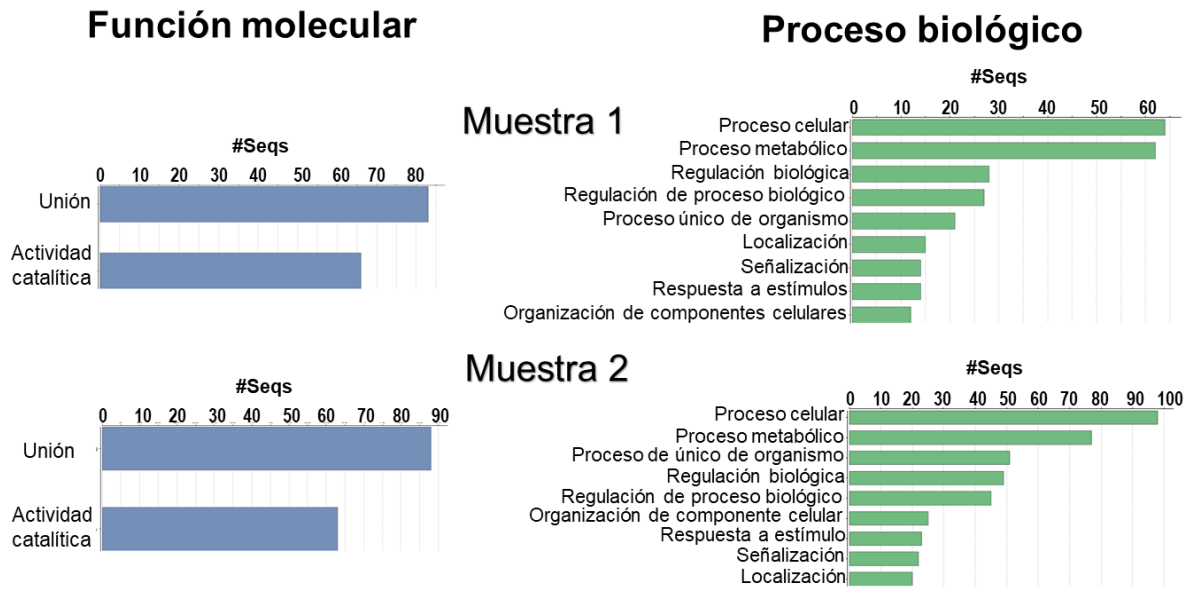


Figura 16. Perfil funcional de las proteínas de *T. solium* identificadas por espectrometría de masas en la matriz proteica de los corpúsculos calcáreos. El análisis de ontología génica de las proteínas de huésped se realizó considerando la función molecular (gráficos de la izquierda) y proceso biológico (gráficos de la derecha) usando la base de datos de Blast2GO (NCBI).

Otro enfoque para identificar proteínas del huésped en la matriz de los CC mediante western-blot usando anticuerpos contra albúmina e IgG porcinas ya que constituyen las proteínas del huésped más abundantes en fluido y tejidos del cisticerco (Fig. 17). Los resultados mostraron que ambas proteínas están presentes en la matriz de forma intacta, ya que ambas proteínas se detectaron en el corrimiento electroforético correspondientes a su peso molecular total de 67 kDa, en el caso de la albúmina, y de 50 kDa, en el caso de las IgG; que corresponden a las cadenas pesadas. La cadena ligera de las inmunoglobulinas no se detectó (Fig.17).

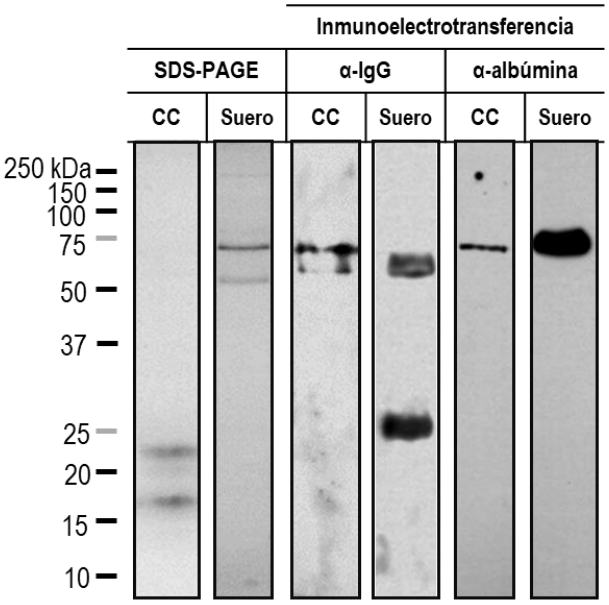


Figura 17. Identificación de IgG y albúmina en la matriz proteica de los corpúsculos calcáreos (CC) del cisticerco de *T. solium*. Se obtuvo la fracción proteica de la matriz orgánica de corpúsculos calcáreos para realizar ensayos de western-blot. En los dos primeros carriles se muestra el gel de SDS-PAGE de la fracción proteica de la matriz orgánica de los CC y de un suero de cerdo, teñidos con azul de Coomassie. En los dos carriles siguientes se muestran los western-blot de la fracción proteica de los CC y del suero de cerdo, puestas a reaccionar con un anticuerpo anti-IgG. Finalmente, en los dos últimos carriles se muestran los western-blot de ambas fracciones (CC y suero de cerdo) con un anticuerpo anti-albúmina.

VII. Discusión

El propósito inicial de este trabajo fue aclarar el destino de las proteínas del huésped que son incorporadas en grandes cantidades hacia los tejidos del cisticerco. Se puso especial énfasis en el análisis del procesamiento de las inmunoglobulinas, ya que junto con la albúmina sérica constituyen las proteínas del huésped más abundantes en el fluido y en el tejido del cisticerco. Varios estudios han reportado la presencia de proteínas del huésped dentro del FV, o el tejido de la forma larvaria de varios céstodos, como *Echinococcus granulosus (*Kagan *et al.,* 1963, Chordi *et al*., 1965; Coltorti *et al*., 1975; Hustead *et al*., 1977b; Shapiro Z, 1992; Zeghir-Bouteldja *et al*., 2017); *T. crassiceps* (Hustead *et al*., 1977a, 1977b; Ambrosio *et al*., 1994; Aldridge *et al*.,2006), *T. taeniaformis* (Hustead *et al*., 1977a, 1977b, Hayunga *et al*., 1989;), *T. saginata* (Machnicka *et al*., 1986) y *T. solium* (Navarrete-Perea *et al*. 2014, 2017). Sin embargo, el uso o destino final de las proteínas del huésped permanece poco estudiado; en el caso de la albúmina se ha propuesto que participa en la regulación de la presión osmótica en el fluido vesicular o como recolector de especies reactivas de oxígeno en *T. crassiceps* (Aldrige *et al*., 2006), mientras que la hemoglobina, la haptoglobina y la ferritina se ha propuesto que desempeñan un rol para el parásito similar al que desempeñan en los tejidos del huésped, como chaperonas de hierro (Navarrete-Perea *et al*. 2016).

Una hipótesis inicial planteada en este proyecto doctoral fue que las proteínas del huésped eran internalizadas, para ser degradadas como una fuente de aminoácidos, contribuyendo a la síntesis *de novo* de proteínas del cisticerco. Para rastrear los productos de degradación de las proteínas internalizadas, se realizaron cultivos de cisticercos de *T. crassiceps* en presencia de IgG de ratón y GFP marcadas metabólicamente con leucina tritiada (Leu-^{3}H). La etiqueta radiactiva nos permitió rastrear la incorporación de este aminoácido en las proteínas del cisticerco ya que es uno de los aminoácidos más abundante en todos los organismos y el cisticerco de *Taenia solium* es incapaz de sintetizarlo (Tsai *et al*., 2013). Por lo tanto, si las proteínas marcadas (IgG o GFP) eran degradadas hasta aminoácidos libres, la Leu-^{3}H sería incorporada en las proteínas del cisticerco sintetizadas *de novo*. Para favorecer el uso de los aminoácidos a partir de las proteínas marcadas, se

diseñó un medio al que se denominó Medio Mínimo para Cisticercos, el cual carece de aminoácidos libres y está sólo suplementado con albúmina, esto para evitar el estrés osmótico de los cisticercos.

Contrario a lo esperado de acuerdo a la literatura, ninguna de las dos proteínas mostró indicios de ser degradada significativamente, y, por lo tanto, solo una pequeña cantidad de leucina-^{3}H, proveniente de la IgG murina y de la GFP marcadas metabólicamente, fue detectada. A pesar de que se logró detectar incorporación de leucina-^{3}H en proteínas sintetizadas *de novo* por los cisticercos en cultivo, la cantidad fue insignificante. Por otro lado, solo una parte mínima de la proteína marcada (IgG o GFP) que se les había agregado a los cisticercos en cultivo, parecía estar degradada como se observó en los ensayos de fluorografía (Fig. 12). Puesto que se incorporaron cisticercos a los que se les adicionó leucina-^{3}H, bajo las mismas condiciones de cultivo, pudimos comprobar que los cisticercos mantenían una gran actividad biosintética (evidenciada por las múltiples bandas detectadas por fluorografía) en las condiciones de cultivo. Por lo tanto, fuimos capaces de concluir que la ausencia de proteínas sintetizadas *de novo* en los cultivos con IgG-^{3}H y GFP-^{3}H no se debía a una baja actividad biosintética debida a las condiciones del cultivo de los cisticercos (Fig. 12).

El que no haya proteínas sintetizadas con leucina-^{3}H a partir de la IgG y GFP marcadas, aunado a la baja degradación detectada de las mismas, sugiere que las proteínas del huésped no son una fuente principal de aminoácidos. En su lugar, los cisticercos parecen preferir absorber aminoácidos libres del ambiente, los cuales están presentes en la circulación sanguínea y en otros fluidos del huésped. Se ha reportado la presencia de aminoácidos libres en el fluido hidatídico y no se descarta lo mismo para el del cisticerco de *Taenia* spp (Celik et al., 2001, Juyi et al., 2013).

Nuestro grupo reportó hace tiempo la incorporación inespecífica de inmunoglobulinas por los cisticercos de *T. crassiceps* (Ambrosio et al., 1994). Recientemente mostramos que las IgG incorporadas por el cisticerco de *T. solium* mantienen su actividad de anticuerpo (Navarrete-Perea *et al.*, 2017). En este estudio, se confirmó el último hallazgo también para las IgG purificadas de los

tejidos de los cisticercos de *T. crassiceps*, que mostraron reconocimiento de varios antígenos parasitarios en el rango de 12 a 250 kDa, en ensayos de western-blot. De forma interesante se observaron más bandas reconocidas por los anticuerpos en el FV que en tejido, lo que sugiere que los complejos inmunes incorporados son de anticuerpos que reconocen en mayor medida antígenos parasitarios presentes en FV.

Los CC son concreciones minerales ampliamente distribuidas en los céstodos; se ha propuesto que podrían estar involucradas en la osmoregulación, neutralización de productos ácidos, o simplemente como reservorio de material inorgánico (Von Brand et al., 1965; Grau *et al*. 1965). En el caso de *T. solium*, los CC se forman en la luz de los canales proto-nefridiales (Vargas-Parada et al., 1999 a, b). Es bien conocido que incorporan sales de Ca^{++}, Mg^{++}, Mn^{++} y Cu^{++}, pero principalmente de fosfato de calcio, que son depositadas sobre una matriz orgánica compleja compuesta de proteínas, carbohidratos y lípidos (von Brand et al., 1960).

La composición de la matriz orgánica de los CC ha sido poco estudiada. Un reporte de nuestro grupo describe la presencia de proteínas antigénicas intactas, así como una proteína de unión a calcio (Zurabian *et al*., 2005). En el presente proyecto doctoral exploramos la composición de los CC a través de espectrometría de masas, lo que permitió identificar y cuantificar más de 600 proteínas en cada muestra. Un hallazgo sorprendente derivado del análisis de los datos fue que aproximadamente el 70% de las proteínas identificadas correspondían a proteínas del huésped, lo cual plantea el asunto de la composición de la matriz de los CC a un nivel diferente, como un posible mecanismo para el almacenamiento o la disposición de las proteínas del huésped incorporadas. Es decir, que los CC parecen constituir “basureros” o “depósitos” de proteínas no degradadas; entre ellas las proteínas del huésped.

Como un acercamiento adicional para explorar la composición proteica de los CC, realizamos ensayos de western-blot para la identificación de proteínas del huésped, en especial albúmina e IgG, las cuales fueron reconocidas en el extracto de CC. De forma interesante, nuestros resultados mostraron que ambas proteínas tenían el tamaño molecular esperado, lo que sugiere que permanecieron intactas en la matriz

de los CC. Lo cual sugiere que también otras proteínas del huésped que son depositadas en los CC, ya sea de forma íntegra o parcialmente degradada, podrían ser depositadas en los CC. Los datos de este estudio aunados a los hallazgos previos acerca del alto contenido de proteínas del huésped en los tejidos del cisticerco, casi un 20%, sugieren que el cisticerco orienta la incorporación de proteínas del huésped hacia los CC (Navarrete-Perea *et al*., 2014). La abundancia de las proteínas del huésped en los CC sugiere un papel fisiológico, por ejemplo para nuclear el depósito de sales, o como un depósito de estas proteínas. Sin embargo, se trata de un primer hallazgo que merece ser investigado ya que los mecanismos implicados aún se desconocen; solo existe un estudio que sugiere la posible participación de las fasciclinas como "puente" de interacción entre los CC y otras proteínas (Chun-Seob *et al*. 2017).

En nuestro grupo de trabajo hemos descrito que las proteínas del huésped incorporadas podrían tener una función para el cisticerco similar a la que tienen en los tejidos del huésped (Navarrete-Perea *et al.*, 2016, 2017) o como se describe en este trabajo, estas proteínas podrían desempeñar un rol como componentes de la matriz proteica en el proceso de deposición de minerales en los CC.

Las proteínas identificadas en la matriz de los CC fueron muy variables entre ambas muestras. Las diferencias podrían deberse a que cada muestra fue obtenida de muchos cisticercos de un solo cerdo y hasta donde sabemos, la variabilidad entre individuos en el proceso de formación de los CC no ha sido explorada a fondo. Otras posibilidades para explicar la alta variabilidad en la composición proteica incluyen factores como el tiempo de infección, la etapa en la que se encontraban los parásitos, el estado inmune del huésped, entre otras (Von Brand *et al*., 1960; Fleury *et al.,* 2016).

Nuestro hallazgo de la diversidad y cantidad de proteínas del huésped internalizadas por los cisticercos de *T. crassiceps* y *T. solium* no tiene antecedente en la literatura. Se trata de un ejemplo inédito de "intimidad" molecular huésped-parásito que, evidencia una interacción altamente compleja y una complementación fisiológica y funcional. Es concebible que los cisticercos captan y retienen proteínas

intactas del huésped y aprovechan su función original para sus propias necesidades, hasta que simplemente las desechan depositándoles en los CC o almacenándolas en ellos. Finalmente, nuestros hallazgos también confirman y expanden las funciones hasta ahora asignadas para los CC de los metacéstodos taénidos; parece claro que desempeñan un papel fisiológico mucho más importante que el propuesto anteriormente como almacén de materiales de desecho, incluyendo proteínas intactas del huésped.

VIII. Conclusiones

1. El uso de las inmunoglobulinas G del huésped internalizada por los cisticercos de *Taenia crassiceps*, como una fuente de aminoácidos es mínimo; la degradación de IgG al interior de los tejidos del cisticerco es extremadamente limitada.
2. El destino principal de las proteínas del huésped internalizadas por el cisticerco de *Taenia solium,* son los corpúsculos calcáreos; actúan como componente de la matriz orgánica sobre la cual se depositan las sales de calcio.
3. Las proteínas del huésped llegan a representar hasta un 70 % del contenido proteico de los corpúsculos calcáreos. Se identificaron más de 700 especies de proteínas. Este hallazgo demuestra una extraordinaria complejidad proteica de la matriz orgánica de los corpúsculos calcáreos.
4. Nuestros hallazgos confirman y expanden las funciones hasta ahora asignadas a los corpúsculos calcáreos; desempeñan un papel de almacén de materiales de desecho, incluyendo proteínas del huésped.

IX. Bibliografía

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X. Anexos

Publicaciones

1)

Flores-Bautista J, Navarrete-Perea J, Fragoso G, Flisser A, Soberón X, Laclette JP. Fate of uptaken host proteins in *Taenia solium* and *Taenia crassiceps* cysticerci. Biosci Rep. 2018 Jul 6;38(4). doi: 10.1042/BSR20180636.

2)

Navarrete-Perea J, Isasa M, Paulo JA, Corral-Corral R, **Flores-Bautista J**, Hernández-Téllez B, Bobes RJ, Fragoso G, Sciutto E, Soberón X, Gygi SP, Laclette JP. Quantitative multiplexed proteomics of *Taenia solium* cysts obtained from the skeletal muscle and central nervous system of pigs. PLoS Negl Trop Dis. 2017 Sep 25;11(9). doi: 10.1371/journal.pntd.0005962.

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Research Article

Fate of uptaken host proteins in *Taenia solium* and *Taenia crassiceps* cysticerci

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During the study of host–parasite relationships in taeniid parasite diseases, including cysticercosis and hydatidosis, reports have described the presence of host proteins in the cyst fluid and tissue of metacestodes. However, the fate or role of host elements inside the parasite remains barely explored. After the publication of genomes of four cestode species, it became clear that these organisms possess a limited biosynthetic capability. The initial goal of the present study was to determine if uptaken host proteins could be a source of essential amino acids for cysticerci. To track the utilization of uptaken proteins, we added metabolically labeled IgG-^{3}H and GFP-^{3}H to the culture medium of *Taenia crassiceps* cysticerci. Incorporation of labeled amino acid was evaluated by fluorography in cysticerci extracts. Our results showed that the use of uptaken proteins by cysticerci as a source of amino acids appeared negligible. Exploring alternative fates for the host proteins, proteomic analysis of the protein matrix in calcareous corpuscles was carried out. Since *T. crassiceps* does not contain calcareous corpuscles, proteomic analyses were performed in corpuscles of *Taenia solium* cysticerci. Our results demonstrated that host proteins represented approximately 70% of protein content in the calcareous corpuscles. The presence of the two major uptaken host proteins, namely albumin and IgG, was also demonstrated by Western blot in the matrix of corpuscles. Our findings strongly suggested that the uptake and disposal of host proteins involve calcareous corpuscles, expanding the physiological role of these mineral concretions to a far more important level than previously proposed.

Introduction

During the last decades, considerable advances for understanding host–parasite relationship in taeniid helminths have been achieved using a murine model of cysticercosis based on *Taenia crassiceps* [1]. This parasite has the advantage of its facility for maintenance under laboratory conditions, through intraperitoneal passage of cysticerci from infected to healthy mice [2,3]. The analysis of four tapeworm genomes has revealed highly simplified and host-dependent organisms [4]. Taeniids show a very limited biosynthetic metabolism, acquiring sugars, most amino acids (L, K, H, I, M, F, T, W, V, S, and P), nucleosides and fatty acids from the host, to produce its own macromolecules [4]. In contrast, these taeniids have great capability to uptake nutrients; cysticerci absorb and consume large quantities of glucose through transporters TGTP1 and TGTP2 and store the excess as glycogen [5]. A similar phenomenon occurs with the acquisition of fatty acids and cholesterol from the host environment [6,7]. Amino acid absorption in *T. crassiceps* was reported several decades ago, through the proposal of three mechanisms specific for neutral, basic, and acidic amino acids [8,9]. Analysis of the taeniid genomes also revealed the presence of coding genes for amino acid transporters [4].

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Cysticerci are larval forms possessing a fluid-filled vesicle; the presence of host proteins in the vesicular fluid (VF) of cysticerci is a well-known fact [10-15]. We have also reported that host proteins might represent 11–13% of the protein content in the vesicular fluid of swine cysticerci, with albumin and immunoglobulins being the most abundant proteins [16]. More recently, using high-throughput proteomics, we identified 891 proteins of host origin from a total of 4259 that were identified and quantified in a *Taenia solium* cysticerci whole protein extract [17]; thus, host proteins represent 20% of total parasite protein species. The biological role and fate of the uptaken host proteins have barely been studied. Uptake of host albumin has been proposed to be involved in the maintenance of osmotic pressure [14]. In the case of uptaken haptoglobin and hemoglobin, the parasite appears to take advantage of their normal function in the host for its own benefit, we have proposed that these and other host iron chaperons are used by cysticerci to fulfill its iron requirements [18]. Uptake of immunoglobulin has been proposed as a mechanism of immune evasion and even as a source of amino acids [19].

Since host proteins are abundant in cysticerci tissues, the aim of the present study was to elucidate their fate in the larval tissue, using *T. solium* and *T. crassiceps* cysticerci. Initially, we evaluated if uptaken metabolically radiolabeled host immunoglobulin G (IgG-leucine^{3}H) acted as a source of essential amino acids in *T. crassiceps* cysticerci. For this, we tracked the incorporation of one essential amino acid (leucine-^{3}H) as a building block for the synthesis of cysticerci own proteins. For comparison, we used another metabolically radiolabeled protein that is not related to the parasite, the green fluorescent protein (GFP-leucine-^{3}H). Our results showed that the use of uptaken proteins as a source of amino acids was remarkably low by cysticerci. Searching for an alternative fate for host proteins, we carried out proteomic analyzes of calcareous corpuscles (CC) in *T. solium*. CC are widely distributed in larval and adult forms of cestodes; these structures are mineral deposits of phosphate salts onto an organic matrix of proteins, polysaccharides and lipids, involved in gathering toxic metabolites and other materials into an inert structure [20,21]. Our results showed that host proteins constitute the main component, up to 70%, of the protein matrix. The presence of the two major uptaken host proteins, namely albumin and IgG, in the matrix of the corpuscles was also demonstrated by Western blot. Thus, a major fate for the uptaken host proteins seems to be its coalescence within these mineral concretions. Our results expand the physiological role of calcareous corpuscles and raise questions as to the design of cysticerci as biological devices.

Materials and methods

Biological specimens

For the *in vitro* protein uptake assays, *T. crassiceps* (ORF strain) cysticerci maintained through intraperitoneal passage in female mice BALB/cAnN strain were used [1]. Cysticerci were collected from the peritoneal cavity of infected mice after humanitarian killing and washed several times with sterile phosphate buffered saline, pH 7.4 (PBS) before *in vitro* culture. All procedures involving mice were carried out in accordance to the institutional guidelines for the care and use of laboratory animals (CICUAL permit No. ID199).

For the isolation of calcareous corpuscles, *T. solium* cysticerci were dissected from skeletal muscle of naturally infected pigs, after humanitarian killing, in accordance with institutional guidelines from the School of Veterinary Medicine and Zootechnia, UNAM. The inflammatory capsules surrounding cysticerci were removed and the parasites were washed several times with ice-cold PBS and frozen (without PBS) at −70°C until use.

Metabolic labeling of mouse IgG and recombinant GFP

A murine hybridoma producing monoclonal IgG antibodies (against an unrelated antigen) was grown in RPMI-1640 medium (Biowest) supplemented with 10% fetal bovine serum (FBS). A new culture of this hybridoma was initiated at a density of 1×10^{5} cells/ml in a medium added with leucine-^{3}H (Leu-^{3}H) at 5 μCi/ml (Perkin-Elmer) and maintained for 14 days. After centrifugation of hybridoma cells, the supernatant was collected and mixed 1:1 (v/v) with sodium phosphate buffer (20 mM), pH 7.4. Isolation of the metabolically labeled mouse IgG (IgG-^{3}H) was carried out by affinity chromatography through a column of protein G coupled to Sepharose 4B (Sigma-Aldrich Co). Western blot assays using crude extracts of *T. crassiceps* and *T. solium* cysticerci reacted against the supernatant of the hybridoma showed no recognition of any parasite's protein (not shown).

Metabolically labeled green fluorescent protein (GFP-^{3}H) was produced by growing a recombinant strain of *Escherichia coli* [22]. Bacteria were grown in Luria-Bertani medium containing 5 μCi/ml of Leu-^{3}H (Perkin-Elmer) for 24 h. Bacteria were harvested and suspended in lysis buffer of guanidine 5 M, Tris 10 mM pH 7.4 and complete protease inhibitors cocktail (Roche), sonicated 3 min (six passes of 30 s) within an ice bath and centrifuged for 30 min at 4000 ***g***. Isolation of the GFP was carried out by affinity to nickel, through a HiTrap IMAC FF column (GE Healthcare), according to the manufacturer's instructions.

The specific activity of the IgG-^{3}H and GFP-^{3}H was determined by dissolving aliquots of each one in Tritosol [23] and evaluating the amount of radioactivity in a liquid scintillation counter (Beckman Coulter LS6500). Radiolabeled proteins were also evaluated by fluorography [24]. Briefly, 10 μg of extracts were resolved by SDS-PAGE, the gels were soaked twice for 30 min with dimethyl sulfoxide (DMSO) and then in 20% (w/w) PPO (2,5-diphenyloxazole) for 3 h. Finally, DMSO was removed with water and the gel was dried and exposed to an X-ray film at −70°C for about 10 days before development.

In vitro culture of *T. crassiceps* cysticerci in the presence of IgG-^{3}H and GFP-^{3}H

Groups of 20 cysticerci were precultured for 3 days in ten volumes of a minimal medium for cysticerci (MMC) (Supplementary File S1) containing the salt base of RPMI-1640 without amino acids. Preculture medium was replaced every 24 h. For the evaluation of the uptake and use of metabolically labeled proteins, cysticerci were cultured during nine more days in 1 ml of MMC supplemented with 2% albumin and 500 μg of IgG-^{3}H (430,125 CPM) or GFP-^{3}H (9225 CPM) at 37°C with an atmosphere of 5% CO_2. In order to evaluate the biosynthetic activity of the parasites under culture conditions used in these experiments, another group of cysticerci, from the same stock, was cultured in identical medium added only with 30 μCi of Leu-^{3}H.

At the end of each culture, cysticerci were collected and washed as above. The VF was obtained by cutting the bladder wall with a scalpel on a Petri dish and mixed 1:1 (v/v) with lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 10 mM Tris) and protease inhibitors cocktail. A tissue extract (TE) was obtained by homogenization of cysticerci in a lysis buffer using a Benchmark D1000 homogenizer. The homogenate was centrifuged for 15 min at 14000 ***g*** and the supernatant collected. The TE, VF, and culture medium (CM) collected were frozen at −70°C until use. To ascertain if radioactivity had been incorporated into newly synthesized cysticerci proteins, 100 μg of each extract were resolved by SDS-PAGE using 10% polyacrylamide gels and processed for fluorography as described above, exposing the dried gels for approximately 30 days.

Functional activity of the uptaken mouse IgG

A crude extract was obtained through homogenization of cysticerci in PBS (1:1, v/v) adding the protease inhibitor cocktail. Extracts were centrifuged 15 min at 14000 ***g*** and the supernatant collected. The total extract was applied to a Protein G-Sepharose 4B column. Bound IgG was eluted using 0.1 M glycine, pH 2.3, and immediately neutralized with Tris 1 M, pH 11. Fractions containing the bound IgG were concentrated using an Amicon system (10 kDa cutoff) and washed several times using PBS, pH 7.4. The purified IgG was quantified by the noninterfering protein assay kit (GBiosciences) and tested for antibody activity against TE and VF by Western blot using rabbit anti-mouse IgG coupled to peroxidase (Sigma-Aldrich) as secondary antibody. The antigen–antibody reaction was developed using a West-Femto chemiluminiscence kit (Thermo Scientific) following manufacturer's instructions.

Calcareous corpuscles protein extracts from *T. solium* cysticerci

The CC were isolated after homogenization of *T. solium* cysticerci in PBS as above. This species of parasite allows isolation of enough corpuscles for proteomic analysis. Two stocks of cysticerci obtained from naturally infected pigs were used; the homogenates were centrifuged at 14000 ***g*** for 30 min at 4°C to sediment CC in the bottom of the tube. Cell debris was removed by resuspending pellets in ultrapure water (Milli-Q, Millipore Co) followed by centrifugation; this rinsing procedure was repeated at least eight times until clean whitish pellets of CC were obtained. The two pellets were resuspended separately in 0.5 ml of 0.1 N HCl to remove proteins that are not part of the matrix but adsorbed onto the CC surface during cysticerci homogenization. Finally, CC were centrifuged and then dissolved in 2 ml of 0.1 N HCl. Dissolved CC material was concentrated using ultrafiltration Amicon vials (3 KDa cutoff) and resuspended in 150 μl of buffer containing 7 M urea, 2 M thiourea, 4% CHAPS, and 10 mM Tris. Protein content was quantified by noninterfering protein assay (GBiosciences).

Western blot of calcareous corpuscles protein extracts

Samples of 15 μg of each *T. solium* cysticerci CC protein extract were resolved in 15% SDS-PAGE and transferred onto a nitrocellulose membrane. In the case of porcine serum, 5 μg were applied to the lane. Membranes were blocked using 10% skim milk in PBS and reacted with sheep α-albumin (Abcam ab186525) and then with a rabbit α-sheep-IgG (Abcam ab6747), or with a rabbit polyclonal α-mouse IgG serum (A9044 Sigma-Aldrich).

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Mass spectrometry of the calcareous corpuscles protein extracts

Prior to analysis, CC protein extracts were reduced using 10 mM dithiothreitol (DTT, BioRad) for 1 h and alkylated with 15 mM iodoacetamide (BioRad) for 30 min. Iodoacetamide was quenched using 15 mM DTT. Each sample was digested with modified porcine trypsin and prepared for LC/MS/MS. Samples were injected into an Acquity UPLC-BEH-C18 column (Waters, Milford, MA), which was coupled in-line with a LTQ Orbitrap XL (Thermo Fisher Scientific, Waltham, MA) equipped with an electrospray ion source. The samples were equilibrated in solvent A containing 100% H_2O and 0.1% formic acid and eluted through a gradient with solvent B: 100% acetonitrile and 0.1% formic acid (see below). A sample of 3 μl of trypsin digested proteins was trapped using pre-column (Symmetry® C18, 5 μm, 180 μm × 20 mm, Waters), which was then switched in-line onto a 10-cm capillary UPLC column (100 um ID BEH-C18 1.7 μm particle size). The column was enclosed in a column heater operating at 35°C. After loading, peptides were separated with a 60-min gradient at a flow rate of 400 nl/min. The gradient was as follows: 3–50% Solvent B, 30 min; 50–85%, 1 min; 85%, 7 min and 3%, 22 min. Eluted peptides were directly electrosprayed into the mass spectrometer through a standard coated silica tip (NewObjective, Woburn, MA).

The LTQ was operated in data-dependent acquisition mode to automatically alternate between a full scan in a range of m/z 400–2000, and the three most intense ions were sequentially isolated and fragmented using collision-induced dissociation (CID). CID was performed with helium as collision gas at normalized collision energy of 40% and 10 ms of activation time. Data acquisition was controlled by Xcalibur 2.0.7 software (Thermo Fisher Scientific). Tandem mass spectra, obtained from the LTQ Orbitrap XL, were extracted by Proteome Discoverer version 1.3 (Thermo Scientific) and obtained peptide sequences were searched for Sequest against *T. solium* and *Sus scrofa* genome databases to identify the proteins of origin.

All files generated by Sequest were searched with the following parameters: Peptide tolerance was allowed to be 1.6 Da, fragment mass tolerance was ±0.8 Da, and a maximum two missed cleavages allowed. Carbamidomethylation of cysteine was set as static modification and methionine oxidation was set as variable modification.

MS data analysis

Relative protein abundance, represented by normalized spectral abundance factors, was calculated by the number of spectral counts (SpC) identifying a protein, divided by the protein's length, divided by the sum of SpC for all proteins in the experiment [25]. Gene ontology (GO) analysis for the host (*Sus scrofa*) was performed using the PantherGO algorithm [26]. Taenia proteins were annotated and analyzed for GO with Blast2GO [27].

Results

Uptake and use of metabolically labeled IgG and GFP by *T. crassiceps* cysticerci maintained under *in vitro* culture

For the protein uptake assays, *T. crassiceps* cysticerci were used to take advantage of their ready availability. IgG-^{3}H and GFP-^{3}H were produced separately under *in vitro* conditions. Purity of the two metabolically labeled proteins resulted > 81% as estimated by densitometry of Coomassie Blue stained gels (Figure 1); IgG-^{3}H was observed as the two-characteristic heavy (50 kDa) and light (25 kDa) chains (Figure 1A), an additional band was observed at 150 kDa, which apparently corresponded to the whole immunoglobulin. GFP-^{3}H was also observed at the expected (27 kDa) size with additional bands of lower abundance (Figure 1B). Tritium labeling of the two proteins was detected by fluorography of the dried gels (Figure 1C,D).

To track the uptake of host proteins and their use as a source of amino acids by *T. crassiceps* cysticerci, IgG-^{3}H or GFP-^{3}H was added to the culture medium where cysticerci were maintained *in vitro* for 9 days. The TE, VF, and CM protein extracts were resolved by SDS-PAGE (Figure 2B). Radioactive label of the proteins in the extracts was determined by fluorography. In the case of cysticerci cultures in the presence of IgG-^{3}H, the expected heavy and light chains were clearly observed at approximately 50 and 25 kDa in TE; the two bands were not detected in VF and CM (Figure 2A). Additional bands of lower size were barely observed underneath the 50 kDa band, suggesting a marginal degradation of the metabolically labeled IgG. Similarly, fluorography of cysticerci extracts cultured in the presence of GFP-^{3}H showed the band corresponding to complete GFP (27 kDa) and several weak bands also observed in the purified GFP, also consistent with a limited proteolytic activity in cysticerci tissue. Again, the major 27 kDa band was not observed in VF or CM. In contrast with the lack of detection of IgG and GFP in the VF, SDS-PAGE of the same extracts showed the abundant presence of a 67 kDa protein in VF, consistent with BSA (Figure 2B), suggesting that this protein was uptaken from the culture medium, strongly supporting the concept that this protein acts as an osmotic regulator for cysticerci [14].

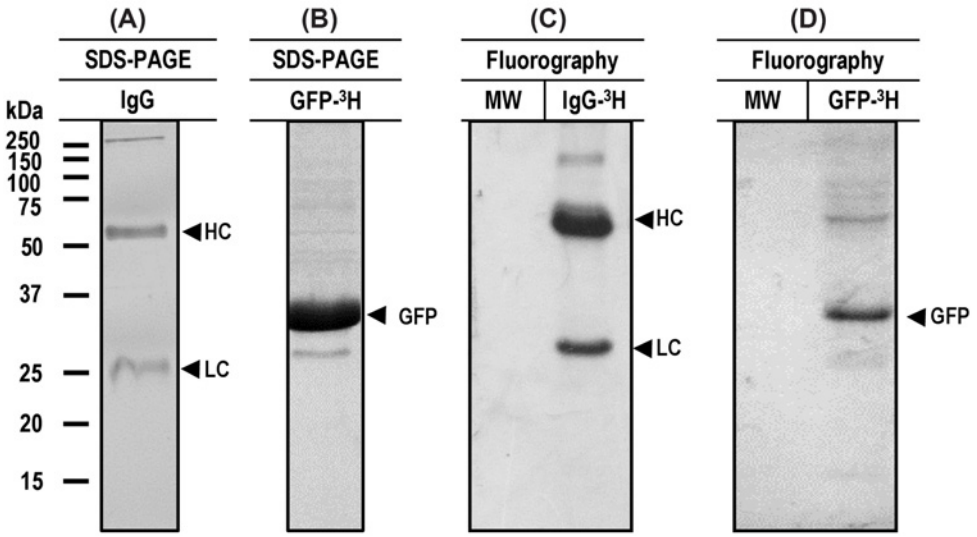

Figure 1. Metabolically labeling and purification of mouse IgG and GFP

After separate *in vitro* cultures of mouse hybridoma and recombinant bacterial cells, the metabolically labeled IgG and GFP were purified by affinity chromatography using columns with protein G and Nickel Sepharose 4B respectively. The purity of IgG (**A**) and GFP (**B**) was evaluated by SDS-PAGE. The radioactive labeling was evaluated by fluorography on SDS-PAGE treated gels. In both cases, radioactive labeling of IgG heavy and light chains (**C**) and GFP (**D**) was detected in the expected molecular sizes; MW, molecular weight marker.

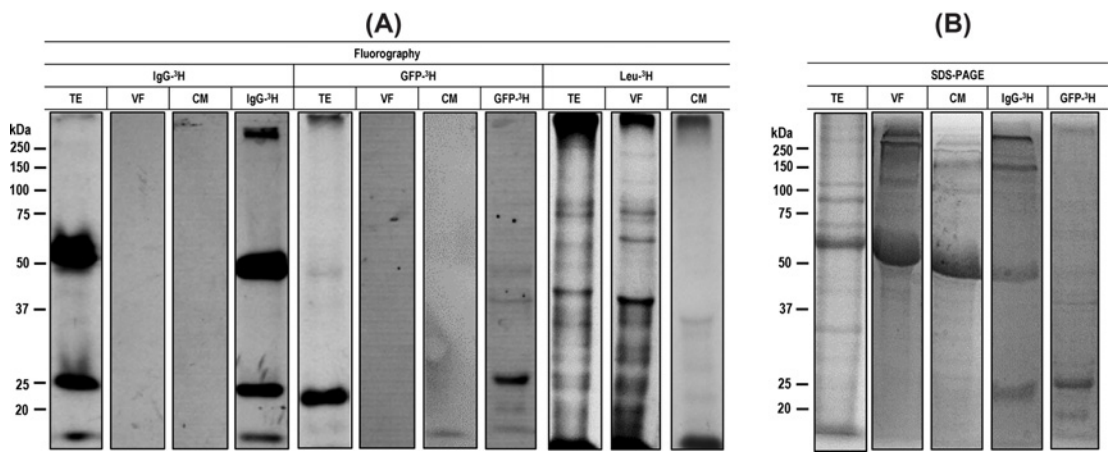

Figure 2. Electrophoresis and fluorography of protein extracts from *T. crassiceps* cysticerci maintained under *in vitro* culture in the presence of IgG-^{3}H or GFP-^{3}H

Cysticerci were cultured in the presence of IgG-^{3}H or GFP-^{3}H and a paired group of cysticerci was cultured with Leu-^{3}H for 9 days. After harvesting the cysts, samples of TE, VF, and CM were obtained. (**A**) All extracts were resolved in 10% SDS-PAGE and treated for fluorography. The X-ray films were exposed to dried gels for approximately 30 days. (**B**) SDS-PAGE patterns of cysticerci's extracts stained with Coomassie blue.

The *in vitro* maintenance during 9 days in a minimal culture medium posed the question as to the physiological state of cysticerci. To ascertain if the larvae remained capable for protein biosynthesis, a group of cysticerci was maintained *in vitro* in the same culture medium added with Leu-^{3}H. The protein extracts obtained from this culture showed numerous radioactive bands ranging from 25 to more than 250 kDa, indicating that cysticerci retained a high biosynthetic activity under the culture conditions used in these experiments (Figure 2A).

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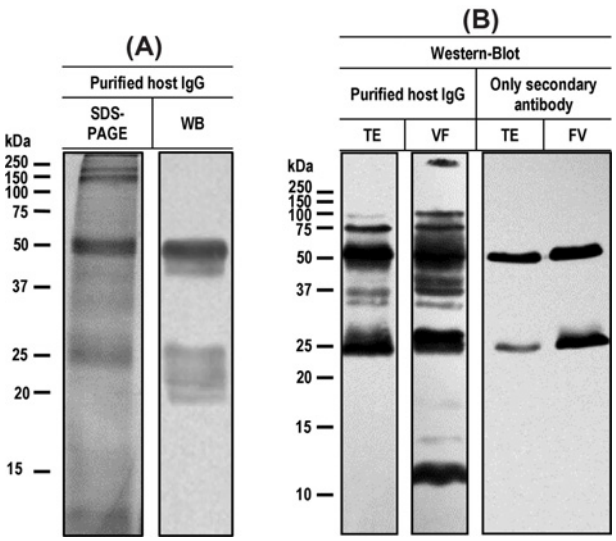

Figure 3. Purification and functional assays of the uptaken host IgG in protein extracts of *Taenia crassiceps* cysticerci

(**A**) Purity of the bound IgG was evaluated by SDS-PAGE and Western blot using an anti-mouse IgG coupled to HRP. (**B**) Antibody function of the purified mouse IgG was evaluated through Western blot. Samples of *T. crassiceps* TE and VF were resolved through SDS-PAGE and then transferred to a nitrocellulose membrane. The IgG purified from cysticerci's total protein extracts was used as primary antibody followed by a rabbit anti-mouse IgG coupled to HRP, as secondary antibody. The secondary antibody alone was also tested as control.

Functional assays of host IgG purified from *T. crassiceps* cysticerci's crude extracts

To evaluate if uptaken host immunoglobulins maintained an antigen binding activity, as recently described for *T. solium* [17], mice IgG was purified from a saline crude extract of *T. crassiceps* cysticerci (Figure 3). The purity of the isolated mouse IgG was evaluated by SDS-PAGE and Western blot, using rabbit anti-mouse IgG, the heavy and light chains of the IgG were readily detected (50 and 25 kDa) (Figure 3A). Antibody activity testing was also carried out by Western blot: tissue extracts and VF were resolved by SDS-PAGE and transferred onto a nitrocellulose membrane, then, membranes were incubated with IgG purified from *T. crassiceps* cyst; several bands were recognized (Figure 3B).

Proteome profile of the *T. solium* calcareous corpuscles

The well-known abundance of immunoglobulins in cysticerci tissue and fluid contrasted with its marginal utilization as a source of amino acids observed above. An alternative fate for host proteins inside cysticerci was investigated. It is well known that CC are mineral concretions of phosphate salts deposited onto an organic matrix of proteins, polysaccharides, and lipids. Formation of CC has been proposed in taeniids as a mechanism of gathering toxic metabolites and other materials into an inert structure [20,21]. In spite of the fact that all above analyses were carried out using *T. crassiceps*, to facilitate the proteome analysis of CC we decided to use *T. solium* cysticerci that produce manageable amounts of CC. Our assumption was that the physiological role of these calcareous concretions, in closely related species of taeniid tapeworms, was alike. It is worth remembering that little is known about the protein composition of the CC organic matrix [28], therefore, we decided to analyze the protein composition of CC through mass spectrometry as well as through Western blotting using specific antibodies to host proteins (IgG and albumin).

Protein extracts of CC were obtained from two batches of *T. solium* cysticerci and then processed for mass spectrometry. From the peptide sequences obtained, a total of 636 and 760 proteins were identified in the two samples of CC, through search in the *T. solium* and *Sus scrofa* genome databases (Supplementary File S2). A remarkable finding was that approximately 70% of the proteins identified in both CC samples, 412 and 508, corresponded to host proteins, whereas only 224 and 252 proteins corresponded to *T. solium* in the first and second sample respectively. Variability of proteins identified in the two CC samples was considerably high, only 111 (73 host and 38 parasite) proteins were shared (Supplementary File S2); this means that 75–80% of the identified proteins were variable in the two samples of CC. However, GO analysis on the 636 and 760 host and parasite proteins identified in the two CC

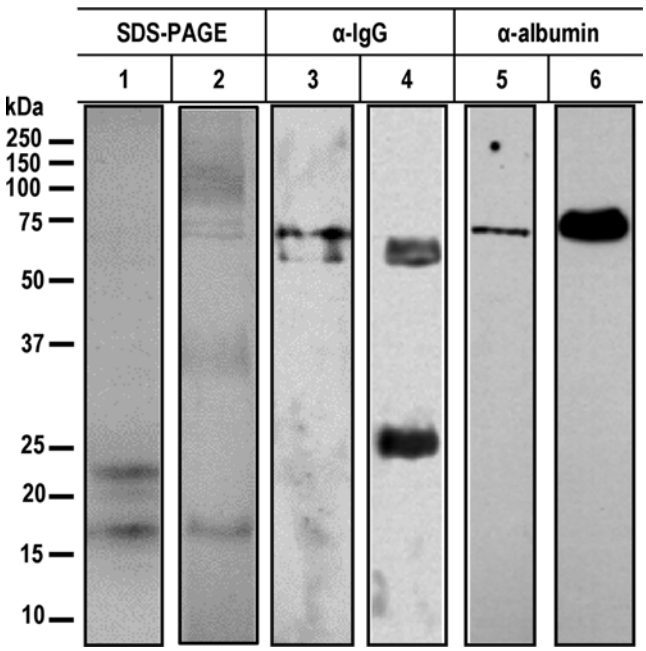

Figure 4. Immunological identification of host IgG and albumin in the protein matrix of calcareous corpuscles of *T. solium* cysticerci

(1) Coomassie Blue staining of a protein extract of (15 μg) CC and (2) filver staining of porcine serum (5 μg). Western blot of protein extracts of CC (3 and 5) and porcine serum (4 and 6) reacted with polyclonal antisera directed against mouse IgG coupled to HRP (3 and 4) or a sheep α-albumin and then with a rabbit α-sheep-IgG coupled to HRP (5 and 6).

samples resulted in similar patterns of function (Supplementary Figure S1 and S2). For example, included in the 20 most abundant host and parasite proteins were those involved in transport mechanism in mitochondria (mitochondrial thiamine pyrophosphate carrier, S-adenosylmethionine mitochondrial carrier), transcription regulation (KRAB A domain containing protein, PC4 and SFRS1-interacting isoform X2, zinc finger 182 isoform X1), and regulation of cytoskeleton (twitchin, arfaptin-1 isoform X1, microtubule-associated 6 isoform X1, formin 2 isoform X7) (Supplementary File S3).

Another approach to the analysis of host proteins in the CC protein matrix was through Western blot using specific antibodies against porcine albumin and IgG (Figure 4). Results showed that both proteins were incorporated into CC protein matrix at least partially intact; albumin was detected in a 67 kDa band whereas IgG showed only a 50 kDa band corresponding to the heavy chain; the light chain was not detected.

Discussion

The initial goal of the present study was to elucidate the fate of the abundant uptaken host proteins in taeniid larval tissue, particularly immunoglobulins, as a source of amino acids for newly synthesized cysticerci proteins or their disposal as waste in calcareous corpuscles. Several studies have reported the presence of host proteins inside the vesicular fluid and tissue of taeniid larval stages including *Echinococcus granulosus* [29-34], *T. crassiceps* [10,13,14,32], *T. taeniaformis* [10,12,32], *T. saginata* [11], and *T. solium* [16,17]. Surprisingly, the final use or fate of these host proteins remains poorly studied; albumin has been proposed to regulate the osmotic pressure in the vesicular fluid and/or scavenge reactive oxygen species in *T. crassiceps* [14], whereas hemoglobin, haptoglobin, and ferritin have been proposed to play a role for the parasite as iron chaperons, similar to the role they play in the host tissues [17,18]. In the present study, we cultured cysticerci in the presence of metabolically labeled IgG or GFP. Leu-^{3}H allowed to track the incorporation of this amino acid residue onto cysticerci proteins because it is an abundant amino acid in all organisms and taeniid cysticerci are unable of synthetizing it [4]. Therefore, if the metabolically labeled proteins (IgG or GFP) were degraded up to free amino acids, the Leu-^{3}H should be incorporated into *de novo* synthetized cysticerci proteins. A defined and minimal culture medium (MMC), without free amino acids, was specifically formulated here to favor the use of the residues released after degradation of the labeled protein. Cysticerci were precultured in the

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absence of free amino acids and proteins, in order to favor the release of host proteins in the freshly dissected cysticerci, as previously reported [14]; then, cysticerci were cultured in the same medium in the presence of one of the metabolically labeled proteins, only supplemented with albumin, to avoid cysticerci's osmotic stress.

Unexpectedly, no new protein containing leucine-^{3}H appeared to be synthesized out of the metabolically labeled IgG or GFP. If cysticerci incorporated Leu-^{3}H from IgG or GFP into newly synthesized proteins, the amount was negligible and could not be detected by the methodology used in the present study. In fact, only a marginal part of IgG and GFP appeared degraded in our fluorography assays. It is worth mentioning that cysticerci maintained a high biosynthetic activity under the 9 days of *in vitro* culture used here, as shown in experiments where groups of cysticerci were maintained *in vitro* in the same culture medium added only with Leu-^{3}H (Figure 2A).

Both findings suggest that digestion of IgG and GFP (and possibly other proteins), as a mechanism to obtain free amino acids for protein biosynthesis, seems to be negligible. Instead, cysticerci appeared to show a preference to absorb free amino acids from the environment; free amino acids are present in blood plasma and other body fluids at similar levels. High levels of free amino acids have been reported in hydatid fluid [35,36].

We reported long ago, the unspecific uptake of mouse immunoglobulin by *T. crassiceps* cysticerci [13]; more recently, we showed that host IgG uptaken by *T. solium* cysticerci maintain their antibody activity [17]. In this report, this finding for mouse IgG purified from *T. crassiceps* cysticerci was confirmed; several cysticerci's antigens in the range of 12–250 kDa were clearly recognized by the affinity-purified uptaken IgG antibodies. In fact, several antigenic parasite bands were highly recognized by the purified host IgG in VF, suggesting that uptaken host antibodies play a role in diminishing the exposure of relevant parasite antigens.

Calcareous corpuscles are mineral concretions widely distributed in cestodes that might be involved in osmoregulation, neutralization of metabolic acidic products, or simply as reservoirs of inorganic material [37,38]. In the case of *T. solium*, CC are formed in the lumen of protonephridial ducts [20,21] and made up of mineral salts such as Ca, Mg, Mn and Cu, mainly calcium phosphates, deposited on a complex organic matrix composed of proteins, polysaccharides, and nuclei acids [39]. The composition of the organic matrix of CC has been poorly studied; an early report describes the presence of intact antigenic proteins as well as a calcium binding protein [28]. In this report, we explored the protein composition of CC through mass spectrometry. Over 600 proteins were identified in each sample. Surprisingly, approximately 70% of the identified proteins corresponded to host proteins. This finding raises the issue of the protein composition of the CC matrix to an entirely different level; perhaps CC can be conceived as a mechanism for the storage/disposal of the uptaken host proteins.

As an additional approach to explore the function of the host uptaken proteins, we carried out assays for the identification of the proteins in CC extracts through Western blot. Albumin and IgG were readily identified using specific antibodies; interestingly, both proteins appeared in the expected molecular size, indicating that both proteins remained intact in the CC matrix. This suggests that, at least, part of host proteins is enrooted toward CC without being degraded. The mechanism of this process remains entirely unknown.

We have described that uptaken host proteins might play a role for cysticerci, similar to the role they play in host tissues [16,17], or as described here, they might play a role as components of the protein matrix for the process of mineral deposition in CC. The present study, together with our previous findings of the notably high content of host protein species in the tissues and fluid of taeniid metacestodes, up to ∼21%, possibly align the uptake of host proteins with their disposal through CC [17].

Proteins that we identified in CC samples were extremely variable; cysticerci used for the isolation of CC came from different naturally infected pigs bred in small rural communities in remote areas of Mexico. Differences could be due to the fact that each sample was made up of many cysticerci from a single pig. To our knowledge, individual variability in the process of CC formation between individual cysticerci has not been explored. Other possibilities to explain the high variability in the protein composition of the CC includes factors such as time of infection, stage of the parasite, immune status of the host etc. [39,40].

Finally, we propose that the uptake, utilization, and disposal of host proteins clearly depict an unmatched host–parasite molecular intimacy in cysticercosis. Moreover, CC might play a physiological role far more important to the previously proposed. It appears that cysticerci uptake at least some intact host proteins and take advantage of their original function in the host tissues until they are simply caught up by salt deposition in the CC.

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Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

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Author Contribution

J.F.-B. and J.N.-P. performed the experiments. J.F.-B., J.N.-P., and J.P.L. designed the experiments and analyzed data. G.F. provided biological specimens. X.S. aided with proteomic analysis. J.F.-B. and J.P.L. drafted the manuscript. G.F., J.N.-P, A.F., X.S., and J.P.L. revised the draft before submitting.

Abbreviations

CC, calcareous corpuscles; CPM, counts per minute; CID, collision-induced dissociation; CM, culture medium; MMC, minimal medium for cysticerci; SpC, spectral counts; TE, tissue extract; VF, vesicular fluid.

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PLOS | NEGLECTED TROPICAL DISEASES

RESEARCH ARTICLE

Quantitative multiplexed proteomics of *Taenia solium* cysts obtained from the skeletal muscle and central nervous system of pigs

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Abstract

In human and porcine cysticercosis caused by the tapeworm *Taenia solium*, the larval stage (cysts) can infest several tissues including the central nervous system (CNS) and the skeletal muscles (SM). The cyst's proteomics changes associated with the tissue localization in the host tissues have been poorly studied. Quantitative multiplexed proteomics has the power to evaluate global proteome changes in response to different conditions. Here, using a TMT-multiplexed strategy we identified and quantified over 4,200 proteins in cysts obtained from the SM and CNS of pigs, of which 891 were host proteins. To our knowledge, this is the most extensive intermixing of host and parasite proteins reported for tapeworm infections.Several antigens in cysticercosis, *i.e.*, GP50, paramyosin and a calcium-binding protein were enriched in skeletal muscle cysts. Our results suggested the occurrence of tissue-enriched antigen that could be useful in the improvement of the immunodiagnosis for cysticercosis. Using several algorithms for epitope detection, we selected 42 highly antigenic proteins enriched for each tissue localization of the cysts. Taking into account the fold changes and the antigen/epitope contents, we selected 10 proteins and produced synthetic peptides from the best epitopes. Nine peptides were recognized by serum antibodies of cysticercotic pigs, suggesting that those peptides are antigens. Mixtures of peptides derived from SM and CNS cysts yielded better results than mixtures of peptides derived from a single tissue location, however the identification of the 'optimal' tissue-enriched antigens remains to be discovered. Through machine learning technologies, we determined that a reliable immunodiagnostic test for porcine cysticercosis required at least five different antigenic determinants.

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Author summary

Human and porcine cysticercosis caused by *Taenia solium* is a parasite disease still endemic in developing countries. The cysts can be located in different host tissues, including different organs of the central nervous system and the skeletal muscles. The molecular mechanisms associated with the tissue localization of the cysts are not well understood. Here, we described the proteome changes of the cysts obtained from different host tissues from infected pigs using quantitative multiplex proteomics. We explored the diversity of host proteins identified in the cyst's protein extracts and we also explored the immune-localization of several host-related proteins within the cysts, and propose their possible function. We identified several proteins and antigens enriched for a given tissue localization. Several synthetic peptides designed from these tissue-enriched antigens were tested trough ELISA. Using a combination of peptide mixtures and machine learning technologies we were able to distinguish non cysticercotic and cysticercotic pig's sera. The tissue-enriched proteins/antigens could be useful for the development of improved immunodiagnostic tests capable of discriminate the tissue-localization of the cysts.

Introduction

Human and porcine cysticercosis caused by the larval stage of *Taenia solium*, is acquired by the ingestion of this parasite's eggs. After activation by several gastrointestinal agents, the oncospheres penetrating the intestinal wall later establish in different tissues and organs including the skeletal muscles (SM) and the brain. In humans, establishment of cysts in the central nervous system (CNS) causes neurocysticercosis (NC), a serious and pleomorphic disease that can become highly debilitating [1]. Heterogeneity of human NC has been associated, at least in part, with the number and localization of the cysts in the CNS [2], as well as to many other factors including a complex immune response directed to a number of cyst's antigens [3, 4, 5, 6].

The molecular factors associated with the tissue localization of the *T. solium* cysts remain poorly understood [7]. Other pathogenic microorganisms (*S. pneumoniae*, *Campylobacter jejuni*, *Escherichia coli*, *Trypanosoma brucei*, etc.), show tissue preference linked to a number of specific pathogen's proteins [8, 9, 10, 11, 12].

Information available on proteomics changes of flatworm parasite infections is limited. However, we know that parasites respond to hormones, cytokines and other host's molecules [13]. The availability of several tapeworm genomes [14] has allowed to detail this complex host-parasite cross-communication including insulin, EGF/FGF, TGF-b/BMP, among others (for an updated review see [15]). Insulin responsiveness has been described for *Schistosoma mansoni*, *Taenia crassiceps* and *Echinococcus multilocularis* [16, 17, 18]. The differential effects of steroid hormones during parasite infections is also well documented [19]. Some parasites also have the ability to respond to host cytokines; for example, *S. mansoni* has receptors to TNF-α and TGF-β and proteomic and genomic changes have been reported in response to those cytokines [20, 21, 22].

The advent of high throughput proteomic techniques greatly widens our power to approach these old questions in molecular helminthology. In this context, body fluids of the host may affect proteome expression of infectious agents, for example, *E. coli* growing in a media supplemented with urine show a differential proteome signature [23]. Furthermore, several proteomic changes of *Streptococcus pyogenes* have been reported in response to serum supplementation

[24]. The molecular factors associated with the tissue localization of helminth parasites within the host tissues has been less explored; in the case of *Trichinella spiralis* several changes have also been reported between parasites isolated from different host tissues [25]. However, important advances in helminth proteomics, including metacestode cystic/vesicular larval forms, have been reported [26–29].

It is conceivable that the host tissue's molecular environment modulates the protein expression of pathogens, including parasites. Accordingly, specific proteomic profiles of parasites could be associated with a certain tissue localization.

Understanding the proteome changes of parasites in different host tissues, can provide insights not only on the molecular networking occurring in complex host parasite relationships, but it could also be useful for the design of more effective vaccines, drugs, as well as for the improvement of available diagnostic procedures.

Here we benefited from isobaric quantitative proteomics to elucidate the proteomic changes of *T. solium* cysts obtained of SM and CNS of pigs. A protein profile was found associated with each tissue localization, allowing the identification of 42 tissue-enriched antigens and the design of 14 synthetic antigenic peptides that were evaluated for antibody recognition using infected and uninfected pig's sera. Our results indicated that an optimal immunological diagnosis for porcine cysticercosis requires at least five different epitopes from several tissue-enriched antigens. A remarkable finding was the conspicuous and abundant presence of host proteins in the protein extracts of the cysts; 891 host proteins were identified and quantified. We present initial findings suggesting that several intact host's proteins might play a significant role in tapeworm's physiology.

Materials and methods

Protein extracts

Cyst's isolation and total protein extracts for quantitative proteomics. Cysticercotic pigs were humanely sacrificed by certified veterinary doctors in accordance with institutional protocols from the Institute for Biomedical Research and the School of Veterinary Medicine and Zootechnics at the Universidad Nacional Autónoma de México. *T. solium* cysts were dissected immediately after sacrifice from the skeletal muscle and the central nervous system of three experimentally and two naturally infected pigs, using only cysts in the vesicular stage, all the cysts included in this study had a similar appearance (size ≈ 1 cm, transparent bladder wall and crystalline vesicular fluid). The cysts were obtained under aseptic conditions, inflammatory capsules surrounding the cysts were carefully and completely removed to reduce the host contaminant material (except those bound to the cyst's surface), the larvae were washed several times with ice cold PBS, pH 7.3. Time elapsed between the sacrifice and the cyst's isolation was no longer than 90 min. From each tissue and pig, five whole cysts from each animal and tissue were homogenized in 8M urea in 50 mM HEPES pH 8.0, complemented with protease inhibitors (Complete, Roche) using a polytron homogenizer. The extracts were centrifuged 15 min at 14,000 x g and the supernatants were collected. Afterwards, all supernatants were reduced (10 mM DTT for 1 h) and then alkylated (15 mM iodoacetamide) for 30 min in the dark; excess iodoacetamide was quenched using 15 mM DTT. A graphic explanation is provided in S1 Fig.

Antigenic extracts for ELISA and western blotting assays. The insoluble fraction of cysts tissue was obtained as described previously [30]. The vesicular fluid (VF) was obtained from skeletal muscle cysts from naturally infected pigs; the bladder wall was sectioned using a scalpel and the released VF from several cysts was pooled and diluted 1:2 using 50 mM Tris pH 7.3, complemented with protease inhibitors.

Total saline extracts for IgG purification. Cysts were obtained from the skeletal muscle of naturally infected pigs (n = 3), homogenized in PBS, pH 7.3, supplemented with 0.45 M of NaCl and protease inhibitors using a Polytron and centrifuged at 14,000 rpm for 15 min.

Protein digestion and TMT labelling

Methanol–chloroform precipitation of the reduced and alkylated protein extracts was performed prior to protease digestion. Samples of 400 μg of each protein extract were resuspended separately in 100 μL of 8 M urea in 50 mM HEPES, pH 8.2. After solubilization, the protein extracts were diluted to 4 M urea with 50 mM HEPES, pH 8.2, and digested at RT for 3 h with endoproteinase Lys-C (Wako, Japan) at 5 ng/μL. The mixtures were then diluted to 1 M urea with 50 mM HEPES, pH 8.2, and trypsin was added at a 50:1 protein-to-protease ratio. The reaction was incubated overnight at 37˚C and stopped by the addition of 100% TFA to a final pH < 2. Peptides were desalted using 50 mg tC18 SepPak solid-phase extraction cartridges (Waters, Milford, MA) and lyophilized. Desalted peptides were resuspended in 100 μL of 200 mM HEPES, pH 8.2. Peptide concentrations were determined using the microBCA assay (Thermo Fisher Scientific, Waltham, MA). One-hundred micrograms of peptides from each sample was labeled with TMT reagent. TMT-10 reagents (0.8 mg, from Thermo Fisher Scientific) were dissolved in anhydrous acetonitrile (40 μL), of which 10 μL were added to the peptides along with 30 μL of acetonitrile (final acetonitrile concentration of approximately 30% (v/v)). The labeling reaction proceeded for 1 h at room temperature and then was quenched with hydroxylamine (Sigma, St. Louis, MO) to a final concentration of 0.3% (v/v). The TMT-labeled samples were mixed equally, vacuum centrifuged to near dryness, desalted using 200 mg solid-phase C18 extraction cartridge (Sep-Pak, Waters), and lyophilized.

Off-line Basic pH Reversed-Phase (BPRP) fractionation

The TMT-labeled peptides were fractionated using BPRP HPLC. An Agilent 1100 pump equipped with a degasser and a photodiode array (PDA) detector (set at 220 and 280 nm wavelength) from Thermo Fisher Scientific (Waltham, MA) were used. Peptides were subjected to a 50 min linear gradient from 5% to 35% acetonitrile in 10 mM ammonium bicarbonate pH 8 at a flow rate of 0.8 mL/min over an Agilent 300 Extend C18 column (5 μm particles, 4.6 mm ID, and 220 mm in length). Beginning at 10 min of peptide elution, fractions were collected every 0.38 min into a total of 96 fractions, which were consolidated into 24, of which 12 nonadjacent samples were analyzed. Samples were dried via vacuum centrifugation. Each eluted fraction was acidified with 1% formic acid and desalted using StageTips [31], dried via vacuum centrifugation, and reconstituted in 4% acetonitrile, 5% formic acid for LC–MS/MS analysis.

Liquid chromatography and tandem mass spectrometry

All mass spectrometry data were collected on an Orbitrap Fusion mass spectrometer (Thermo Fisher Scientific, San Jose, CA) coupled to a Proxeon EASY-nLC II liquid chromatography (LC) pump (Thermo Fisher Scientific). Peptides were eluted over a 100 μm inner diameter micro-capillary column packed with ∼0.5 cm of Magic C4 resin (5 μm, 100 Å, Michrom Bioresources) followed by ∼35 cm of Accucore resin (2.6 μm, 150 Å, Thermo Fisher Scientific). For each analysis, we loaded ∼1 μg of the peptide mixture onto the column. Peptides were separated using a 90 min gradient of 6–26% acetonitrile in 0.125% formic acid at a flow rate of ∼350 nL/min. The dynamic exclusion duration was set at 90 s, with a mass tolerance of ±7 ppm. Each analysis used the multinotch MS3-based TMT method [32] on an Orbitrap Fusion mass spectrometer, which has been shown to reduce ion interference compared to MS2 quantification. The scan sequence began with an MS1 spectrum (Orbitrap analysis;

resolution 120000; mass range 400–1400 m/z; automatic gain control (AGC) target 2×10^5; maximum injection time 100 ms). The 10 most-abundant MS1 ions of charge states 2–6 were fragmented, and multiple MS2 ions were selected using a Top10 method. MS2 analysis was composed of collision induced dissociation (quadrupole ion trap analysis, AGC 4×10^3; normalized collision energy (NCE) 35; maximum injection time 150 ms). Following acquisition of each MS2 spectrum, we collected an MS3 spectrum as described previously [32], in which multiple MS2 fragment ions were captured in the MS3 precursor population using isolation waveforms with multiple frequency notches. MS3 precursors were fragmented by high energy collision-induced dissociation (HCD) and analyzed using the Orbitrap (NCE 55; AGC 5×10^4; maximum injection time 150 ms, resolution was 60,000 at 400 Th).

Data analysis

Instrument data files were processed using a SEQUEST-based in-house software pipeline [33]. Spectra were converted from.raw to mzXML using a modified version of ReAdW.exe. A database containing all predicted ORFs for entries from the parasite (*T. solium* genome database; http://www.genedb.org/Homepage/Tsolium; downloaded March 31, 2015) and the host (*Sus scrofa* database; http://www.uniprot.org/proteomes/?query=taxonomy:9823; downloaded March 31, 2015) was used. This database was concatenated with another database composed of all protein sequences in the reverse order. Searches were performed using a 50 ppm precursor ion tolerance for total protein level analysis. The product ion tolerance was set to 0.9 Da. These wide mass tolerance windows were chosen to maximize sensitivity besides SEQUEST searches and linear discriminant analysis [34, 35]. TMT tags on lysine residues and peptide N termini (+229.163 Da) and carbamidomethylation of cysteine residues (+57.021 Da) were set as static modifications, while oxidation of methionine residues (+15.995 Da) was established as a variable modification.

Peptide-spectrum matches (PSMs) were adjusted to a 2% false discovery rate (FDR) [35]. PSM filtering was performed using a linear discriminant analysis, as described previously [33], while considering the following parameters: XCorr, ΔCn, missed cleavages, peptide length, charge state, and precursor mass accuracy. For TMT-based reporter ion quantitation, we extracted the signal-to-noise (S/N) ratio for each TMT channel and found the closest matching centroid to the expected mass collapsed to a 1% peptide FDR and then collapsed further to a final protein-level FDR of 1%. Moreover, for protein assembly, principles of parsimony were used to produce the smallest protein set, necessary to account for all observed peptides. Proteins were quantified by summing reporter ion counts across all matching PSMs using in-house software, as described previously [36]. Briefly, a 0.003 Th window around the theoretical m/z of each reporter ion (126, 126.1278 Th; 127N, 127.1249 Th; 127C, 127.1310 Th; 128N, 128.1283 Th; 128C, 128.1343 Th; 129N, 129.1316 Th; 129C, 129.1377 Th; 130N, 130.1349 Th; 130C, 130.1410 Th; 131, 131.1382 Th) was scanned for ions, and the maximum intensity nearest the theoretical m/z was used. PSMs with poor quality, MS3 spectra with TMT reporter summed signal-to-noise ratio less than 387, or no MS3 spectra were excluded from quantitation [32]. The RAW files will be made available upon request. Protein quantitation values were exported for further analysis in Excel, Perseus 1.5.2.4 and GraphPad prism v6. Proteins with more than three missing channels were discarded, in the case of identifications based in a single peptide, that peptide was present in at least 7 samples. The selection of the tissue-enriched proteins (Fig 1) was based on the comparison of fold changes between CNS and SM cysts using a multiple T-test and Benjamini-Hochberg correction with a 5% of FDR (there were 5 CNS samples, unfortunately, one sample of CNS cysts was discarded at the end, due to poor data quality).

A)

Total peptides	Unique peptides	Total proteins	Total MS/MS	Total MS3	Protein FDR	Peptide FDR	Quantified proteins	4,259	*Taenia solium*	3,368
58,748	36,184	5,022	227,738	227,773	1.99% (50)	0.22% (64)			*Sus scrofa*	891

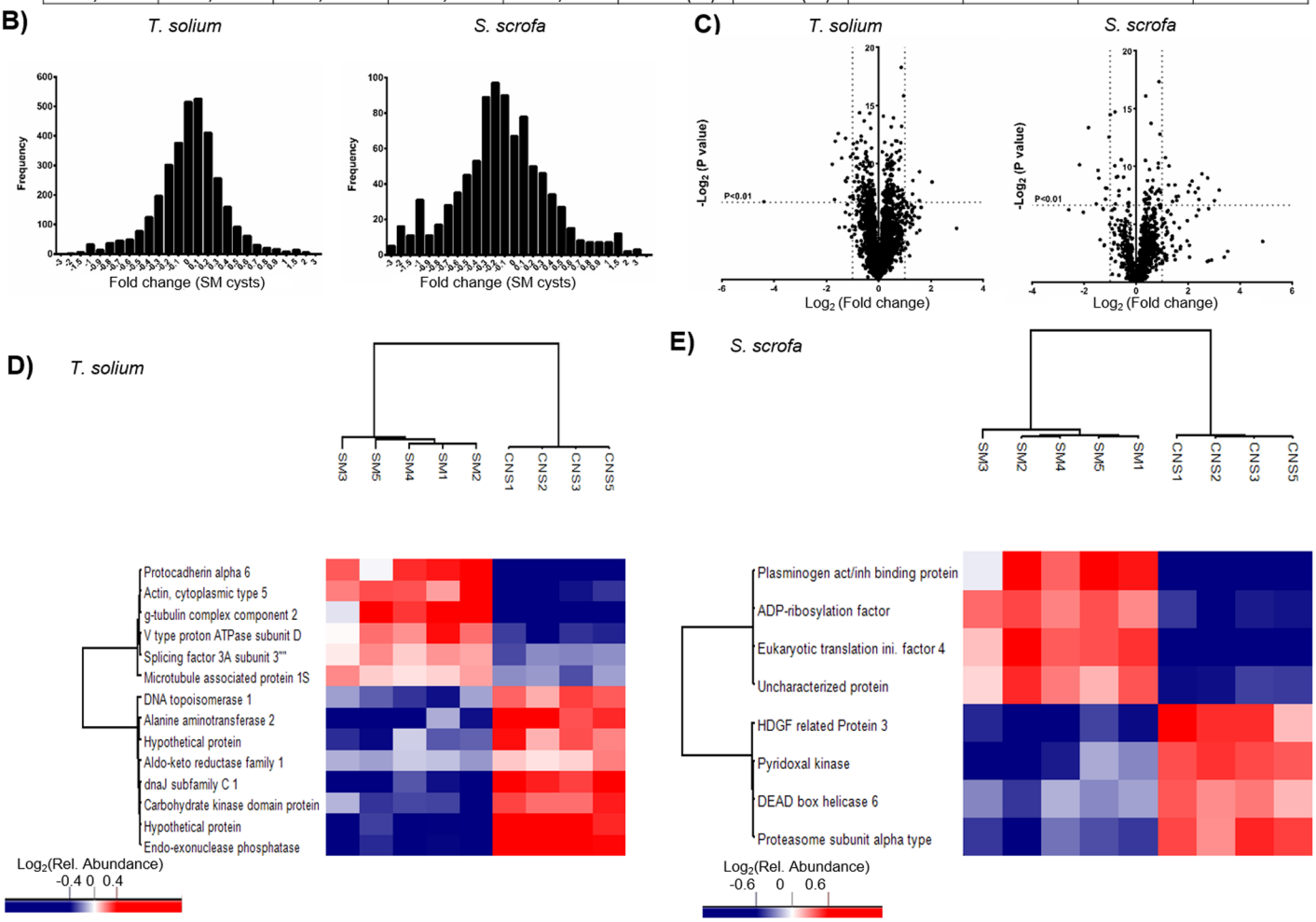

Fig 1. Proteomic analysis of *Taenia solium* cysts. A) Table summarizing peptide and protein quantification from the nine samples. Proteins were collapsed to a final protein-level FDR < 2%. B) Fold change distributions of proteins of skeletal muscle (SM) cysts. C) Volcano plots showing P-value and the Log_2 (fold change CNS cysts/SM cysts) of proteins of SM and CNS cysts. D) Heat map showing the signature for *T. solium* proteins of SM and CNS cysts. E) Heat map showing the signature for the host (*Sus scrofa*) proteins quantified in protein extracts of SM and CNS cysts.

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Peptide selection for antibodies detection

Proteins with a P-value <0.01 (n = 261) and proteins without changes (lowest coefficient of variation, n = 50) were chosen to predict their antigenic regions. A detailed explanation is found in the S2 Fig. Initially, the antigenicity algorithm [37] and the B cell epitope algorithm [38] were used to quantitatively estimate the proteins with the higher antigenicity. Only the proteins predicted by both algorithms with a high percentage of antigen/epitope were selected (n = 42). The peptide selection was based on the following criteria: length of at least 15 amino acids (average size of predicted antigenic regions = 14.1); coincidence in the prediction of at least 5 amino acids by both algorithms, and at least, 10 amino acids should be predicted by one of the algorithm. The resulting peptides were submitted to an algorithm that was trained with a set of synthetic peptides of proven utility in diagnostic procedures as well as with a set of peptides that were not useful [39]; peptides with the highest probability of recognition by

antibodies were selected. A total of ten peptides were selected (one from each protein); 4 peptides derived from proteins that were abundant in SM cysts, 4 from CNS cysts and 2 from proteins that did not show change in both tissues. All peptides were purchased from GenScript (USA).

Protein sequence analysis

Proteins from the host (*Sus scrofa*) were annotated using the PantherGo algorithm [40, 41]; in the case of *T. solium* proteins, only proteins with a P-value<0.01 were submitted to Argot2 algorithm [42–44] using a threshold of 200. Disulfide bonds, N-linked glycosylation sites, transmembrane regions, signal peptides, and GPI-anchoring sites were predicted for selected proteins using several algorithms [45–56].

Experimental validation of theoretically predicted antigenic peptides

The insoluble fraction of *T. solium* cysts, the VF and the synthetic peptides were tested by ELISA. Briefly, 1.5 μg of the insoluble fraction and of the VF, as well as 500 ng of each synthetic peptide were used to coat separate wells of microtiter plates. After overnight incubation at 4˚C with mild agitation, the plates were washed, blocked for 2 h with 1% albumin in PBS-0.05% Tween 20 (PBST) and incubated with different pig sera, diluted 1:200 in PBST and incubated overnight at 4˚C. A HRP coupled anti-pig IgG hyperimmune serum was used (diluted 1:4,000) as secondary antibody. The reaction was developed using OPD (0.4 mg/mL) for about 3–5 min and stopped with 3N HCl. Absorbance at 492 nm was determined in a Multiskan FC (Thermo-Fisher Scientific).

Purification of pig IgG from a protein extract of *T. solium* cysts

The total saline extract was passed through a column of Protein G coupled to Sepharose 4B. The bound IgG was eluted using 0.1 M glycine pH 2.3 and immediately neutralized with Tris 1M, pH 7.3. Fractions containing the bound IgG were concentrated using an Amicon system (10 kDa cutoff) and washed several times using PBS, pH 7.3. The purified IgG was quantified by Non-Interfering protein assay (GBiosciences).

Functional testing of the pig IgG purified from the cysts extracts

The IgG purified from the cysts protein extracts was tested for antibody activity through conventional ELISA and western-blotting procedures. For ELISA, microtiter plates were separately coated using 1.5 μg of VF or the insoluble protein fraction of cyst tissue (see above) in carbonate buffer, pH 9.6. After overnight incubation at 4˚C with mild agitation, the plates were washed three times using PBST and blocked using 1% albumin in PBST for 1 h at room temperature. After another washing cycle, the plates were incubated overnight at 4˚ C with the IgG fraction purified from the *T. solium* cysts. A pool of sera from 15 cysticercotic pigs was also used in similar assays for comparison. Dilutions for both the IgG purified from the cysts and the pool of sera from the infected pigs are shown below. After washing, the plates were incubated with a HRP-coupled rabbit anti-pig IgG hyperimmune serum (diluted 1:1000) for 1h at room temperature. The reaction was developed using OPD (0.4 mg/mL) for about 3–5 min and stopped with 3N HCl. Absorbance at 492 nm was determined in a Multiskan FC (Thermo-Fisher Scientific).

In the case of western-blotting, 20 μg of the VF and the insoluble fraction were resolved through 12% SDS-PAGE and transferred onto a nitrocellulose membrane. The membranes were blocked overnight using 10% of skim milk in PBS and incubated with 10μg/mL of the IgG purified from *T. solium* cysts in 10% skim milk at room temperature. After three washings

using PBS-Tween 0.1%, the membrane strips were incubated with the rabbit anti-pig IgG secondary antibody (diluted 1:85,000) for 2h at room temperature. The antigen-antibody reaction was developed using a West femto chemiluminiscence kit (Thermo) following the manufacturer's instructions.

Multi antigenic peptide testing

For each subset of *k* antigenic peptide measures and n subjects, predictive accuracy was measured by Leave One Out Cross Validation. Here, *n* independent training/testing procedures using a Support Vector Machine (SVM) were performed. Each training set consisted in all except one individual value, and testing set being the individual left out. Accuracy is computed as the fraction of times each individual test was correctly classified for that particular selection of *k* peptides. Source code for SVM implementation is found in S1 Data and is contained in scikit-learn package [SKLEARNREF] with default parameters [57].

Tissue immune-localization studies

Cysts (CNS and SM) were fixed in Zamboni solution. Afterwards, all samples were dehydrated and embedded in paraffin. Heat-mediated antigen retrieval was performed on 5-μm sections, using a 0.1 M sodium citrate solution (pH 6.0) in a high-pressure sterilizer (120˚C for 5 min) and endogenous peroxidase was consumed by incubation with 0.3% (v/v) H_2O_2 in PBS for 10 min at RT. Afterwards, the tissue section on slides were washed three times with PBS and maintained in a blocking solution (0.1% BSA in PBS, Sigma-Aldrich) for 10 min. After washing with PBS, the slides were incubated overnight with the primary antibody (the list of antibodies used could be found in S1 Table), diluted 1:50, in PBS-0.1% BSA, at 4˚C. After washing several times with PBS, the tissue sections were incubated with the corresponding second antibody (HRP-conjugated) 1:1000 for 30 min at 37˚C. Peroxidase activity was visualized by incubating the samples for 2 min with 3-diaminobenzidine tetrahydrochloride (DAB, MP Biomedicals). Reaction was stopped with water, and sections were counterstained with hematoxylin, dehydrated, cleared, and mounted with resine (Gold Bell). The single labeled sections were examined and photographed under light microscopy (Nikon Eclipse 80i) using a digital color video camera (Nikon Digital Sigth). The second antibody controls could be found in S3 Fig.

Data availability

All relevant information are within the manuscript or supplementary material. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [58] partner repository with the dataset identifier PXD00527. The source code for the support vector machine is available in the S1 Data; protein and peptide identifications can be found in S2 and S3 Data; the subset of highly antigenic proteins can be found in S4 Data; the functional annotation of the tissue-enriched proteins in S5 Data and several cestode proteome comparison in S6 Data.

Western-blotting of host proteins in the cysts protein extracts

Briefly, 50 μg of VF and insoluble fraction of cysts tissue were resolved by 12% SDS-PAGE, the gels were transferred onto nitrocellulose membranes. The membranes were blocked overnight using 10% of skim milk in PBS and incubated with the primary antibody diluted 1: 3000 in 10% skim milk at room temperature (S1 Table). After three washings using PBS-Tween 0.1%, the membrane strips were incubated with the corresponding secondary antibody diluted 1:50,000 in PBST. The antigen-antibody reaction was developed using a West femto chemiluminiscence kit (Thermo) following the manufacturer's instructions.

Proteome comparisons

We compared our proteomic dataset with several comprehensive proteomic studies performed in *Echinococcus granulosus* [26], *E. multilocularis* [27], *Mesocestoides corti* [28] and the theoretical secretome of *T. solium* [59]. In the case of *Echinococcus* and *Mesocestoides* proteomes, the sequences of the identified proteins in those studies were obtained from WormBase (www.parasite.wormbase.org/). After, those sequences were blasted against the *T. solium* database (http://www.genedb.org/blast/submitblast/GeneDB_Tsolium). Then, the top-ranked protein was considered the *T. solium* homologue of a certain *Echinococcus* or *Mesocestoides* protein (the complete list can be found in S6 Data).

Ethics statement

The Animal protocol was revised and approved by the Ethical Committee for the Care and Use of Laboratory Animals at the Institute for Biomedical Research, Universidad Nacional Autónoma de México, under the license number ID 199 which follows the guidelines stated by the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Results

High-throughput proteomics of *T. solium* cysts

The proteome of the larval phase of *T. solium* was a complex mixture of parasite and host proteins. Using a TMT-multiplexed strategy, we were able to identify and quantify over 4,200 proteins across the nine cyst samples. Among these proteins, 3,368 were identified as parasite proteins, whereas 891 proteins were of host origin (Fig 1A). To our knowledge, this is the largest number of identified and quantified proteins in a multiplex assay for a cestode parasite to date. All the proteins were found across all the samples. More than 99.4% of proteins (including host or parasite) were identified in the nine samples. However, in the case of proteins TsM_000997700 and TsM_000195700, single peptides were only present in 8 samples and absent in only one. The protein changes observed between parasites obtained from different hosts and tissues were relatively discrete; the large majority of the identified and quantified cyst proteins remained at similar levels of expression, i.e., more than 3,200 proteins were found within a fold change of -1:1 (Fig 1B and 1C) for the SM and CNS cysts of the five pigs analyzed. Quantified host proteins were more variable between cysts from different tissues than between cysts from different pigs, thereby supporting the reproducibility of protein changes across the study (Fig 1B and 1C).

However, several parasite and host proteins were enriched for certain tissue localization of the cysts. For example, protocadherin alpha 6 ($P = 0.0003$), actin type 5 ($p = 0.0002$), a component of the γ-tubulin complex ($P = 0.0001$), a subunit of the splicing factor 3A ($P<0.0001$) and a protein associated with microtubules ($P = 0.0001$), were more abundant for cysts dissected from SM. On the other hand, proteins significantly associated to cysts dissected from the CNS of the pigs included a DNA topoisomerase 1 ($P = 0.0001$), alanine aminotransferase ($P = 0.0002$), an aldo-keto reductase ($P = 0.0002$), dnaJ protein ($P = 0.0001$), a protein containing a carbohydrate kinase domain ($P<0.0001$) and two hypothetical proteins ($P<0.0001$ and $P = 0.0003$) (Fig 1D). Two groups of host proteins were also found enriched for each tissue localization of the cysts (Fig 1E).

Host proteins in the cysts

Several studies consistently detected intact host proteins in protein extracts of Taeniid parasites [30, 60–64]. In a recent report, we identified 17 host proteins in the vesicular fluid of *T.*

solium cysticerci [63]. The results reported here, to our knowledge, are by far the largest set of host proteins reported within the tissues and fluids for any cestode parasite, suggesting a highly complex and close contact between the porcine and the cysts proteins.

It has been proposed that the host proteins are up-taken through a non-specific mechanism such as fluid phase endocytosis [60]. In our dataset, the host proteins were more variable than cysts proteins, this could be associated with a differential composition of the cysts microenviroment, CNS vs SM (S2 and S3 Data). Gene ontology analysis allowed determining that a diversity of host proteins were present; categories included metabolic enzymes involved in pathways like glycolysis or fructose/galactose metabolism, as well as signaling proteins including those participating in the integrin and ubiquitin-proteasome system. Functions of the uptaken host proteins included RNA binding, chaperones, oxidoreductases, ribosomal and isomerases (Fig 2A), those pathways were also enriched for the skeletal muscle proteome of the

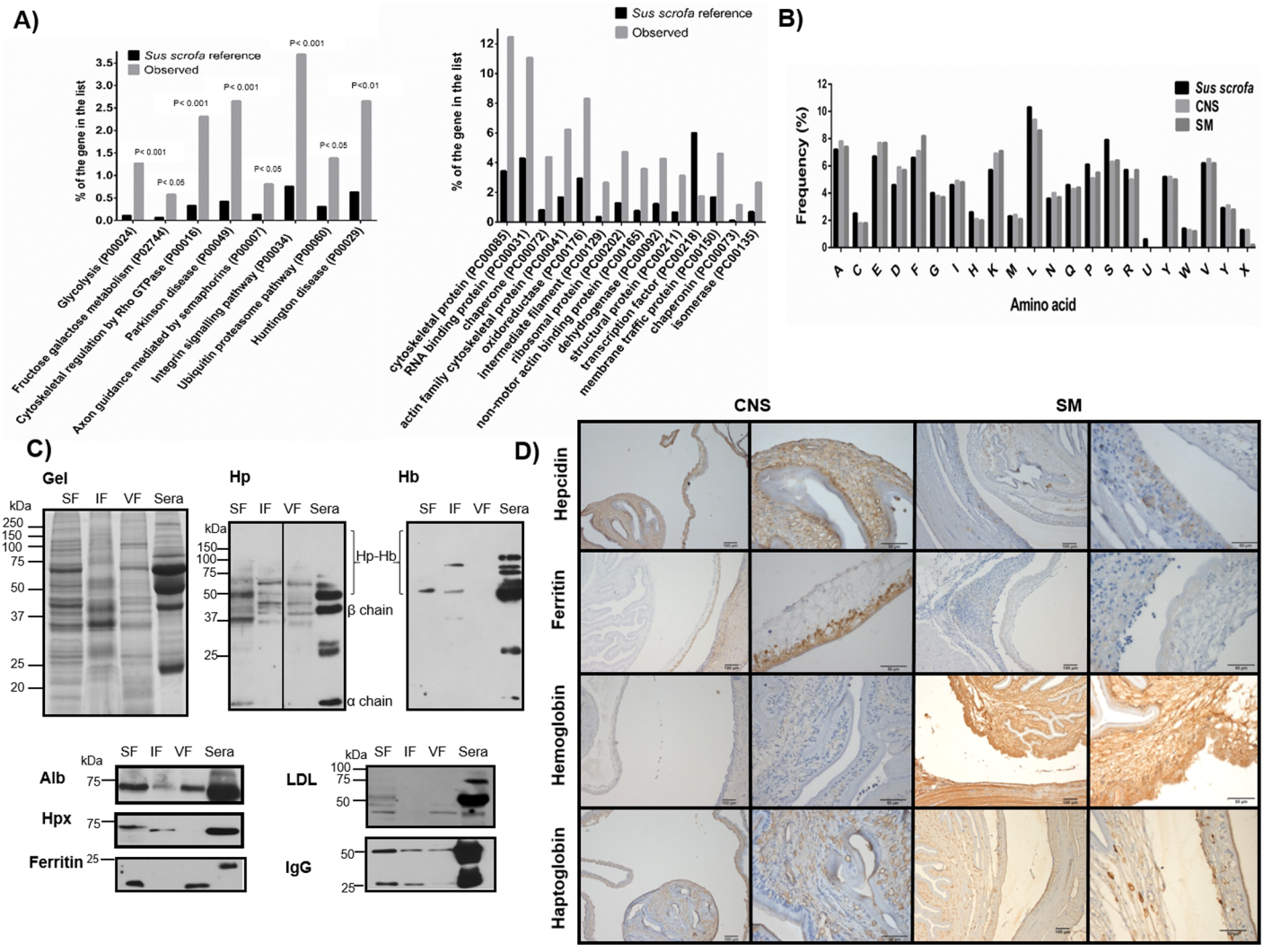

Fig 2. Annotation, composition and localization of host proteins in *T. solium* cysts. A) Significantly enriched host proteins in cysts extracts: metabolic pathways (categories with a P value<0.05) and molecular functions (categories with a P value <0.001). B) Amino acid composition of the host proteins associated with the central nervous system (CNS) and the skeletal muscle (SM) localization of the cysts (P value< 0.05), for comparison is shown the amino acid composition of the whole *Sus scrofa* proteome. C) Using specific antibodies several host proteins were identified in cyst's protein extracts. D) Immuno-localization of host proteins related to iron metabolism in cysts obtained from the CNS and SM; scale bars in the first and third column of images correspond to 100 μM whereas in the second and fourth columns correspond to 50 μM.

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pigs [65], suggesting that the up-taken host proteins reflect the composition of the cysts micro-environment (S5 Data).

It has been proposed that the cysts use the up-taken host proteins, including immunoglobulins, as a source of amino acids [66, 67]. The amino acid composition of the host proteins resulting from cysts obtained from CNS and SM was very similar; the biggest differences were found for aspartic acid and serine, reaching only an increase of 1.2% and a decrease of 1.5%, respectively (compared with *S. scrofa* proteome). The similar amino acid composition of up-taken host proteins in CNS and SM cysts is consistent with the concept of an unspecific mechanism for the host protein uptake by the cysts (Fig 2B).

We also explored the question of the host proteins tissue localization. Tissue localization studies were carried out to determine the distribution of several host proteins within the cyst's tissues. We selected host proteins related to iron metabolism in the host: haptoglobin (Hp), hemoglobin (Hb), hemopexin (Hpx), hepcidin and ferritin; other host proteins such as LDL, albumin and IgG, were used for comparison. Two experimental approaches were employed: cyst protein fractionation followed by western blotting and immuno-localization in tissue-sections. Through western blotting we found the majority of host proteins in the vesicular fluid, as well as in the soluble fraction of cyst's tissue (Fig 2C). Abundant host proteins such as IgG and albumin were detected in the three protein fractions tested (soluble and insoluble fraction of cyst´s tissue and in the vesicular fluid). Hemopexin (Hpx) was found in the tissue's extracts (soluble and insoluble fractions) and was not detected in the vesicular fluid; in the case of the insoluble fraction of cysts tissue, the immuno-reactive band was detected at the same molecular weight as the positive control (serum) and in the soluble fraction a band with a slightly increased molecular weight was detected. LDL was found present in the cyst's tissue soluble protein extract and scarce in the vesicular fluid (Fig 2C).

In the case of the host proteins related to iron metabolism, the most abundant ones found in the cyst's tissues were Hp and Hb; for Hp several bands were recognized (most of the bands were shared with the positive control), but others only appeared in the cysts extracts. In the case of Hb, immunoreactive bands were detected in the cysts tissue. After immune-histochemical analysis we found a conspicuous distribution of Hp and Hb in the cyst's tissues, particularly in tissue surrounding the spiral canal of the invaginated scolex. Hepcidin and ferritin were also found in the cyst's subtegumentary tissue, but in the case of ferritin in cysts extracts, the immuno-reactive bands had lower molecular weight than the band in serum (Fig 2C and 2D). All tested host proteins (with the exception of ferritin) were detected in their expected molecular weight in gels, suggesting that at least a fraction of the protein is intact.

This idea was explored further using as a model the uptaken host IgG. IgG was located on the outer surface of cysts in both CNS and SM cysts, with a more intense signal in SM cysts (Fig 3A). This localization was consistent with a previous report [68]. To explore the antibody activity of the uptaken host IgG; we purified host IgG from total saline extracts of SM cysts through affinity chromatography using protein G (Fig 3B). The purity of the isolated pig IgG was evaluated by SDS-PAGE and western blot (Fig 3C). Heavy and light IgG chains were detected at the expected molecular weights (50 and 25 kDa), suggesting that pig IgG was uptaken intactly. Two assays were performed to test the recognition of *T. solium* antigens by the purified pig IgG: ELISA and western blot using vesicular fluid (VF) or the insoluble fraction of the cysts as parasite antigens and the purified IgG as primary antibody (Fig 3D). The purified pig IgG reacted both, with the VF and the insoluble fraction of cysts antigens in a saturable and specific way. For western blotting, both antigenic fractions, VF and the insoluble fraction reacted with the purified IgG from the cysts (as shown in Fig 3E). As expected, several bands were recognized and were shared with the band obtained using sera from cysticercotic pigs (Fig 3D), indicating that uptaken host immunoglobulins retained their antigen-binding activity.

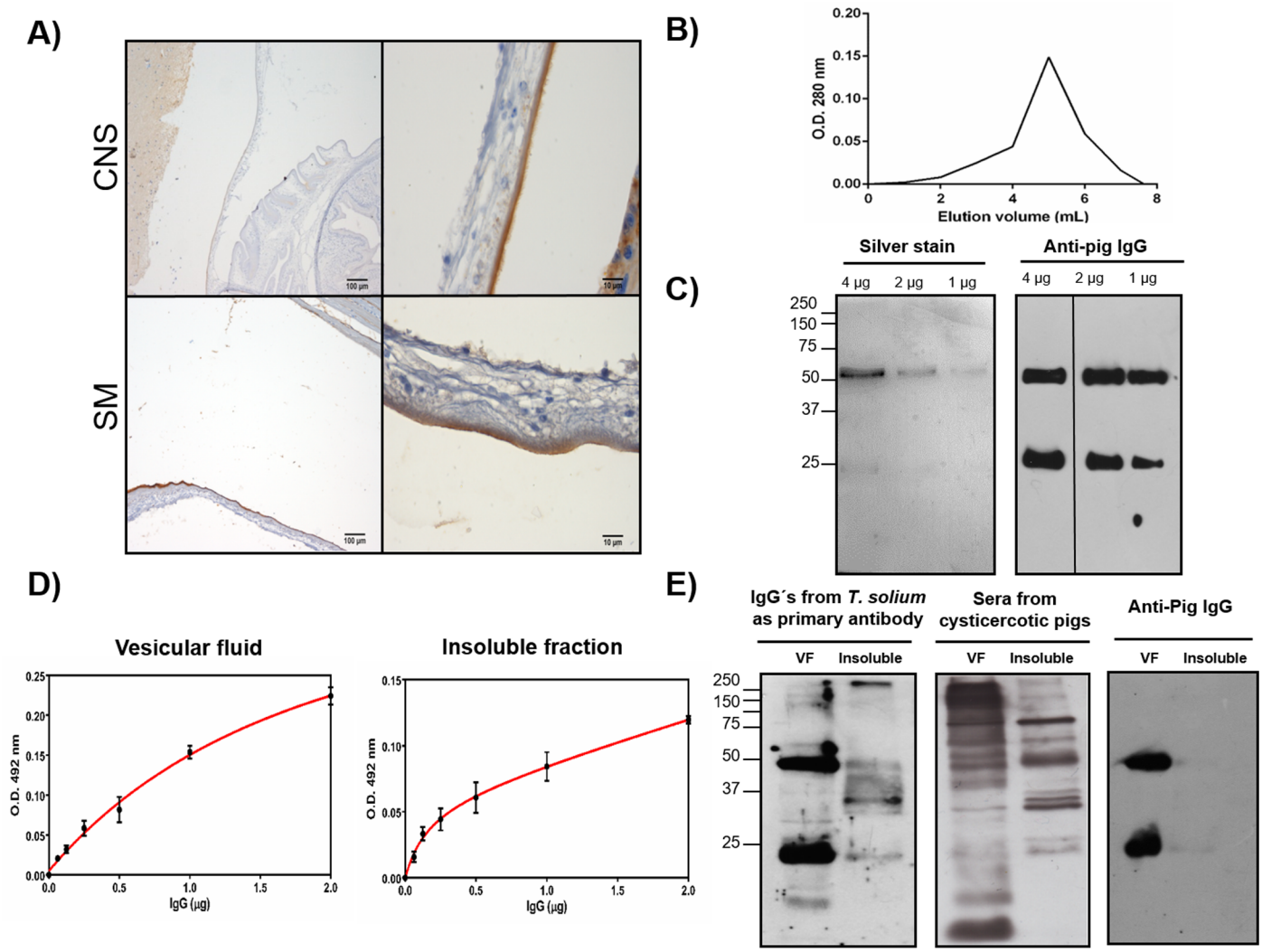

Fig 3. Immuno-localization and functional evaluation of host immunoglobulins purified from protein extracts of *Taenia solium* cysts. A) Immuno-localization of host IgG in cysts obtained from the central nervous system and skeletal muscle of pigs. B) Immunoglobulins present in total protein extracts of cysts were purified using a Sepharose 4B column coupled with Protein G. C). The purity of the bound IgG was evaluated by SDS-PAGE and western blotting using an anti-pig IgG coupled to HRP. D) The antibody activity of purified IgG was evaluated by ELISA. Samples of *T. solium* vesicular fluid and insoluble fraction were used to coat 96 well microtiter plates. Afterwards, the purified IgG was incubated overnight at 4˚C under slow agitation. Then, the antigen-antibody reaction was developed using a colorimetric method. E) Recognition of cysts proteins by the purified IgG through western blotting. Samples of *T. solium* vesicular fluid and insoluble fraction were resolved through SDS-PAGE and then transferred onto a nitrocellulose membrane. The purified IgG from cysts protein extracts was used as primary antibody (for comparison sera from cysticercotic pigs was also included) and an anti-pig IgG coupled to HRP was used as secondary antibody. Lower right panel shows the reaction with the secondary antibody alone as control.

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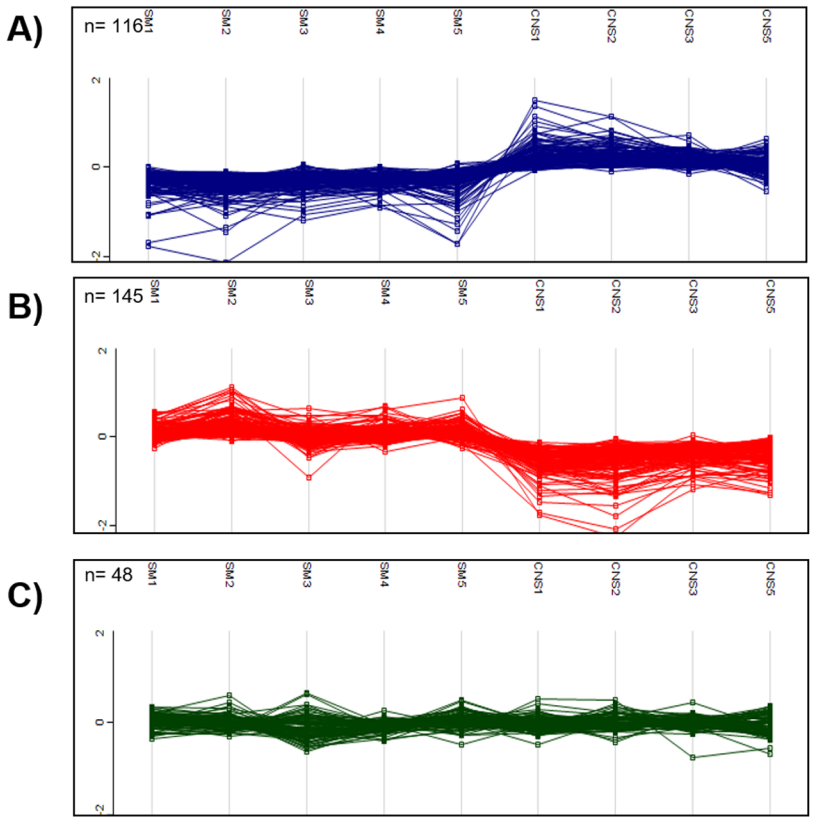
Proteomic changes of *T. solium* cysts obtained from SM and CNS

To explore the molecular functions and biological processes of the *T. solium* proteins identified for each tissue localization, we performed a *k*-means clustering analysis using a subset of cysts proteins with a significant fold change (P-value<0.01, n = 261). Two major clusters were identified, one for each tissue localization (Fig 4). For CNS cysts (Fig 4A), 116 proteins were abundant for parasites of this tissue localization (compared with SM parasites). These proteins were associated with metabolic processes, transport and phosphorylation. In the case of SM cysts (Fig 4B), methylation, signal transduction and microtubule-based processes were the most



A) Biological process	No.	Molecular function	No.
Metabolic process	15	Transferase activity	14
Transport	12	ATP binding	9
Phosphorylation	10	Hydrolase activity	9
Proteolysis	5	Zinc ion binding	9
Cell adhesion	4	Nucleotide binding	7

B) Biological process	No.	Molecular function	No.
Methylation	5	Hydrolase activity	13
Microtubule-based process	5	Zinc ion binding	13
Signal transduction	5	Actin binding	12
Phosphorylation	4	ATP binding	12
Mitotic nuclear division	3	RNA binding	6

C) Biological process	No.	Molecular function	No.
Phosphorylation	5	ATP binding	6
Ubiquitin related processes	3	Hydrolase activity	6
Mitochondrial processes	3	Transferase activity	5
ATP hydrolysis coupled proton transport	2	Kinase activity	4
GTP processes	2	Ubiquitin related processes	4

Fig 4. *k*-means clustering and associated biological/molecular functions for proteins of skeletal muscle (SM) and central nervous system (CNS) cysts. Cluster associated with the CNS (A), SM localization (B) and 48 constitutive proteins with the lowest coefficient of variation (C). The proteins included have a P-value <0.01 (n = 261). Only the most frequently observed categories and molecular functions (obtained using Argot2 algorithm) are show on the right panel. The y axis shows the Log_2 (relative abundance).

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frequently observed categories. For comparison, 48 proteins with the lowest coefficient of variation that remained in similar levels between cysts from different tissues were selected, including proteins associated with ubiquitination, phosphorylation and mitochondrial processes (Fig 4C, S5 Data). The relevance of those pathways in explaining both preferential localizations of the cysts, in terms of adaptation and survival within the host tissues, deserves future study.

As mentioned above, several host and parasite proteins were associated with the tissue localization of the cysts. To investigate more thoroughly that possibility, several previously described antigens (reviewed in [69]) were queried for in our database. As shown in Fig 3, several relevant antigens in cysticercosis (*i.e.*, paramyosin, GP50 and a calcium binding protein), were more abundant in SM cysts than in CNS cysts (Fig 5A). Moreover, we mined our database to search for other antigens enriched for each tissue localization. The case of the tetraspanin family appeared to be especially interesting as tetraspanins are relevant antigens in schistosomiasis [70] and hydatidosis [71]. Our results showed the expression of five members of the tetraspanin family; of these, two proteins were enriched in the CNS localization and one in the SM localization of the cysts, while the other two have no detectable changes. These proteins: TsM_000744700 and TsM_001075800 were subsequently analyzed (Fig 5B). Initially, the large extracellular loop of these proteins was deduced using several algorithms (see Materials and Methods). In those tetraspanins, the protective domain (the protein region associated with protection in vaccination trials) is located within the large extracellular loop [70, 71]. Therefore, this portion of the protein was analyzed for antigenicity using algorithms for B cell epitope-

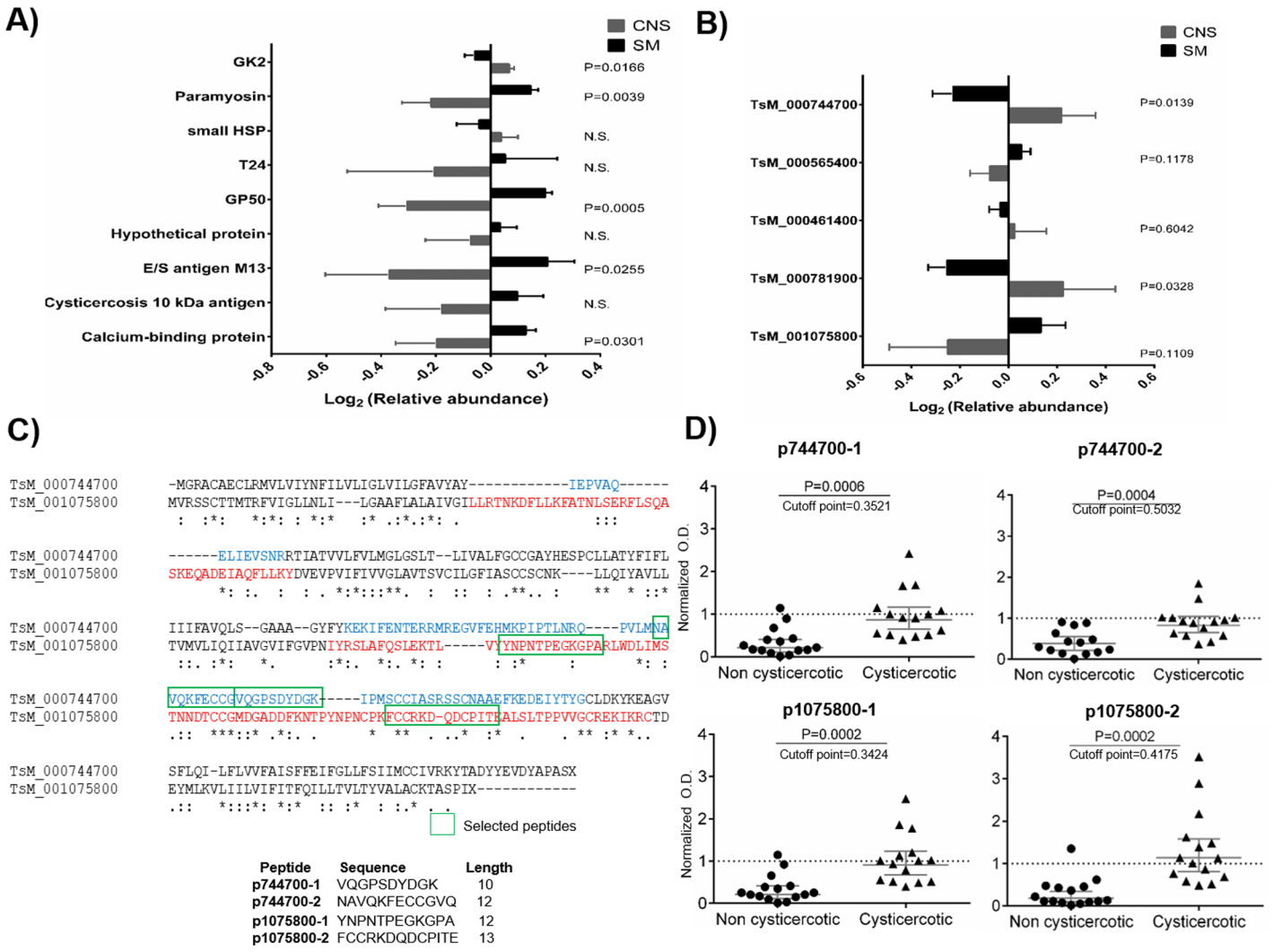

Fig 5. Protein abundance of several well-studied antigens in *T. solium* cysts antigens. A) Relative abundance of cyst's antigens obtained from the skeletal muscle (SM) and central nervous system (CNS); B) Relative abundance of tetraspanin proteins quantified in our proteomic analysis for SM and CNS cysts; C) Alignment of SM- and CNS-abundant tetraspanins. The extracellular loops are bold in red and blue, selected peptides are marked by a square. Listed at the bottom are the selected peptide sequences and length. D) Recognition of the synthetic peptides by non cysticercotic and cysticercotic pig sera. The normalized optical density (O.D.) is the result of dividing each individual O.D. by the cut-off value (mean value of non cysticercotic pigs plus two standard deviations). P-values are shown at the top of each figure.

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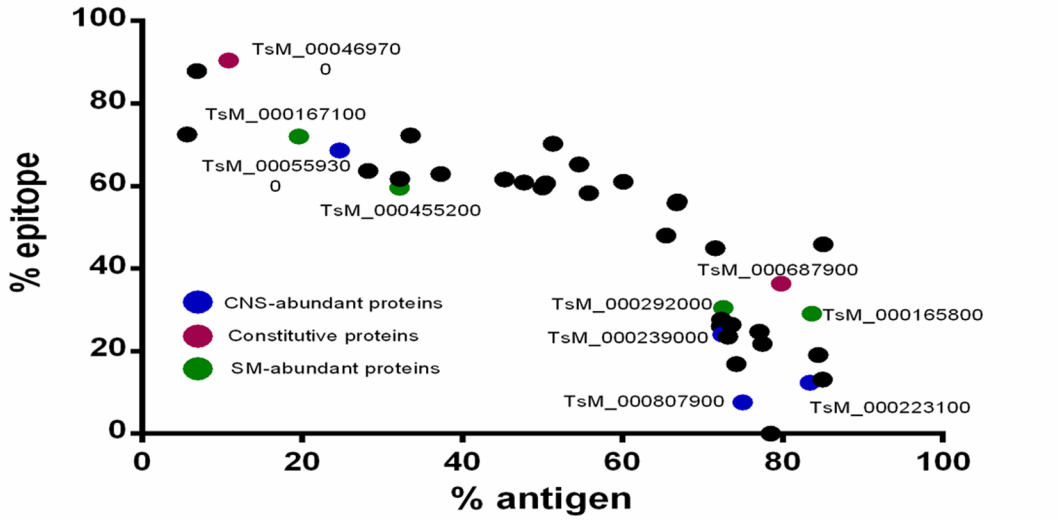
prediction. Two peptides were chosen from each protein and synthesized through a commercial service (TsM_000744700: VQGPSDYDGK, NAVQKFECCGVQ; TsM_001075800: YNPNT-PEGKGPA, FCCRKDQDCPITE) (Fig 5C). To explore if these four peptides (p744700-1 and 2; p1075800-1 and 2) were recognized by antibodies in the sera of cysticercotic pigs, two groups of 15 sera (cysticercotic and non cysticercotic) from pigs bred in rural endemic areas were used. As shown in Fig 5D, the four peptides were recognized by several infected animals, with the peptide FCCRKDQDCPITE (p1075800-2) having the strongest antibody recognition.

The differential abundance of several antigenic proteins (GP50s, paramyosin, E/S protein M13, calcium-binding protein, tetraspanins, etc.) between SM and CNS cysts, provides evidence about the presence of antigens that were enriched for SM or CNS cysts. Our next goal was the identification of highly antigenic tissue-enriched proteins; here we defined a tissue-

A)



B)

Protein	*T. solium* accession number	Fold Change CNS	Peptide	Peptide designation	Hydrophobicity	Hydropathicity	Hydrophilicity	Charge
Hypothetical protein	TsM_000559300	2.98	TLRQAPQIANPRARE	p559300	-0.42	-1.18	0.47	2
Hypothetical protein	TsM_000807900	1.55	PERVPKRVETFYADVIAPW	p807900	-0.16	-0.4	0.11	0
Palmitoyltransferase ZDHHC24	TsM_000223100	0.84	YCLRCETVRPPRAHHCSQCDV	p223100	-0.32	-0.59	0.12	2
Protein of unknown function DUF1620	TsM_000239000	0.56	LDPILGVPLNARTLKPAAAAE	p239000	-0.02	0.39	-0.06	0
Cystatin B (Stefin B)	TsM_000687900	0	TGQEPSEVKITEVSR	p687900	-0.29	-0.97	0.68	-1
Hypothetical protein	TsM_000469700	0	SEETYEHVEHARRPSRRPP	p469700	-0.55	-2.15	0.99	1
Hypothetical protein	TsM_000292000	-0.58	ERHCNAPQKHHARCDGHPHPS	p292000	-0.43	-1.92	0.49	3.5
Hypothetical protein	TsM_000167100	-0.6	GENTQHYKYRVGEKEK	p167100	-0.5	-2.3	0.9	1.5
Pinin	TsM_000455200	-0.67	RRFVANGAPPSVFSR	p455200	-0.24	-0.31	0.05	3
Hypothetical protein	TsM_000165800	-1.3	EGCKSRKGCSCEPGKCACV	p165800	-0.29	-0.48	0.61	2

Fig 6. Selected peptides from skeletal muscle (SM) and central nervous system (CNS) abundant cysts proteins. A) Proteins predicted with high antigenic and/or epitope content. B) Ten proteins were selected: four SM (green) and four CNS (blue) abundant proteins. Two constitutive proteins were also chosen. One peptide was then selected for each protein.

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enriched protein as one with differential abundance between CNS and SM cysts (p value< 0.01). The high antigenicity was defined using B cell epitope and antigenicity predictors, see Materials and Methods. First, we selected 261 proteins with a significant fold change (P value< 0.01) and 48 proteins with the lowest coefficient of variation, for comparison. Those proteins were analyzed through several antigenicity algorithms (see Materials and Methods and S2 Fig). Several proteins were predicted as strongly antigenic by one algorithm (antigen/epitope content $\geq$ 70%). Another group of proteins was also predicted as highly antigenic by both algorithms, although with a lower antigen/epitope content (50%) (Fig 6A).

Using this approach, 40 highly antigenic proteins were identified (the complete list of proteins can be found in S4 Data). To experimentally test the reliability of our antigenic prediction, 10 proteins were selected (Fig 6A and 6B). Of these proteins, four were enriched for CNS cysts, four were enriched for SM cysts and two proteins with a low coefficient of variation between CNS and SM cysts, none of these proteins had been studied before in *T. solium*. Then, a single epitope was selected for each protein (selected epitopes had to be predicted by both

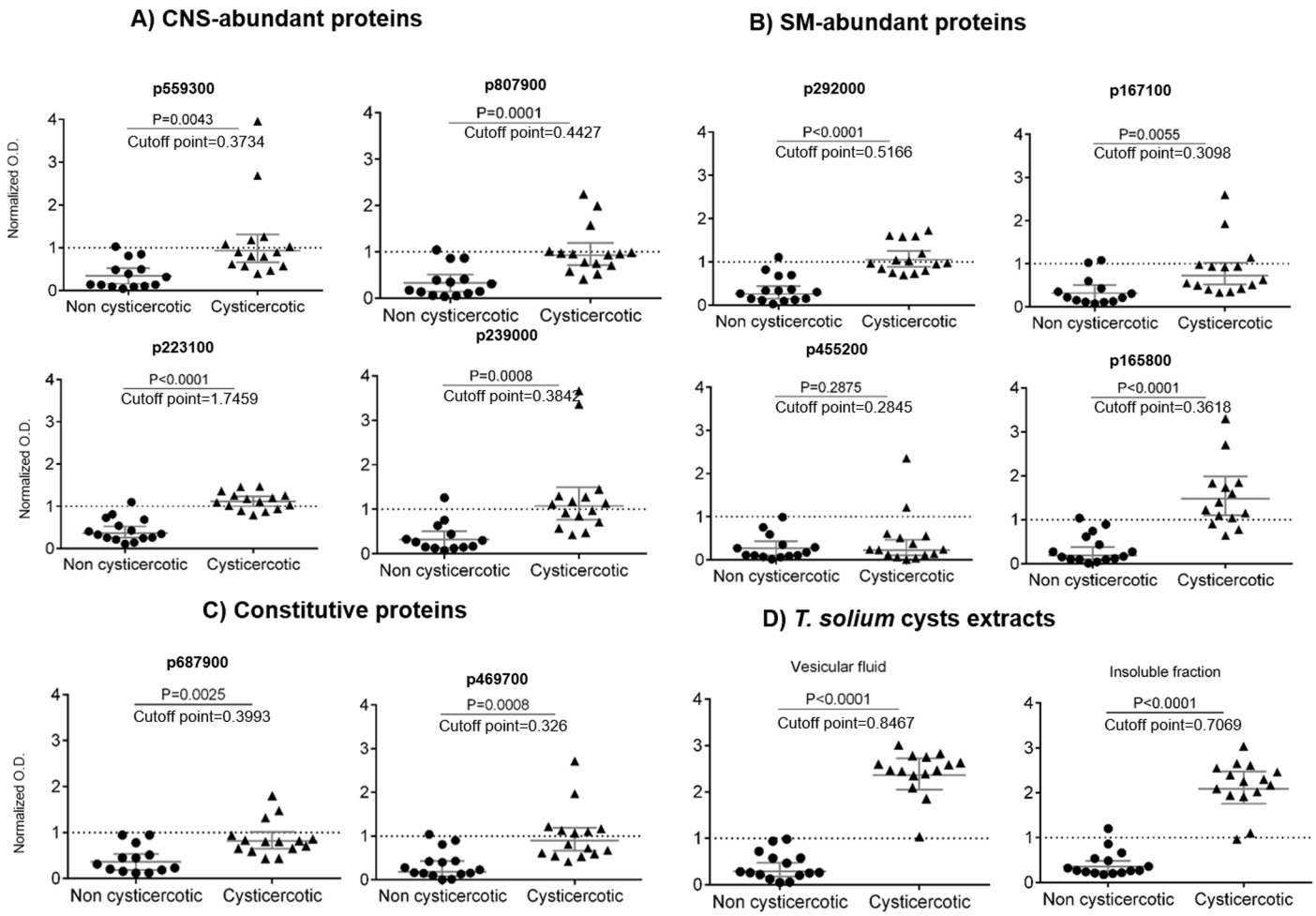

Fig 7. *Taenia solium* peptide recognition by cysticercotic and non cysticercotic pig sera through ELISA. A) Peptides from central nervous system, B) skeletal muscle abundant proteins, C) constitutive proteins and D) cysts protein extracts. Microtiter plates were coated with the peptides or extracts and incubated with sera from non cysticercotic (n = 15) and cysticercotic (n = 15) pigs, all bred in rural endemic areas. The normalized optical density (O.D.) is the result of dividing each individual O.D. by the cut-off value (mean value of non cysticercotic pigs plus two standard deviations). P-values are shown at the top of each figure.

https://doi.org/10.1371/journal.pntd.0005962.g007

algorithms). After the synthesis of the antigenic peptides (Fig 6B), they were evaluated for antibody recognition by ELISA, using the same two groups of sera mentioned above. For a peptide to be considered as a valid antigen, the difference between the recognition of the cysticercotic and the non cysticercotic pig sera had to be statistically significant.

Nine of 10 peptides (with the exception of one based on pinin) were significantly recognized by the sera from cysticercotic, in comparison with the sera from non cysticercotic pigs (Fig 7). These data, suggest that the proteins from which the peptides were originated are immunologically recognized in porcine cysticercosis; then we have identified several tissue-enriched antigens; interestingly, a peptide that was enriched for SM (p165800) and other for CNS (p223100) cysts, produced the highest difference between the cysticercotic and non cysticercotic pig sera (Fig 7A and 7B). The recognition by the IgG present in the sera of cysticercotic pigs, suggested that there is a subset of tissue-enriched antigens for cysts located in different host tissues. However, whether the rest of the predicted antigenic proteins are valid antigens in cysticercosis requires further screening.

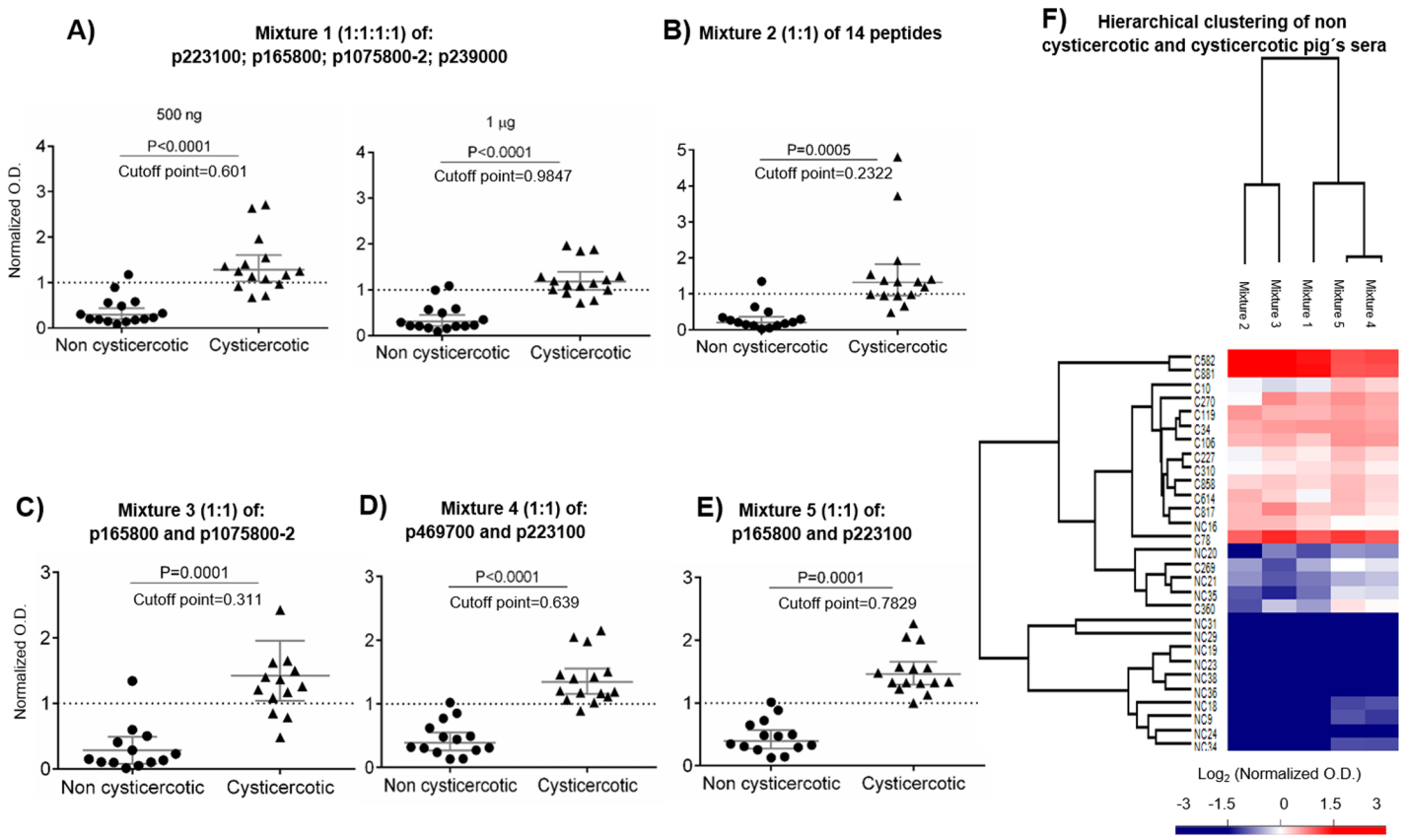

Fig 8. Peptide mixtures as potential diagnostic agents for *Taenia solium* cysticercosis. Several peptide mixtures were used: A) The best four individual peptides (using 500 ng or 1 µg to coat each microtiter plate well). The rest of the peptides combinations were used at 500 ng. B) Mixture of all 14 peptides synthesized in this study, C) Mixture of the peptides derived of skeletal muscle (SM) abundant proteins, D) Mixture of one peptide of a central nervous system (CNS) abundant protein and one of a constitutive protein and E) Mixture of peptides from SM and CNS cysts. The normalized optical density was calculated by dividing each individual O.D. by the cut-off value (mean value of non cysticercotic pigs plus two standard deviations). P-values are shown at the top of each figure. F) Heat map showing the individual response to antigenic peptide mixtures. The normalized optical density was transformed using Log_2. White represents values near to the cut-off point, red represents values over the cut-off point (positive samples) and blue represents values below the cut-off point (negative samples).

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Diagnostic potential of tissue-enriched antigens

The tissue-enriched antigens could be exploited for the improvement of current diagnostic tools for cysticercosis. A diagnostic test for cysticercosis would ideally include antigenic determinants for each possible tissue localization of the cysts, as well as antigens that are not affected by the tissue-localization of the cysts. To explore the diagnostic potential of those tissue-enriched antigens, several combinations of peptides were used in mixtures. The initial mixture was made using the peptides that were previously found to produce the highest optical densities, when tested separately with the same cysticercotic pig sera: p223100, p165800, p1075800-2 and p239000 (1: 1: 1:1); two concentrations were employed (Fig 8). As shown in Fig 8A, the lowest concentration produced better results. However, not all sera from the cysticercotic animals produced a significantly positive reaction when compared with the non cysticercotic pig's sera. Other combinations of synthetic peptides were also tested, including a mixture of the 14 peptides that produced the worst performance (Fig 8B). We also tested combinations of peptides from SM-abundant proteins (Fig 8C), or/and SM constitutive proteins (Fig 8D). Interestingly, when the mixture included the best peptide for each tissue localization, p223100

for CNS and p165800 for SM, 14 out of 15 cysticercotic and non cysticercotic pig sera were clearly differentiated (Fig 8E). However, we were not able to improve the performance obtained using the two crude protein extracts from the cysts, indicating that the 'ideal' antigenic subset will require further investigation.

In this study, each pig showed a differential response for each peptide and peptide mixture (Fig 8F); the idiosyncrasy of individual humoral host immune responses against *T*. *solium* cyst antigens is well known [3], as it is in other infectious diseases [72–74]. In addition, the considerable genetic/antigenic variation between cysts obtained from different endemic areas it is frequently reported [75–77].

The immuno-diagnosis of an infectious disease is often performed using a single antigen, i.e., using a single protein/peptide or antibody to discriminate between healthy and infected hosts. In the case of infections caused by *E*. *granulosus*, the use of AgB and 8 kDa proteins have been tested as diagnostic agents. However, new methodological approaches are needed for parasite infections such as schistosomiasis, echinococcosis and cysticercosis, to discriminate between infected hosts with low-parasite loads [1, 2; 78, 79].

A novel approach involves the machine-learning models that have proven useful in the diagnosis and prediction of several diseases. Several algorithms have been developed for the diagnosis of breast [80], colorectal [81] and non-small cell lung cancer [82]. Distinct antigenic response patterns (ARP) may constitute better representations of the pathogen's fingerprints than single-peptide responses. Thus, a multi-antigenic peptide testing (MAPT) can identify such ARP for each infection.

To explore the viability of a MAPT using our synthetic peptides, we constructed an antigen response space (ARS), where each individual is represented by a single point. The position of each individual point (one pig's serum) depends of its antigenic response to several peptides. For k antigenic peptides considered, k OD measures determine the individual position in ARS. In this sense, an ARP can be defined as a particular region in ARS where all infected hosts are present. Machine-learning algorithms can be directly used to identify boundaries of such regions. It should be noticed that a given ARS could represent a corresponding antigenic peptide subset.

To explore the potential of the synthetic peptides to define an ARS for pig's cysticercosis, all possible combinations of peptides were evaluated. Analysis was performed taking 14 single peptide measures, 5 peptide mixtures measures, and a combination of both. Thus, 16,383, 31 and 524,287 possible ARS representations were evaluated in each case. Evaluations were performed with leave-one-out cross validation and a support vector machines as a classifier (see Materials and Methods). Number of errors achieved (expressed as percentage) by the best and worst combination for all possible number of peptides were used simultaneously for ARS constructions. As depicted in Fig 9A–9C, the use of peptide combinations usually produced better results than individual peptides. For comparison, we produced an image of ARS using peptide combinations and cysts protein extracts (Fig 9D and 9E). Using this approach we were able to discriminate infected versus non-infected pigs. This discrimination resulted in a similar performance to the one obtained using complex crude cysts extracts (Fig 9D and 9E).

Based on our current preliminary results, developing an accurate immunodiagnostic test for cysticercosis, requires a number of specific antigens from cysts of different tissue localizations within the host, as well of antigens from cysts obtained from different geographical areas. In addition, the use of novel-analytical tools such as machine-learning models, can efficiently discriminate between healthy and infected hosts.

In contrast, we tested this approach using sera from non-cysticercotic (n = 8) and neurocysticercotic patients (n = 12). As expected, our ARS approach produces better results than using a single peptide or peptide mixture. However, sensitivity was about 75% (S4 Fig) indicating

A)

	Number of individual peptides													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Best	10	6.67	10	6.67	6.67	10	10	10	10	10	10	10	10	10
Worst	100	70	46.7	46.7	43.3	40	40	36.7	36.7	33.3	30	23.3	23.3	10

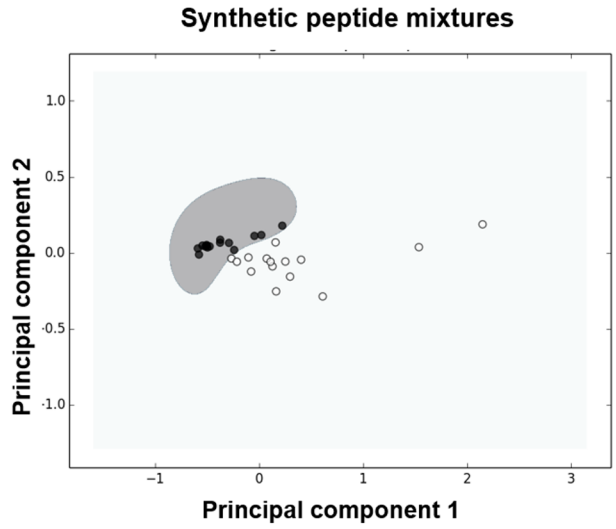
B)

	No. of peptides mixtures				
	1	2	3	4	5
Best	3.33	3.33	3.33	3.33	3.33
Worst	23.33	10	13.33	6.67	3.33

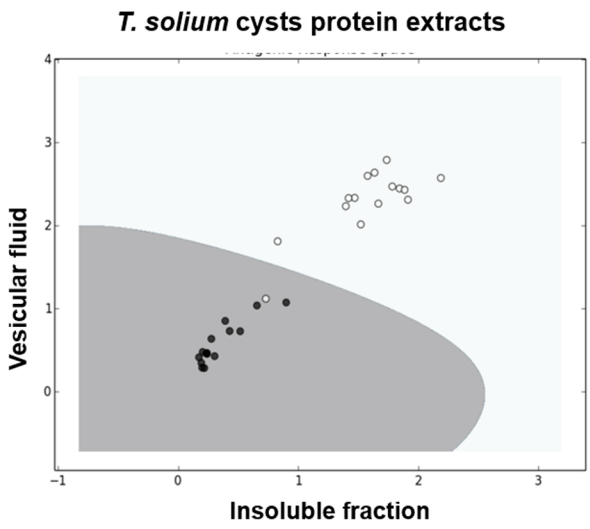
C)

	Combined use of individual peptides and peptide mixtures																		
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
Best	3.33	3.33	3.33	3.33	3.33	3.33	3.33	3.33	3.33	3.33	3.33	3.33	3.33	3.33	3.33	3.33	3.33	3.33	6.67
Worst	100	70	46.67	46.67	43.33	43.33	40	40	40	40	40	30	26.67	23.33	16.67	16.67	13.33	10	6.67

D)



E)



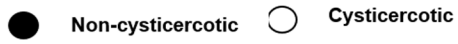


Fig 9. Antigenic response space. The raw optical densities were analyzed using machine-learning methodologies. We determined the performance of (A) individual peptides measurements, (B) peptide mixtures measurements and (C) the combination of both strategies. Best/worst refers to the percentage of error (taking into account our n = 30; an error of 10 implies that three pig sera were not discriminated). Visual representation of the antigenic response space for multiple antigen testing (D) and *T. solium* cysts protein extracts (E).

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that selection of adequate peptides/proteins for diagnosis in humans will require separate studies.

Discussion

Several studies have recently focused on deciphering the proteome in *Taeniid* parasites. The proteomes of the whole larva, the protoscolex, the pre-adult stage and some immunogenic proteins have being characterized for *Echinococcus spp*. [83–87]. The proteome of *T. solium* has been less explored, although the composition of the excretion/secretion products [88], the proteins of activated oncospheres [89] and a small group of immunogenic proteins have been reported [62]. Moreover, an algorithm for the identification of unique mass spectra for *Taeniid* parasites has been developed [90]. In addition to the fact that these are still initial efforts, a relevant aspect that remains uncharacterized refers to the proteomic changes associated with the tissue localization of the cysts in the host. Knowing these changes might be essential to

understand the tissue preference of the cysts. Although *T. solium* cysts can establish in a variety of tissues they appear to show preference towards the skeletal muscles and several locations in the central nervous system, including the brain.

In this report, we describe the proteome of *T. solium* cysts obtained from CNS and SM of infected pigs. We used state-of-the-art quantitative isobaric proteomics to identify and quantify more than 4,200 proteins in a single assay. This is the largest number of identified and quantified proteins so far described for a cestode parasite. A challenging finding is the high amount of host proteins in all crude extracts of *T. solium* cysts [60–63]. The presence of intact host proteins in the extracts from cestode parasites has been known for six decades [60–64, 91]. Some recent reports on *Echinococcus spp*. proteomics have also identified a variety of host proteins in the protein extracts of this cestode, for example, 43 proteins were identified in the cysts fluid [27] and up to 293 proteins were identified as excretion/secretion (E/S) products [59]. In our study, we were able to quantify 891 proteins of host origin, the highest number of host proteins identified for a cestode parasite, which brings back the interest on the role of host proteins in the cyst's physiology. However, if *Taenia spp*. contains more host proteins than *Echinococcus spp*. remains to be elucidated; both studies were performed using different proteomic (as well as sampling) strategies. Here, we benefited from high throughput and state-of-the-art multiplexed proteomics that enabled us to identify a significant number of cyst and host proteins.

Herein, one out of each five proteins identified resulted of host origin. These identified host proteins are involved in a number of metabolic, physiologic, signaling and regulatory processes for the pig. It is worth remembering that the *T. solium* genome revealed a greatly simplified organism, lacking a number of metabolic processes (biosynthesis of amino acids, fatty acids, etc.) as a result of its evolutionary adaptation to parasitism [14]. It is conceivable that the host proteins present in the cysts (associated with metabolic and signaling functions) could play a role for the parasite, beyond being a mere source of amino acids. In the case of *E. granulosus*, the identified host proteins were also associated with metabolic processes, response to stimuli and regulation of biological processes [26]. It is conceivable that some host proteins retain their function and could play a role on metacestode physiology.

In order to explore this idea, we carried out functional assays and tissue localization studies for a group of host proteins. For example, highly abundant host proteins like albumin and IgG were found in all protein fractions obtained from the cysts, indicating that their presence is ubiquitous in parasite's tissues and fluid. Another example were LDL and hemopexin, which were found in the cyst's tissue but were scarce in the vesicular fluid. Cestode parasites have a reduced capacity for lipid biosynthesis [14]. In the case of *Schistosoma mansoni* (trematode), several proteins have been identified as LDL-binding proteins [92, 93] and LDL was found associated with parasite's tegument [94]. In our study, we found the LDL protein associated with cysts tissue. However, if *Taenia spp*. parasites have a subset of specialized proteins to bind and uptake LDL from the host remains to be seen.

Hpx was abundant in the cysts tissue extracts, while scarce in the VF; interestingly, in the soluble fraction of cysts tissue, the Hpx band showed a slight increase in the apparent molecular weight, while in the insoluble fraction the band was detected at the same molecular weight as in the control serum. In the case of Hp and Hb, several bands were detected (especially for Hp); this finding can be explained by the presence of Hp in different forms: the free form, as well as in complexes with Hb. Furthermore, some bands could be the result of Hp/HpHb complex degradation by cysts proteases. We have recently described that intact and functional Hp are present in the cyst's tissue and could be associated with the iron uptake by the cysts [64]. Here we widen the scope of our investigation on the possible involvement of host proteins in the cyst's iron metabolism. We carried out tissue localization studies for several host proteins

associated with the iron metabolism (hepcidin, ferritin, hemoglobin and haptoglobin). The four proteins were detected in the cyst's tissue, being Hb and Hp the most abundant and widely distributed within the cysts, suggesting that a relevant portion of iron uptake by the larvae might be supported by these host proteins.

Hepcidin is a master regulator of iron metabolism produced by the liver and its active form is a peptide of 25 amino acids. Hepcidin binds to ferroportin and induces its lysosomal degradation, thus decreasing the iron export by the target cell [95]. However, hepcidin signaling appears to be restricted to mammals [96]; at least, no reports about a cestode homologue ferroportin are available; future studies are need to explore the role (if any) of hepcidin in cestodes biology. On the other hand, ferritin is considered the major iron storage protein [97]. Using specific antibodies, we found ferritin present in the subtegumentary tissue of cyst bladder wall, suggesting that it could also play a role for the parasite, the bands that were detected in protein extracts of cysts had a decrease in the molecular weight (compared with the control). However, after searching the *T. solium* genome database for ferritin, we found that the cyst has two homologues with a very similar predicted molecular weight, therefore, host and parasite ferritins appears to be undistinguishable in molecular size (≈20 kDa). Therefore, it is possible that the immuno-localized ferritin is a combination of host and cysts ferritin stocks. Whatever the source of ferritin is, its localization in close contact with the host tissue suggests that cysts accumulates specialized molecules for iron storage.

Albumin appears to be involved in the maintenance of parasite's osmotic pressure in the vesicular fluid, fulfilling a similar function to the role it plays for the host [61]. In addition, immunoglobulins have been proposed as a source of amino acids for the cysts [66, 67].

We determined the antigen-binding activity of host IgG purified from total protein cyst's extracts through ELISA and western blotting, testing their ability to react with cysts antigens using two protein crude extracts. Purified IgG from the cysts showed a clear antibody activity through the recognition of several antigenic bands (those bands were also shared when sera from cysticercotic pigs were used), suggesting that at least a part of the uptaken host IgG were specific antibodies directed against cysts proteins.

Several other host proteins could also retain their function. Many other uptaken host proteins could play a physiological role, for the parasite. It could also be that potentially deleterious host proteins are simply removed from the host-parasite interface. Ascertain if the host proteins play functions in the physiology of the cysts is an open area of research that could disclose a number of unexpected results.

With respect to the *T. solium* cysts proteins identified and quantified in our proteomic assays, the changes found between CNS and SM cysts were discrete; more than the 90% of the identified and quantified proteins (>3,100) were grouped within a fold change of -1 and 1. A tissue-enriched protein pattern (including cysts and host proteins) was associated to each cysts tissue localization. These protein patterns could represent a homeostatic adaptation to the biochemical conditions in different tissue environments (SM vs CNS). Our dataset was compared with others previous proteomic reports for helminths [26–28; 59]. From our dataset, 167 proteins were considered excretion/secretion proteins (compared with the *T. solium* theoretical secretome [59]) (S6 Fig, and S6 Data). In addition, almost 30% of the *T. solium* gene products were considered 'hypothetical', meaning that those genes could not be functionally annotated. Among those hypothetical proteins, the expression of 357 proteins was validated here (S6 Fig and S6 Data). After comparison with the proteomes of *E. granulosus*, *E. multilocularis* and *M. corti*, 14 proteins were common to the four cestodes (S6 Data); those proteins included: a 14-3-3 family member, a fatty acid binding protein, a protein with EGF domain, a lactate dehydrogenase, etc. Indicating that the proteome of cestode parasites are usually highly complex mixtures of parasite and host proteins. The relative consistence of high amounts of host proteins

and the relevance of those common proteins identified between the four cestodes need future investigation.

After this initial characterization, several antigens were found enriched in the SM localization of the cysts (paramyosin, a calcium-binding protein, E/S antigens, etc.).

Similarly, other proteins belonging to different families (tetraspanins and GP50s antigens) were also differentially found between CNS and SM cysts. Tetraspanins are integral membrane proteins directly exposed to the host [70, 71] and have been considered vaccine candidates in several helminth infections [98–100]. However, since tetraspanins are highly polymorphic, vaccination trials have produced controversial results [101]. For *T. solium*, the tetraspanin family has been poorly characterized, only one member (T24) with a good performance as a diagnostic antigen [102], has been described. In this report, five members of the tetraspanin family were quantified; two were associated with the CNS localization of the cysts and one with the SM localization. To investigate if those proteins are antigens during cysticercosis, two peptides were chosen from the amino acid sequence of either TsM_000744700 or TsM_001075800. The four peptides were recognized by antibodies occurring in the sera of naturally infected pigs (four pigs showed a significant recognition of both proteins). The strongest recognition was associated with p1075800-2, derived from the SM-enriched tetraspanin. Regarding *T. solium* T24 tetraspanin, glycosylation strongly influenced its antibody recognition [102]. It remains to be seen whether the antibody-recognition of the tetraspanins studied here can be increased using recombinant and glycosylated forms.

The diagnostic antigen GP50, is a GPI-anchored glycoprotein with affinity to *Lens culinaris* lectin, this protein is a promising candidate agent for the immunodiagnosis of NC. Nevertheless, it showed a poor performance when sera from patients having a single viable cyst in the CNS were tested [103, 104]. Two GP50 proteins were found among our proteomic data (S5 Fig). The "canonical" GP50 was associated with the SM localization of the cysts, while a "truncated" form was slightly increased in the CNS cysts. The truncated CNS-abundant GP50 lacked a predicted GPI-anchoring site, in addition to less disulfide bonds and predicted N-linked glycosylation sites. A diagnostic test based on the combination of these two GP50s deserves further study.

We hypothesized that several tissue-enriched antigenic proteins can be used as markers for the tissue localization of cysts. This idea was explored through a combination of theoretical and experimental approaches. Initially, 261 proteins with significant fold changes were selected for epitope prediction using two algorithms; 40 proteins with high antigenic or high epitope content were found enriched either in SM or CNS cysts. Ten proteins were then selected for experimental testing through the synthesis of a single antigenic peptide chosen for each protein. All peptides were recognized by antibodies in the sera of infected animals, but with great variability. When tested with sera from cysticercotic and non-cysticercotic pigs, 9 out of 19 peptides were significantly recognized (p value < 0.01) by the sera from infected animals. The only exception was the peptide derived from a pinin: p455200. These results support the existence of tissue-enriched antigens. The immunodiagnostic potential of those peptides was also tested: peptides were used alone or in mixtures for recognition by the same group of cysticercotic and non-cysticercotic pig's sera. The best results were obtained with several peptide combinations; in fact, the best combination included peptides derived from SM and CNS-enriched cysts proteins. This can only be considered as an initial study about the potential utility of tissue-enriched antigens; future studies are being conducted using the full recombinant proteins to increase the sensitivity of the immunodiagnostic tests.

A number of antigens have been tested as diagnostic agents. We hypothesized that testing multiple antigens, could produce a better strategy to distinguish between healthy and cysticercotic pigs. A machine-learning strategy was employed and 540,701 combinations were analyzed. Peptide mixtures produced better results than individual peptides. Using five mixtures

of peptides allowed to discriminate between cysticercotic and non cysticercotic sera. Our results suggested that, at least five different antigenic determinants are required in order to develop an efficient diagnostic test, able to differentiate between the two groups of sera. Unfortunately, as those synthetic peptides were tested with sera from naturally infected pigs, we do not have relevant information about those infections, such as primary vs secondary infection, co-infections, and nutritional status.

The tissue-dwelling, larval phase of cestodes is usually characterized by host immunomodulatory activities [105]. The observation that some SM and CNS enriched proteins were recognized by antibodies present in the sera of infected animals, could have implications on our understanding about the modulation of the host immune response by cestode parasites.

Conclusions

High-throughput proteomics using a TMT-multiplexed strategy allowed the identification and quantification of over 4,200 proteins across the nine samples of *T. solium* cysts. The *T. solium* cyst's proteome constitutes a mixture of host and parasite proteins (one of each 5 proteins were of host origin). *T. solium* cysts obtained from either naturally or experimentally infected pigs have several proteins and antigens enriched for SM or CNS localizations. The identified host proteins were highly diverse and were involved in a number of metabolic and signaling processes. Through immuno-localization studies carried out for several host proteins, we found that they are localized in a variety of cysts tissues (tegumentary or subtegumentary tissues in the bladder wall or in the scolex). Other host proteins were detected in the vesicular fluid. Here, we also showed that several host proteins are uptaken intactly, as an example, IgG retained their antige-binding activity. Exploring the functional activity of host proteins in the cyst's tissue certainly deserves further studies.

The parasite's antigens that were found enriched for a certain tissue, could be used for the design of highly effective immunodiagnostic methods by combining the peptides/proteins derived from SM and CNS enriched antigens. Using several peptide mixtures and machine-learning models we were able to distinguish between cysticercotic and non cysticercotic pigs with an efficiency that is comparable to the current diagnostic methods using complex cyst's crude extracts, however, the appropriate subset of tissue-enriched antigens remains to be identified. Development of an optimal immunodiagnostic test for human and porcine cysticercosis requires the use of SM and CNS enriched antigens; variation of those antigens in the cysts isolated from different endemic areas remains to be analyzed, though. This assessment could be crucial for the improvement of the current diagnostic tests. Characterization of the antigenic proteins enriched with each tissue localization of the cysts, is worth studying not only for the design of more efficient immunological tests for human and porcine cysticercosis, but also because they could be involved in complex tissue specific immunomodulatory processes.

Supporting information

S1 Fig. Proteomic strategy. Groups of 5 cysts each were dissected either from the skeletal muscle or the central nervous system of five cysticercotic pigs. The five cysts in each group were dissected from the same animal. Afterwards, protein extracts were obtained from each group of cysts and digested using LysC/Trypsin; the peptides were desalted, dried and labelled using TMT reagents. After labelling, the peptides were combined in a single tube and fractionated by basic pH reverse phase liquid chromatography; 12 non-adjacent pooled fractions were analyzed in an Orbitrap Fusion mass spectrometer.
(TIF)

S2 Fig. Selection of antigenic peptides. More than two hundred proteins of *Taenia solium* cysts were analyzed to identify their antigenic regions. The antigenic regions were quantitatively estimated using two algorithms. Only the proteins (42) with the highest content of antigens/epitopes were furtherly screened. Only peptides that were predicted by both algorithms and subsequently analyzed using LBTOPE algorithm were considered, as a result, only one peptide was selected for each protein.
(TIF)

S3 Fig. Immunohistochemistry controls. Paired section of central nervous system and skeletal muscle cysts were incubated only with the secondary antibody.
(TIF)

S4 Fig. Antigenic response space using human sera. The raw optical densities were analyzed using machine-learning methodologies. A) Individual peptide testing. B) Antigenic response space using human sera, also was include a peptide named p678000 (HSTCQSCTKCPPGQGAEKPC) associated with the skeletal muscle localization of the cysts.
(TIF)

S5 Fig. Sequence alignment, antigenicity and abundance of two *Taenia solium* GP50 proteins. A) Sequence alignment of the two GP50 proteins characterized here and the GP50 previously described [103]. Signal peptide sequence, GPI-anchoring site, N-glycosilation site and disulphide bond prediction are indicated on the sequence. Each GP50 was associated with a tissue localization of the cysts (B and C); D) Antigenicity and B-cell epitope predictions are shown for each protein using Kolaskar and Bepipred algorithms (see materials and methods for further details).
(TIF)

S6 Fig. Cestode proteome comparisons. *Taenia solium* proteome was compared with the reported proteomes of *Echinococcus granulosus*, *E. multilocularis* and *Mesocestoides corti*. A) Venn diagram showing than only 14 proteins are consistently found expressed between those four parasites. B) Protein ID (referred to the *T. solium* genome database) and names of the 14 proteins shared between those four organisms. C) Comparison between the *T. solium* theoretical secretome and the proteome described here for *T. solium* cysts obtained from skeletal muscle and central nervous system. 187 ES proteins (out of 807 reported in the secretome were shared in both lists. D) Hypothetical protein expression; a total of 357 hypothetical proteins (without functional annotation) were identified in our study (referred to the *T. solium* genome database).
(TIF)

S1 Table. List of antibodies used. Antibody reactivity, clonality, vendor and catalogue number of the antibodies used for western blotting and immunohistochemistry.
(PDF)

S1 Data. Source code for the support vector machine.
(PY)

S2 Data. Proteins identified in the extracts of *Taenia solium* cysts dissected from skeletal muscle and central nervous system of infected pigs, identified and quantified by mass spectrometry.
(XLSX)

S3 Data. Peptides of identified in the extracts of *Taenia solium* cysts dissected from skeletal muscle and central nervous system of infected pigs, identified and quantified by mass

spectrometry.
(XLSX)

S4 Data. Highly antigenic proteins enriched for skeletal muscle or central nervous system.
(XLSX)

S5 Data. Functional annotation of the tissue-enriched proteins of *Taenia solium* cysts located in the skeletal muscle and central nervous system, as well as the functional annotation of a subset of constitutive proteins and the skeletal muscle pig proteome obtained from Zhang *et al* 2015.
(XLSX)

S6 Data. Cestode proteome comparisons. The proteome of *Taenia solium* was compared with the reported proteomes *of Echinococcus granulosus*, *E. multilocularis* and *Mesocestoides corti*.
(XLSX)

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