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MINISTERIO DE CIENCIA E INNOVACIÓN

DIRECCIÓN GENERAL DE INVESTIGACIÓN

PROYECTOS I+D, ACCIONES ESTRATÉGICAS Y ERANETS

INFORME FINAL

Investigador Principal	JORDI ALBEROLA DOMINGO
Título del Proyecto:	PROTEINAS RECOMBINANTES PRODUCIDAS EN LARVA DE INSECTO EN DIAGNOSTICO, TRATAMIENTO Y PREVENCION DE LEISHMANIASIS
Organismo:	UNIVERSIDAD AUTÓNOMA DE BARCELONA
Centro:	FACULTAD DE VETERINARIA
Departamento:	FARMACOLOGÍA, TERAPÉUTICA Y TOXICOLOGÍA
Fecha de Inicio:	01/01/2009
Fecha de Finalización:	31/12/2009

Fecha: Bellaterra a 12 de Marzo de 2010

Ilmo. Sr. Subdirector General de Proyectos de Investigación
C/Ramírez de Arellano. 28071 MADRID.

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A. MEMORIA. Resumen de las actividades realizadas y de los resultados del proyecto en relación con los objetivos propuestos (máximo 2.000 palabras).

Destaque su relevancia científica y/o su interés tecnológico.

En el caso de haber obtenido resultados no previstos inicialmente, indique su relevancia para el proyecto.

En caso de resultados fallidos, indíquense las causas.

Proyecto se consideró original y ambicioso, con potencialidad para proporcionar datos de interés sobre la producción de antígenos de *Leishmania* recombinantes. También se consideró que el IP tenía suficientes trabajos de calidad relacionados con el tema en los últimos años. A pesar de ello, a juicio de alguno de los expertos, se consideró que la redacción era precipitada y parca en algunos detalles y que el presupuesto estaba sobrevalorado. Se recomendó detallar mejor la metodología, por objetivos, precisando hitos a alcanzar con cada paso y facilitando un conocimiento más preciso de la propuesta. Así, se concedió financiación puente por 1 año para que pudiese reelaborarse la propuesta.

Dadas las características de la concesión el único objetivo era reelaborar la propuesta, cosa que se hizo.

La disponibilidad del presupuesto concedido permitió a su vez terminar y publicar temas pendientes del proyecto anterior, del cual el presente era continuación natural. También permitió poner a punto nuevas técnicas relacionadas que permitiesen realizar nuevos proyectos a costes más reducidos pues, a pesar de la opinión de alguno de los expertos sobre la posible sobrevaloración del presupuesto, hay que recordar que trabajar con perros es infinitamente más caro que trabajar con roedores, tanto desde el punto de vista de la compra de los animales como de su estabulación como del personal implicado en su manejo.

Entre las actividades realizadas cabe destacar:

Confirmación de la eficacia protectora de las vacunas *prime-boost* heterólogas, compuestas de ADN y de proteína producida en la plataforma de larvas biorreactores de *Trichoplusia ni* y utilizada sin purificar, contra la leishmaniosis en el modelo experimental de cricetos. En nuestro anterior proyecto habíamos demostrado la capacidad inmunogénica en el mismo modelo.

Demostración de la posibilidad real y utilidad de realizar pruebas de DTH en perro con antígenos sin purificar producidos la plataforma de larvas biorreactores de *Trichoplusia ni*.

Puesta a punto de modelos *in vitro* para el análisis de fármacos contra leishmaniosis para preparar la realización de futuros proyectos.

B. RESULTADOS MÁS RELEVANTES ALCANZADOS EN EL PROYECTO (máximo 60 palabras).

Dentro de los logros del proyecto señalados en el apartado anterior, reseñe los más relevantes hasta un máximo de tres.

1. Confirmación de la eficacia protectora de las vacunas *prime-boost* heterólogas contra la leishmaniosis.

2. Utilidad de los DTH con proteína producida en *Trichoplusia ni*.

C. RESUMEN DE LOS RESULTADOS DEL PROYECTO.

C1. Formación del personal	Nº					
Personal formado	(1)					
Personal formado o en formación que se ha transferido al sector industrial:						
Doctores ( )	Titulados Superiores	( )	Técnicos	( )		
C2. Tesis doctorales	(1)					
C3. Artículos científicos en revistas	( )	nacionales	(6)	internacionales		
C4. Artículos de divulgación en revistas	( )	nacionales	( )	internacionales		
C5. Artículos de revisión en revistas	( )	nacionales	( )	internacionales		
C6. Libros, capítulos de libros y monografías	( )	nacionales	( )	internacionales		
C7. Conferencias en congresos (por invitación)	( )	nacionales	( )	internacionales		
C8. Patentes y otros títulos de propiedad industrial	( )	registrados	( )	en explotación	( )	España ( ) extranjero

C1. FORMACIÓN DE PERSONAL EN EL PROYECTO, describir brevemente.

Se ha iniciado la formación de una persona que ha disfrutado de una Beca de Colaboración del Ministerio. Dicha persona realizará la tesis doctoral en el grupo si se concede el proyecto solicitado.

C2. TESIS DOCTORALES REALIZADAS TOTAL O PARCIALMENTE EN EL PROYECTO
Indicar: Título, nombre del doctorado, Universidad, Facultad o Escuela, fecha de comienzo, fecha de lectura, calificación y director.

Título: Vacunación y diagnóstico de la leishmaniosis visceral mediante proteínas recombinantes de Leishmania infantum producidas en larvas de insecto.
Doctorando: Felicitat Todolí Simó
Universidad: Universitat Autònoma de Barcelona
Facultad: Facultat de Veterinària
Inicio: 2005
Fecha de lectura: 29-10-2009
Calificación: Sobresaliente *cum laude*
Director: Jordi Alberola Domingo y Alhelí Rodríguez Cortés

C3. ARTÍCULOS CIENTÍFICOS EN REVISTAS
Indicar: Autor(es), título, referencia de la publicación, **(adjuntar en formato digital la primera página y aquella en la que se mencione a las entidades financiadoras del proyecto)**

Multi-antigenic heterologous prime-boost is more effective than naked DNA and insect-derived recombinant protein vaccination against experimental visceral leishmaniasis in hamsters.
Todolí T, Rodríguez-Cortés A, Pérez-Filgueira M, Laurenti MD, Gómez-Sebastián S, Rodríguez F, Pérez-Martín E, Escribano JM, Alberola J.
J Infect Dis. Submitted.

Humoral and in vivo Cellular Immunity against the Raw Insect-derived Recombinant Leishmania infantum antigens KMPII, TRYP, LACK and papLe22 in Dogs from an Endemic Area.
Felicitat T, Solano-Gallego L, de Juan R, Morell P, Núñez M, Lasa R, Gómez-Sebastián S, Escribano JM, Alberola J, Rodríguez-Cortés A.
Am J Trop Med Hyg. Reconsideration after Revision.

Dynamics and Predictive Potential of Antibodies against Insect-Derived Recombinant Leishmania infantum Proteins during Chemotherapy of Naturally Infected Dogs.
Todolí F, Galindo I, Gómez-Sebastián S, Pérez-Filgueira M, Escribano JM, Alberola J, Rodríguez-Cortés A.
Am J Trop Med Hyg. 2010 in press.

Leishmania infection: laboratory diagnosing in the absence of a "gold standard".
Rodríguez-Cortés A, Ojeda A, Francino O, López-Fuertes L, Timón M, Alberola J.
Am J Trop Med Hyg. 2010 Feb;82(2):251-6.
PMID: 20134001 [PubMed - indexed for MEDLINE]

Seroreactivity against raw insect-derived recombinant KMPII, TRYP, and LACK Leishmania infantum proteins in infected dogs.
Todolí F, Pérez-Filgueira M, Galindo I, Gómez-Sebastián S, Escribano JM, Rodríguez-Cortés A, Alberola J.
Vet Parasitol. 2009 Oct 14;164(2-4):154-61. Epub 2009 Jun 11.
PMID: 19570612 [PubMed - indexed for MEDLINE]

Anti-Leishmania IgA in urine samples from dogs with clinical leishmaniasis.
Todolí F, Solano-Gallego L, Ojeda A, Quintana J, Lloret A, Roura X, Alberola J, Rodríguez-Cortés A.
Vet Parasitol. 2009 Jan 22;159(1):17-23. Epub 2008 Oct 11.
PMID: 19019547 [PubMed - indexed for MEDLINE]

Al final del documento se adjuntan en formato digital la primera página y aquella en la que se menciona a las entidades financiadoras del proyecto.

C4. ARTÍCULOS DE DIVULGACIÓN EN REVISTAS
Indicar: Autor(es), título, referencia de la publicación.

C5. ARTÍCULOS DE REVISIÓN
Indicar: Autor(es), título, referencia de la publicación, **(adjuntar primera página en formato digital).**

C6. LIBROS, CAPÍTULOS DE LIBROS Y MONOGRAFÍAS
Indicar: Autor(es), título, referencia de la publicación, **(adjuntar en formato digital portada e índice donde figure la información).**

C7. CONFERENCIAS EN CONGRESOS, SIMPOSIOS Y REUNIONES (POR INVITACIÓN)
Indicar: Autor(es), nombre del congreso, lugar de celebración, año.

C8. PATENTES Y OTROS TÍTULOS DE PROPIEDAD INDUSTRIAL
Indicar: Autor(es), título, registro, entidad titular de la patente, año, países, clase.
Indicar cuales están en explotación.

C9. OTROS RESULTADOS EXTRAORDINARIOS NO INCLUIDOS EN LOS APARTADOS ANTERIORES
Indicar Naturaleza y Autor (es). Descríbalo brevemente en un máximo de 50 palabras.

D. CARACTER DE LOS RESULTADOS DEL PROYECTO (señalar hasta dos opciones)

( ) Teóricos	(X) Teórico-prácticos
( ) Prácticos	(X) De inmediata aplicación industrial

E. COLABORACIONES

E1. SI EL PROYECTO HA DADO LUGAR A COLABORACIONES CON OTROS GRUPOS DE INVESTIGACIÓN, coméntelas brevemente.

Con el grupo del Dr. José Ángel Martínez Escribano, del INIA, en el campo de obtención de proteínas en la plataforma de *Trichoplusia ni*. La colaboración es estable.

Con el Dr. Fernando Rodríguez González, el CReSA, en el campo de vacunas de ADN. La colaboración es estable.

E2. SI HA PARTICIPADO EN PROYECTOS DEL PROGRAMA MARCO DE I+D DE LA UE Y/O EN OTROS PROGRAMAS INTERNACIONALES EN TEMÁTICAS RELACIONADAS CON LAS DE ESTE PROYECTO, indique programa, tipo de participación y beneficios para el proyecto.
Mencione las solicitudes presentadas al Programa Marco de la UE durante la ejecución del proyecto, aunque no hayan sido aprobadas.

F. PROYECTOS COORDINADOS [1]
Describa las actuaciones de coordinación entre subproyectos, y los resultados de dicha coordinación con relación a los objetivos globales del proyecto.

[1] A rellenar sólo por el coordinador del proyecto.

G. RELACIONES O COLABORACIONES CON DIVERSOS SECTORES

G1. SI EN EL PROYECTO HA HABIDO COLABORACIÓN CON ENTES PROMOTORES OBSERVADORES (EPO) PARTICIPANTES:

1. Describa en detalle la relación mantenida con los EPO's, y la participación concreta de éstos en el proyecto, especificando, si procede, su aportación al mismo en todos sus aspectos. (Si se ha modificado la relación y/o el apoyo del EPO, en relación con lo previsto a la aprobación del proyecto, descríbalo brevemente).

2. Describa, si procede, las transferencias realizadas al (los) EPO (s) de los resultados obtenidos, indicando el carácter de la transferencia y el alcance de su aplicación.

3. Indique si esta colaboración ha dado lugar a la presentación de nuevos proyectos o si se tiene intención de continuarla en el futuro. En caso afirmativo, describa brevemente cómo va a concretarse.

G2. SI EL PROYECTO HA DADO LUGAR A OTRAS COLABORACIONES CON EL ENTORNO SOCIOECONÓMICO (INDUSTRIAL, ADMINISTRATIVO, DE SERVICIOS, ETC.), NO PREVISTAS INICIALMENTE EN EL PROYECTO, descríbalas brevemente.

H. GASTOS REALIZADOS

H1. GASTOS REALIZADOS EN LA ÚLTIMA ANUALIDAD

Nota: ***Debe cumplimentarse este apartado independientemente de la justificación económica enviada por el organismo.***

1.- Indique el total de gasto realizado en el proyecto:

Concepto	**Total gasto de la anualidad** (€)
Personal	
Costes de ejecución	20.000,-
TOTAL GASTO REALIZADO	24.200,-

2.- Comente brevemente si ha habido algún tipo de incidencia en este apartado que desee reseñar.

H2. GASTOS REALIZADOS DURANTE TODO EL PROYECTO

Nota: ***Debe cumplimentarse este apartado independientemente de la justificación económica enviada por el organismo.***

Euros

1. Gastos de personal (indicar número de personas, situación laboral y función desempeñada)

Total €

2. Material inventariable (describir brevemente el material adquirido)

Total €

3. Material fungible (describir brevemente el tipo de material)

Reactivos , pipetas, tubos

Total 16.077,49

4. Viajes y dietas (describir brevemente)

Asistencia a congreso

Total € 480,16

5. Otros gastos (describir brevemente)
Inscripciones congresos, traducción cientifica

Total €3.442,35

6. Costes indirectos

Total €4.200,-

7. Dotación adicional o complementos salariales, si procede

Total €

TOTAL GASTOS EJECUTADOS DEL PROYECTO € 24.200,-

CON ESTE INFORME DEBERÁ ADJUNTARSE:

Fotocopia de reingreso al Tesoro Público, si procede, de los fondos no utilizados.
Nota: *Debe cumplimentarse este apartado independientemente de la justificación*

I. INFORMACIÓN CORRESPONDIENTE A LA ÚLTIMA JUSTIFICACIÓN DE GASTO.

I1. PERSONAL ACTIVO EN EL PROYECTO DURANTE EL ÚLTIMO PERÍODO DE JUSTIFICACIÓN.

En el cuadro siguiente debe recogerse la situación de todo el personal del o de los Organismos participantes que haya prestado servicio en el proyecto en la anualidad que se justifica, o que no haya sido declarado anteriormente, y cuyos costes (salariales, dietas, desplazamientos, etc.), se imputen al mismo.
Si la persona estaba incluida en la solicitud original, marque "S" en la casilla correspondiente y no rellene el resto de casillas a la derecha.
Indique en la casilla "Categoría Profesional" el puesto de trabajo ocupado, el tipo de contratación: indefinida, temporal, becarios (con indicación del tipo de beca: FPI, FPU, etc.), etc.
En el campo "Función en el proyecto" indique el tipo de función/actividad realizada en el proyecto, (p. e., investigador, técnico de apoyo,…).

Recuerde que:
- En este capítulo sólo debe incluir al personal vinculado a los Organismos participantes en el proyecto. Los gastos de personal externo (colaboradores científicos, autónomos…) que haya realizado tareas para el proyecto debe ser incluido en el capítulo de "Varios".
- Las "Altas" y "Bajas" deben tramitarse de acuerdo con las "Instrucciones para el desarrollo de los proyectos de I+D" expuestas en la página web del MEC.

						Si no se ha incluido en solicitud original:		
Apellido 1	**Apellido 2**	**Nombre**	**NIF/NIE**	**Catgª Profesional**	**Incluido en solicitud original**	**Función en el proyecto**	**Fecha de Alta**	**Observaciones**

I2. GASTOS DE EJECUCIÓN: MODIFICACIONES DE CONCEPTOS DE GASTO CON RESPECTO A LA SOLICITUD ORIGINAL PARA EL ÚLTIMO PERÍODO DE JUSTIFICACIÓN.

Recuerde que los trasvases entre gastos de personal y gastos de ejecución deben tramitarse de acuerdo con las "Instrucciones para el desarrollo de los proyectos de I+D" expuestas en la página web del MEC.

a) Equipamiento:

En el cuadro adjunto, rellene una línea por **cada equipo adquirido** incluido en la justificación de gastos y **no previsto en la solicitud inicial** que dio lugar a la concesión de la ayuda para el proyecto, y justifique brevemente su adquisición. Si se ha adquirido un equipo en sustitución de otro que figuraba en la solicitud de ayuda inicial (por mejorar sus prestaciones, por obsolescencia del anterior…), indíquelo también en la casilla correspondiente.

Identificación del equipo	Importe	Justificación adquisición	Sustituye a ...(en su caso).

b) Viajes/Dietas:

En el cuadro adjunto se justificará la imputación de gasto en viajes y dietas sólo en el caso de que este tipo de gasto **no estuviera previsto en la solicitud inicial**

c) Material fungible:

Se describirá y razonará en el siguiente cuadro la adquisición del material fungible incluido en la justificación de gastos, sólo cuando este tipo de gasto **no estuviera previsto en la solicitud original.**

d) Varios:

Se describirán en el siguiente cuadro los gastos varios más relevantes incluidos en la justificación de gastos y **no previstos en la solicitud original,** justificando brevemente su inclusión. En este apartado se incluirá, entre otros, al personal externo y, en el caso de que el gasto justificado se refiera a colaboraciones científicas, se identificará al colaborador.

FIN DEL INFORME FINAL

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Anti-*Leishmania* IgA in urine samples from dogs with clinical leishmaniasis

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[b] Department of Pathology and Infectious Diseases, Royal Veterinary College, University of London, United Kingdom
[c] Departament Ciencia Animal i dels Aliments, Facultat de Veterinaria, Universitat Autònoma de Barcelona, Spain
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ABSTRACT

Recently, anti-*Leishmania* IgG has been detected in urine samples from *Leishmania*-infected dogs and its concentrations have been correlated with impairment of renal function. The presence and relationship with other anti-*Leishmania* Ig isotypes in urine have not yet been investigated. The current study analyzed the concentrations of anti-*Leishmania* IgA and IgG in sera (Ig-S) and urine (Ig-U) samples by ELISA in 64 untreated dogs with clinical leishmaniasis. All 64 serum samples tested were positive for anti-*Leishmania* IgG. Fifty of them (78.1%) were also positive for anti-*Leishmania* IgA. The results showed the presence of anti-*Leishmania* IgA-U in 38% of the 50 dogs that were positive for specific IgA-S. Thirty-eight of the 64 dogs positive for *Leishmania*-specific IgG-S (59.4%) were also positive for *Leishmania*-specific IgG in urine (IgG-U). The concentrations of anti-*Leishmania* IgA-U were significantly correlated with urine protein/creatinine (uP/C) ratio ($\rho = 0.542$; $P < 0.001$) and with serum biochemical parameters, such as γ-globulins, urea and creatinine. Goldmann–Witmer coefficient (*C* value) indicated that detection of specific IgA in urine samples from dogs with leishmaniasis might not only be due to impairment of filtration of the glomerular barrier but also be due to local production of this isotype, which might reflect a local immunological response to the presence of the parasite in the genitourinary tract. Anti-*Leishmania* IgG-U concentrations were highly correlated with uP/C ratio ($\rho = 0.779$; $P < 0.001$) and *C* value did not support in any case local production of this isotype. IgG isotype might be a more suitable and specific tool to evaluate renal damage due to the lower IgA-U sensitivity and correlation coefficients and evidence of IgA local production. However, dogs found positive for both Ig isotypes in urine presented significantly higher specific IgG-U concentrations and higher uP/C ratios than dogs found positive only for IgG-U, thus suggesting that the first group suffered more severe renal damage. This fact makes it necessary to evaluate the prognosis of dogs showing both anti-*Leishmania* IgA-U and IgG-U in future studies.

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1. Introduction

Canine leishmaniasis (CaL) is a severe and highly prevalent parasitic disease caused by *Leishmania infantum* in the Palearctic and in the Neotropic ecozones. Only 5% to 20% of dogs that become infected develop clinical manifestations (Fisa et al., 1999; Natami et al., 2000;

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with other biochemical markers of renal disease (albumin, urea and creatinine serum concentrations) (Palacio et al., 1995). C value did not support in any case local production of this isotype in genitourinary tract as previously suggested (Solano-Gallego et al., 2003). Therefore, presence of IgG in urine samples seems to be exclusively due to its leakage through the glomerular wall. Our results confirm that detection of anti-*Leishmania* IgG-U could be a more suitable and specific tool to evaluate renal damage than uP/C ratio. Moreover, the presence of anti-*Leishmania* IgG antibodies in urine samples at diagnosis might indicate a poor prognosis of the disease. Further studies during treatment of *Leishmania*-infected dogs are needed.

The results of the current study show the presence of anti-*Leishmania* IgA in urine samples from dogs with clinical leishmaniasis. The origin of this Ig isotype might be due to local production, leakage through the glomerular wall or both. The local production of IgA isotype probably reflects a local immunological response to the presence of the parasite in the genitourinary tract. Because its relationship with renal parameters is lower than that of IgG-U and because local production might be a confusion factor, detection of anti-*Leishmania* IgA in urine samples is not a suitable method for renal damage diagnosis in dogs with leishmaniasis. However, the association between the presence of both specific IgA and IgG and higher uP/C ratios could indicate that anti-*Leishmania* IgA-U detection can be used as a more specific prognostic marker in association with specific IgG in dogs with glomerulonephropathies. Additional longitudinal studies are needed to confirm this hypothesis.

Acknowledgements

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Seroreactivity against raw insect-derived recombinant *KMPII*, *TRYP*, and *LACK Leishmania infantum* proteins in infected dogs

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ABSTRACT

The recombinant proteins KMPII, TRYP, and LACK of *Leishmania infantum* were produced in baculovirus-infected *Trichoplusia ni* larvae and used to analyze the seroreactivity of 165 dog serum samples by the multiple-well ELISA technique (57 infected dogs with clinical signs, 46 naturally infected and 11 experimentally infected; and 108 non-infected dogs, 76 from non-endemic areas and 32 from endemic areas). Recombinant (r) KMPII was the most recognized antigen, as the majority of infected dogs seroreacted against it (0.75). This is the first report of seroreactivity against rTRYP (0.51) and rLACK (0.42) in *L. infantum*-infected dogs, since previous studies using recombinant TRYP and LACK proteins produced in prokaryotic systems failed to detect specific seroreactivity. All non-infected dogs were negative for rTRYP and rLACK, and only one of the 32 from endemic areas seroreacted against rKMPII. The results demonstrate that *L. infantum*-infected dogs develop humoral immunity against rKMPII, rTRYP, and rLACK antigens. There was substantial agreement between crude total *L. infantum* antigen (CTLA)-based ELISA and rKMPII ELISA ($\kappa = 0.664$), although this was higher than that found between the CTLA-based ELISA and rTRYP ($\kappa = 0.427$) or rLACK ($\kappa = 0.343$) ELISA, which can be interpreted as fair and moderate agreement, respectively. Ninety-three percent of the infected dogs analyzed developed specific antibodies against at least one of these three recombinant antigens. When the three recombinant antigen-based ELISA techniques were evaluated in parallel, almost perfect agreement ($\kappa = 0.880$) with CTLA-based ELISA was observed, with a specificity of 0.97 and a sensitivity of 0.93 in relation to CTLA-based ELISA. Further studies using purified recombinant antigens in a single-well test or individually, depending on the objective of the study, are warranted.

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1. Introduction

Zoonotic visceral leishmaniasis (ZVL) is a parasitic disease caused by *Leishmania infantum* (=*Leishmania chagasi* (Mauricio et al., 2000)) in the Palearctic and the Neotropical ecozones. Dogs are both a natural host and the main peridomestic reservoir of the parasite (Dantas-Torres, 2007; Moreno and Alvar, 2002). ZVL is endemic in the Mediterranean basin, where the prevalence of canine infection reaches 67–80% in highly endemic areas (Berrahal et al., 1996; Solano-Gallego et al., 2001). Because of their epidemiological significance, dogs play an important role in controlling transmission of *L. infantum*.

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patients recognized rTRYP (Webb et al., 1998). In the case of rLACK, a higher degree of seroreactivity has been found in humans. In sera from patients with confirmed visceral leishmaniasis, antibodies against LACK were detected in 97% of cases (Maalej et al., 2003). However, the cut-off value of the rLACK ELISA developed in the above-mentioned study is less restrictive than the ELISA described here, because even some healthy control patients tested positive. Our results demonstrate that a humoral response against rTRYP and rLACK is developed in *L. infantum*-infected dogs, although it is less frequent than that observed against rKMPII, as is the case in humans (de Carvalho et al., 2003; Ramirez et al., 1998; Webb et al., 1998). Further studies would be needed to explain why the humoral response against rTRYP and rLACK appears to be restricted to certain individuals. Different populations of infected dogs defined on the basis of seroreactivity against rTRYP and rLACK might be analyzed to investigate the prognostic potential or immunological implications of the development of these specific antibodies.

Previous studies have been unable to detect seroreactivity against rTRYP or rLACK in infected dogs (Fujiwara et al., 2005; Ramiro et al., 2003) using proteins produced in bacteria. It is possible that the rTRYP and rLACK produced in prokaryotic systems lack certain epitopes when compared with the native protein, thus this may explain the inability to detect them. These results highlight the importance of producing recombinant proteins that mimic the tertiary structure of the native protein for the development of accurate immunological techniques. This premise must also be taken into account for successful vaccine design.

Serological reactivity against these three recombinant proteins was independent and complementary, suggesting that each has its own B-cell immunostimulatory epitopes. Interestingly, most infected dogs seroreconized at least one of the three recombinant antigens evaluated. Thus, the combination of rKMPII, rTRYP, and rLACK produced in *T. ni* larvae might be used to develop a low-cost immunological technique for the serodiagnosis of CanL. In this regard, the specificity (0.97; CI: 0.84–1.00) and sensitivity (0.93; CI: 0.86–0.99) of the three ELISAs used in parallel was excellent and not different from that of CTLA-based ELISA, with the advantage of using protein extracts directly from larva containing recombinant antigens with no further purification. In addition, almost perfect agreement was found with the CTLA-based ELISA ($\kappa = 0.880$). Further studies are warranted with purified recombinant antigens, either combined in a single-well test or individually, depending on the objective of the study.

In conclusion, our results demonstrate that rKMPII, rTRYP, and rLACK produced in *T. ni* and used without further purification, are seroreconized by *L. infantum*-infected dogs. The most seroreactive protein was rKMPII, recognized by most of the dogs analyzed. As for rTRYP and rLACK, this is the first report of seroreactivity against them in *L. infantum*-infected dogs. When the three recombinant antigen-based ELISAs were evaluated in parallel, no differences in sensitivity or specificity were found compared with CTLA-based ELISA. These results suggest that the combination of the three recombinant antigens could be useful for the serodiagnosis of CanL, offering an inexpensive diagnostic tool with raw insect-derived proteins without the need of highly specialized laboratory facilities and being applicable in developing countries.

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Leishmania Infection: Laboratory Diagnosing in the Absence of a "Gold Standard"

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Abstract. There is no gold standard for diagnosing leishmaniases. Our aim was to assess the operative validity of tests used in detecting *Leishmania* infection using samples from experimental infections, a reliable equivalent to the classic definition of gold standard. Without statistical differences, the highest sensitivity was achieved by protein A (ProtA), immunoglobulin (Ig)G2, indirect fluorescenece antibody test (IFAT), lymphocyte proliferation assay, quantitative real-time polymerase chain reaction of bone marrow (qPCR-BM), qPCR-Blood, and IgG; and the highest specificity by IgG1, IgM, IgA, qPCR-Blood, IgG, IgG2, and qPCR-BM. Maximum positive predictive value was obtained simultaneously by IgG2, qPCR-Blood, and IgG; and maximum negative predictive value by qPCR-BM. Best positive and negative likelihood ratios were obtained by IgG2. The test having the greatest, statistically significant, area under the receiver operating characteristics curve was IgG2 enzyme-linked immunosorbent assay (ELISA). Thus, according to the gold standard used, IFAT and qPCR are far from fulfilling the requirements to be considered gold standards, and the test showing the highest potential to detect *Leishmania* infection is *Leishmania*-specific ELISA IgG2.

INTRODUCTION

Leishmaniases are endemic in 88 countries on four continents. In terms of global disease burden, leishmaniases are the third most important vector-borne disease, after malaria and lymphatic filariasis, causing 2.4 million disability-adjusted life years lost and 59,000 deaths in 2001.[1] The causative agents are parasitic protozoa of the genus *Leishmania*. Visceral leishmaniasis is caused by species of the *Leishmania donovani* complex: *L. donovani* in the Palearctic and *L. infantum* (= *L. chagasi*[2]) in both the Palearctic and the Neotropical ecozones. *L. infantum* is the etiological agent of a widespread serious zoonotic disease that affects both humans and dogs. Dogs are considered the primary peridomestic reservoir host[3,4] and controlling the dog infection rate reduces the incidence in humans.[5] Sand flies from the genus *Phlebotomus* spp. in the Palearctic and *Lutzomyia* spp. in the Nearctic are the proven vectors of human and canine leishmaniases (CaL).

Prevalence and incidence of the canine infection are important parameters to determine the risk and the ways to control this reemergent zoonosis. The estimation of these parameters depends on the reliable identification of infected dogs.[6] Parasitological examination is a very specific method but lacks sensitivity.[7] Serological methods are more sensitive but may be less specific and the choice of the cut-off value may not be obvious.[6] Moreover, seroprevalence underestimates the true prevalence of infection.[8] Cellular immune response, as measured by lymphoblastogenesis, seems to be specially suited to detect current or previous infection in clinically healthy dogs.[9,10] However, there may be no obvious cut-off. PCR has been confirmed to be a very sensitive and specific technique.[11–13] However, whether PCR is sensitive enough to detect all infected individuals is controversial due to conflicting results and the difficulties to choose the tissue to be sampled.[11,14]

The "gold standard" is the method, or composite of methods, giving results that are regarded as unequivocal classifications. Its use is a necessary prerequisite to examine the diagnostic utility of any test. The only true gold standard for classifying an animal as infected is the isolation of infectious agents or unequivocal histopathological criteria. Unfortunately, there is not such a gold standard for *Leishmania* infection. A way to validate diagnostic tests when lacking a classic gold standard is to take advantage of experimental infections. The strength of this method is that it not only measures the ability of the assay to detect infection, but also allows the kinetics of infection to be followed. In this setting everything is known about every sample collected and about the individual it is collected from. The aim of the present study is to assess the operative validity of several tests used in detection of *Leishmania* infection using samples from experimental infections, a reliable equivalent to the classic definition of gold standard.

MATERIAL AND METHODS

Samples and subjects. Six healthy, 9-month-old, intact, female beagle dogs were used. Dogs were housed in indoor kennels (Isoquimen S.L., St. Feliu de Codines, Spain) with windows covered with delthamethrin-sprayed, double anti-mosquito-nets, and according to the Guiding Principles for the Care and Use of Animals, following the guidelines of the institution's ethics committee.

L. infantum strain MCAN/ES/92/BCN-83/MON-1 obtained from a dog that acquired the infection naturally and had not received treatment was passaged through hamsters. Parasites were then grown in Schneider's insect medium (Sigma, St. Louis, MO), supplemented with 20% fetal calf serum (Gibco, Paisley, Scotland) and 25 $\mu g \cdot mL^{-1}$ gentamicin (Sigma) at 26°C. Parasites in stationary phase were washed and resuspended in phosphate buffered saline (PBS) at 5×10^7 promastigotes per mL. Finally 1 mL was injected by the intravenous route to every dog.[15]

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TABLE 3
ROC curve analysis

	LPA	IgM	IgG1	qPCR-Blood	qPCR-BM	IgA	qProtA	IFAT	IgG	IgG2
Area ROC curve	0.56	0.66	0.73	0.75	0.79	0.81	0.86	0.885	0.89	0,96
Standard error	0.06	0.05	0.05	0.05	0.07	0.04	0.04	0.043	0.03	0.02
95% CI	0.46, 0.65	0.57, 0.75	0.64, 0.81	0.66, 0.83	0.65, 0.90	0.72, 0.87	0.76, 0.93	0.77, 0.95	0.82, 0.94	0.90, 0.99
Significance level	0.2587	0.0017	0.0001	0.0001	0.0001	0.0001	0.0001	0,00	0.0001	0.0001

Groups of means that are not statistically different from each other are indicated by a solid grey horizontal bar.

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Dynamics and Predictive Potential of Antibodies against Insect-Derived Recombinant *Leishmania infantum* Proteins during Chemotherapy of Naturally Infected Dogs

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Abstract. A predictive marker for the success treatment of canine leishmaniasis is required for the application of a more rational therapy protocol, which must improve the probability of cure and reduce *Leishmania* resistance to drugs. We investigated the dynamics and predictive value of antibodies against insect-derived recombinant *L. infantum* proteins rKMPII and rTRYP by using an enzyme-linked immunosorbent assay with retrospective serum samples from 36 dogs during treatment of canine leishmaniasis. In the entire group of dogs, concentrations of antibodies against rKMPII and rTRYP significantly decreased earlier than concentrations of antibodies against crude total *Leishmania* antigen (one versus six months), which suggested that the dynamics of antibodies against recombinant proteins may be useful for assessing clinical improvement after treatment. Interestingly, decreases in antibody concentrations against rKMPII occurred earlier in disease-free dogs than in dogs that remain clinically ill one year after beginning of treatment, which suggested that these antibodies may be useful for predicting disease-free survival one year after the beginning of therapy against cutaneous leishmaniasis.

INTRODUCTION

Canine leishmaniasis is a fatal zoonotic disease caused by *Leishmania infantum* (*L. chagasi*) and transmitted by the bites of infected sand flies of the genera *Phlebotomus* and *Lutzomyia* in the Palearctic and Neotropic ecozones, respectively. In highly endemic foci around the Mediterranean Basin, an infection prevalence ranging from 65% to 80% has been reported in dogs.[1,2] Dogs are natural hosts and the major reservoirs of the parasite that causes human visceral leishmaniasis. Efforts to control canine leishmaniasis are a key factor in reducing transmission to humans and to other dogs.[3,4]

Infected dogs can develop a wide spectrum of *Leishmania*-specific immune responses, ranging from a predominantly cellular immune response (Th1-like) to a predominantly humoral immune response (Th2-like). In this sense, specific antibody levels against *Leishmania* antigen showed a marked correlation with parasite load and clinical status.[5,6] In *Leishmania*-infected dogs that are not able to control the infection, the parasite causes a chronic and systemic disease that culminates in death. The main clinical manifestations include poor general status, generalized muscular atrophy, enlarged lymph nodes, and nonpruritic scaling. Renal failure is the main cause of death in dogs with canine leishmaniasis.[7,8] Hypergammaglobulinemia, hypoalbuminemia, and increased concentrations of urea and creatinine are characteristic biochemical alterations of the disease.

Recently, the vaccines Leishmune® and Leish-Tec®[9,10] were granted marketing authorization against canine leishmaniasis by the Brazilian Ministry of Agriculture and Fisheries, although the Brazilian Ministry of Health recommended they should not be used in the governmental control program until phase III studies have finished. In the absence of a commercially available vaccine in other disease-endemic countries, control of the parasite is restricted to preventing sand fly bites and treating infected dogs. Several agents has been assayed to treat canine leishmaniasis,[11] although the most commonly used is a combination of meglumine antimoniate and allopurinol.[12] Successful chemotherapy has been correlated with a decrease in *Leishmania*-specific antibody levels, restoration of parasite-specific cell mediated immunity, and a reduction of parasite burden.[6,13–17] However, drugs used to treat leishmaniasis have not been able to provide a sterile cure; although temporary clinical remission is normally achieved, relapses are frequent after use of the drug is stopped,[18] thus increasing the risk of parasitic resistance.[19,20]

Research into a marker capable of predicting the success of therapy against *Leishmania* is required to develop a more rational therapy protocol, which must improve the probability of cure and reduce *Leishmania* resistance to drugs. Many studies have reported a significant decrease in levels of specific antibodies (mainly IgG) against crude *Leishmania* antigen in the follow-up of treated infected dogs.[13,14,21] This decrease in concentrations has been described in responsive and in nonresponsive dogs.[8,22] Therefore, seroreactivity against crude total parasite antigen cannot predict the outcome of treatment. However, it is unknown whether the decrease in antibody concentrations is general (all *Leishmania* antigens) or specific. Studies using recombinant proteins might make it possible to investigate this area and exploit the results to develop new and predictive tools to manage this disease.

We recently analyzed the usefulness of enzyme-linked immunosorbent assays (ELISAs) based on insect-derived rKMPII, rTRYP, and rLACK antigens for the serodiagnosis of leishmaniasis in dogs; these assays showed a sensitivity of 93% when used in parallel.[23] The aim of the present study was to describe the dynamics of the antibodies against the two antigens (rKMPII and rTRYP) and to investigate their usefulness in monitoring the therapeutic response and predictive potential in naturally infected dogs treated with meglumine antimoniate and allopurinol.

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at the same time that the first reduction in the number of clinical signs, whereas the decrease in concentrations of antibodies against CTLA occurred at six months. In addition, the proportion of dogs that seroreverted one year after the beginning of treatment to recombinant proteins was significantly higher than those that seroreverted for CTLA. Thus, our results suggest that the dynamics of antibodies against recombinant proteins may be more useful than the dynamics of antibodies against CTLA for assessing clinical improvement after treatment, as described in human patients treated for visceral leishmaniasis. In accordance with our results, antibodies against the recombinant proteins rKMPII[20] and *Lc*TXNPx, a protein similar to TRYP,[20] decreased significantly after treatment of patients with visceral leishmaniasis, conversely to serologic results with crude *Leishmania* antigen.

A significant reduction in concentrations of antibodies against CTLA has been extensively reported in dogs with leishmaniasis after treatment.[8,15,21,22] This reduction is thought to be related to attenuated antigenic stimulation resulting from the decrease in parasite load. Our results might indicate that antigens such as rKMPII or rTRYP could be more sensitive than CTLA in detecting early reduction of *Leishmania* load in treated dogs, although the presence of seropositive dogs for these recombinant proteins at the end of the study could indicate persistence of subclinical infection.[14,15,23] Unfortunately, parasitologic data for these dogs during follow-up or at the end of the study were not available.

Because of the lack of parasitologic data, dogs were split in two groups according to only their clinical status one year after the beginning of therapy. We evaluated the value of rKMPII, rTRYP, and CTLA as predictive markers for long-term disease-free survival in dogs with canine leishmaniasis. One month after treatment was started, concentrations of antibodies against rKMPII had decreased significantly in disease-free survivor dogs. Conversely, in those remaining clinically ill, concentrations of antibodies against rKMPII did not decrease significantly until six months after the beginning of therapy. Thus, the dynamics of antibodies against rKMPII might be an early predictive marker for long-term disease-free survival after therapy for canine leishmaniasis.

Different profiles of antibodies against rKMPII between the two groups of dogs could be caused by different parasite loads related to different immune responses, but further studies are needed to confirm this possibility. Quantification of parasite burden in bone marrow samples by real-time polymerase chain reaction and further immune characterization could be helpful for answering this question. Information from other studies that used bone marrow aspiration showed that there was no sterile cure in most treated dogs, despite clinical improvement.[14,15,21,22] Thus, the possibility of relapse as an epidemiologic risk[24,25] still exists in disease-free survivor dogs, although to a lesser extent. For this reason, it has been recommended to use preventative measures against parasite transmission in animals and to monitor them on a regular basis to assess future clinical relapse.[26]

Concentrations of antibodies against CTLA did not decrease significantly until six months after the beginning of therapy, and this decrease occurred in disease-free survivors and dogs that remained clinically ill. Regarding concentrations of antibodies against rTRYP, although the decrease occurred one month after treatment was started, this decrease was observed in both groups of dogs. Consequently, ELISAs for CTLA[8,22] and rTRYP could not predict long-term clinical success of therapy.

Some parameters have been suggested as predictive markers of clinical outcome in canine leishmaniasis,[8,15,22] although, to date, none has been used in veterinary clinical practice. Dogs showing high levels of IgG against *Leishmania* at diagnosis showed a poor prognosis after chemotherapy.[8] In addition, several studies have reported different dynamics of antibodies against *Leishmania*-specific antigens between responsive and nonresponsive dogs. In accordance with our results, Western blot showed a reduction in the intensity of low molecular mass (12–30 kD) bands in serum samples of dogs that showed clinical improvement after treatment against *Leishmania*. In contrast, persistence of 14-kD, 24-kD, and 29-kD bands has been related to persistence of the parasite and a potential unfavorable prognosis.[22] Interestingly, two studies have described specific seroreactivity against a band of 26 kD, approximately the molecular mass weight of rTRYP, in untreated dogs in the acute phase of disease and in unsuccessfully treated dogs,[27,28] thus suggesting that such seroreactivity can be a marker for active cutaneous leishmaniasis and a potential prognostic marker.

The results reported in the present study suggest that ELISAs based on the insect-derived antigens rKMPII and rTRYP from *L. infantum* may be useful for assessing clinical improvement after treatment of canine leishmaniasis. Also, dynamics of antibodies against rKMPII could be useful for predicting long-term disease-free survival after one year of the beginning of therapy against this parasitic disease in dogs.

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