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ESTUDIOS ESTRATEGICOS PARA LA LUCHA CONTRA LA FASCIOLIASIS HUMANA A NIVEL MUNDIAL

STRATEGIC STUDIES FOR THE FIGHT AGAINST HUMAN FASCIOLIASIS WORLDWIDE

TESIS DOCTORAL INTERNACIONAL

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CERTIFICAN:

Que el licenciado en Farmacia por la Universitat de València D. Ignacio Pérez Crespo ha realizado el trabajo experimental titulado "Estudios estratégicos para la lucha contra la fascioliasis humana a nivel mundial" en el laboratorio del Departamento antedicho en la Facultat de Farmàcia de la Universitat de València bajo su dirección y con el fin de optar al título de Doctor por la Universitat de Valéncia.
Y para que así conste a los efectos oportunos, firman el presente informe en Valencia, a 1 de Julio de 2013.

Fdo:
Prof. Dr. Dr.h.c. Santiago Mas-Coma

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A MI FAMILIA Y AMIGOS

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ABSTRACT

Fascioliasis is a zoonotic parasitic disease caused by *Fasciola hepatica* and *F. gigantica*. Emergence of human fascioliasis prompted a worldwide control initiative including a pilot study in several countries. Given the necessity to characterize the fasciolid populations involved, the overall aim of the present research is, first, the phenotypic characterization of faciolid adults and eggs implicated in human endemic areas, where this initiative has been implemented, through a computer image analysis system (CIAS) applied on the basis of standardized measurements. Second, new immunological techniques and their potential use as immunological diagnostic tests in those areas are evaluated. The specific results have been grouped into five parts. A) Characterization of eggs of both *F. hepatica* and *F. gigantica* for their differential diagnosis. The study revealed that eggs shed by humans show morphological traits different from eggs shed by animals. In humans, *F. hepatica* eggs are bigger and *F. gigantica* eggs are smaller than reported to date from livestock, and their measurements overlap when compared. B) Study of the correlation between egg-shedding and uterus development in *F. hepatica* human and animal isolates. Uterus area (UA) development of adult *F. hepatica* obtained at different days post infection (dpi) in a Wistar rat model with isolates obtained from cattle, sheep, pigs and humans from the endemic human fascioliasis zone of the Northern Bolivian Altiplano was analysed and compared with the number of eggs shed per gram of faeces as obtained through the Kato–Katz technique. This work shows a positive correlation between liver fluke UA and egg production. C) Study of *F. hepatica* phenotypic characterization in Andean human endemic areas. The results showed that two phenotypic patterns could be distinguished in *F. hepatica* adult size: the valley pattern (Cajamarca and Mantaro, Peru) and the altiplanic pattern (northern Altiplano, Bolivia). Furthermore, the Andean valley population and European standard populations presented phenotypic homogeneity. The Altiplano population showed a large size range with a pronouncedly lower minimum size, indicating that uterus gravidity is reached at a smaller size than in valley populations. D) Study of the DRG *Fasciola hepatica* IgG ELISA test as a serological diagnostic tool of human fascioliasis in different epidemiological situations. The study shows that sensitivity and specificity of the DRG assay were 95.3% (95% confidence intervals, 82,9–99,2%) and 95,7% (95% confidence intervals, 92,3–97,5%), respectively. No correlation between egg output and the optical density (OD) values of the test was observed. E) Field evaluation of the MM3 coproantigen detection test for fascioliasis diagnosis and surveillance in human hyperendemic areas of Andean countries. As part of the above-mentioned control initiative, two hyperendemic areas were chosen: Huacullani, Northern Altiplano, Bolivia, representing the Altiplanic transmission pattern with high prevalences and intensities; the Cajamarca valley, Peru, representing the valley pattern with high prevalences but low intensities. It can be concluded that the coproantigen-detection test allows for high sensitivity and specificity, fast mass screening, detection in the chronic phase, early detection of treatment failure or reinfection in post-treated subjects, and is convenient in surveillance programmes. However, this technique falls short when evaluating the fluke burden on its own.

KEY WORDS: *F. hepatica*, *F. gigantica*, adult, uterus, egg, number per gram of faeces, biometry, growth model, principal component analysis, experimental population, natural population, Wistar, human, sheep, cattle, pig, Bolivian Altiplano, Cajamarca valley, Mantaro valley, Bolivia, Peru, Georgia, Egypt, Vietnam, worldwide control initiative, DRG *Fasciola hepatica* test, MM3 coproantigen.

INDICE

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INTRODUCTION

INTRODUCCION

1. INTRODUCTION

1.1. FASCIOLIASIS WORLDWIDE

Fascioliasis is an important human and animal disease caused by the trematode species *Fasciola hepatica* and *F. gigantica*, depending on the causal agent it is a parasitic disease of different epidemiological, pathological and control characteristics. *F. hepatica* has a cosmopolitan distribution, mainly in temperate zones, while *F. gigantica* is found in tropical regions of Africa and Asia. However, the two fasciolid species overlap in large regions (MAS-COMA & BARGUES, 1997). Fascioliasis in livestock has always been recognized as a veterinary problem on a worldwide scale, but in humans the disease was only considered of secondary importance until the end of the 1980´s owing to the relative small number of human reports. Only about 2000 human cases were reported in the 25 years previous to 1990 (CHEN & MOTT, 1990). This scenario changed dramatically after the initiative launched by the World Health Organization at the beginning of the 1990´s. At present, estimates for all continents reach 17 million people to be infected, and this may even be an underestimate when the total lack of data concerning numerous Asian and African countries is considered (MAS-COMA, 2005).

At present, fascioliasis is emerging or re-emerging in numerous regions of Latin America, Africa, Europe and Asia, both in humans and animals (MAS-COMA *et al.*, 2009a), a phenomenon which could partly be related to climate change (MAS-COMA *et al.,* 2008, 2009b). Major human health problems are encountered in Andean countries (Bolivia, Peru, Chile and Ecuador), the Caribbean (Cuba), northern Africa (Egypt), south western Europe (Portugal, France and Spain) and the Caspian area (Iran and neighbouring countries) (MAS-COMA, 2005). Emergence, long-term pathogenicity (VALERO *et al,* 2003, 2006a, 2008) and immunological interactions (BRADY *et al.,* 1999; GIRONES et al., 2007) prompted WHO to include this disease among the so-called neglected tropical diseases (NTDs), which are chronic, debilitating, poverty-promoting and among the most common causes of morbidity in developing countries. Their control and elimination is now recognized as a priority to achieve the United Nations Millennium Development Goals and targets for sustainable poverty reduction (HOTEZ *et al.,* 2007, 2008).

In the Americas, among Andean countries, the highest human fascioliasis prevalence and intensities are encountered in the northern Altiplano of both Bolivia (ESTEBAN *et al.,* 1997; MAS-COMA *et al.,* 1999a) and Peru (ESTEBAN *et al.,* 2002),

as well as in the Cajamarca valley (Peru) (ORTIZ *et al.*, 2000; HILLYER *et al.*, 2001; ESPINOZA *et al.*, 2007; GONZALEZ *et al.*, 2011), and in the Mantaro valley (Peru), (STORK *et al.*, 1973; MARCOS *et al.*, 2004) where *F. hepatica* is the only fasciolid species present (VALERO *et al.,* 1999, 2001) and children and females appear to be the most affected subjects (MAS-COMA, 2005; MAS-COMA *et al.,* 2005, 2009a). Within the human fascioliasis high altitude transmission pattern related to *F. hepatica* transmitted by Lymnaeid vectors of the *Galba/Fossaria* group in Andean countries, two different sub-patterns have been distinguished according to physiographic and seasonal characteristics (MAS-COMA, 2005; MAS-COMA *et al.,* 2009a):

a) the altiplanic pattern, with endemicity distributed throughout an area of homogeneous altitude and transmission throughout the whole year caused by high evapotranspiration rates leading lymnaeid vectors to concentrate in permanent water bodies, e.g. the northern Bolivian Altiplano (MAS-COMA *et al.,* 1999a; FUENTES *et al.,* 1999)

b) the valley pattern, with endemicity distributed throughout an area of heterogeneous altitude and seasonal transmission related to climate, e.g. the Cajamarca valley in Peru (CLAXTON *et al.,* 1997, 1999; GONZALEZ *et al.,* 2011).

In developed countries, patients are diagnosed in hospitals or other health centres usually during the acute phase or at the beginning of the chronic phase. On the contrary, infected subjects detected in surveys in human endemic areas of developing countries are mainly in the advanced stage of chronicity, when not already re-infected due to the high contamination risk, as the liver fluke is able to survive up to 13.5 years in human beings (MAS-COMA *et al.,* 2005). The public health impact of this scenario has reached an importance never expected before, owing to the above-mentioned (i) great pathogenicity of long-term liver fluke infection proven experimentally (VALERO *et al.,* 2003, 2006a, 2008) and (ii) the immune-modulation of fasciolids in the acute phase (BRADY *et al.,* 1999) and their immune suppression effect in chronic and advanced chronic phases (GIRONES *et al.,* 2007), which is in the background of co-infections with other parasitic and infectious diseases (MAS-COMA *et al.,* 2005). In recent years, the availability of a very effective drug against fascioliasis, namely triclabendazole (SAVIOLI *et al.,* 1999), prompted WHO to launch a worldwide initiative against human fascioliasis (WHO, 2007, 2008). This initiative implements control activities against human fascioliasis in endemic areas presenting different epidemiological situations and

transmission patterns (MAS-COMA, 2005; MAS-COMA *et al.,* 2009a). Bolivia and Peru are two of the countries selected for priority intervention due to the high public health problem caused by this disease. Different pilot schemes were designed to assess the best control strategies according to the different epidemiological situations and transmission patterns. The Northern Bolivian Altiplano was chosen as an example of the altiplanic pattern, while the Cajamarca valley was chosen as an example of the valley pattern.

In addition to this, WHO decided in October 2006 to launch a worldwide initiative against this disease (WHO, 2007) based on the success of obtaining a donation of Egaten®, the only highly efficient drug presently available for human treatments (WHO, 2008). After an initial pilot scheme in Vietnam, Egypt, Peru and Bolivia, this initiative is currently being extended to many other countries. Therefore, the capacity to correctly diagnose human fascioliasis becomes crucial. Unfortunately, there are some unavoidable difficulties in human fascioliasis diagnosis in human endemic areas of developing countries. Thus, indirect tests, either serological or using coproantigens, are useless for intensity estimation, are generally expensive and require a minimum of laboratory infrastructure. Furthermore, indirect tests have so far never proved to be able to differentiate between infection by *F. hepatica* and *F. gigantica*, an important aspect considering the higher pathogenicity of the latter due to its larger fluke size but also because of their different epidemiology, transmission and control characteristics. Molecular tests have shown to be useful for differential diagnosis (MARCILLA *et al.,* 2002), but are often unaffordable, too. Therefore, direct coprological analysis for the detection and identification of fasciolid eggs continues to be the most appropriate diagnostic strategy for both detection of infection and estimation of intensity, not only for those human endemic rural areas but also for the implementation of the WHO treatment drive. This conclusion is reached even in spite of the recognized lower sensitivity of egg detection in faecal samples and its uselessness for the diagnosis of patients in the acute phase (HILLYER, 1999), as well as the lack of an accurate relationship between egg counts per gram of faeces and the fluke burden (VALERO *et al.,* 2006b). Moreover, in areas of Africa and Asia where the two fasciolid species overlap, fluke egg size was believed to be the only method allowing the differential diagnosis between *F. hepatica* and *F. gigantica*. The use of serological tests or the lack of a calibrated microscope for measurements with an ocular micrometer explains why subjects diagnosed from areas where both species co-exist are currently referred to as infected by *Fasciola sp.* (MAS-COMA *et al.,* 2005). In such a situation it is surprising that an analysis of the size of eggs shed by humans has never been carried out, so that in fact an extrapolation of the size of the fluke eggs

shed by livestock species has always been used for human diagnosis. In fascioliasis, the usual diagnosis during the biliary stage of infection is based on the classification of eggs found in stools, duodenal contents or bile. However, large variations were first observed in the size of *F. hepatica* eggs in livestock from different geographical locations (TINAR, 1984). Furthermore, it has been experimentally shown that the final host species (sheep, cattle, pig and donkey) decisively influences the size of the *F. hepatica* eggs even within the same endemic area (northern Bolivian Altiplano) (VALERO *et al.,* 2001a). The problem has even been further aggravated by the confirmation of the existence of morphologically intermediate forms between the two fasciolid species and genetic hybrids of both in overlapping areas, endemic for animals (ITAGAKI *et al.,* 2005; SEMYENOVA *et al.,* 2006) and humans in Africa (PERIAGO *et al.,* 2008) and Asia (ASHRAFI *et al.,* 2006; LE *et al.,* 2008). The existence of these intermediate forms has been molecularly verified to be not only common but even the rule in these overlapping areas (BARGUES *et al.,* in prep.) and poses a question mark on whether egg characteristics are suitable as a tool for the differential diagnosis of fascioliasis caused by either species. A concrete example of this problem has been recently emphasized in the diagnosis of fascioliasis in humans (INOUE *et al.,* 2007).

As aforementioned, an alternative to coprological egg detection is the use of immunodiagnostic tests based on the detection of anti-*Fasciola* antibodies and/or coproantigens released by the parasite. Nevertheless, surveys on human fascioliasis have usually been made through various coprological techniques verifying the presence of eggs in stools (VALERO *et al.,* 2009a) and antibody detection tests to confirm the diagnosis of human fascioliasis (HILLYER, 1999). Among these techniques, classical coprological egg detection methods are the most frequently used (CHEN & MOTT, 1990; MAS-COMA *et al.,* 1999b). However, the use of coproantigen detection was applied to diagnose *F. hepatica* infection in patients only in Cuba (ESPINO *et al.,* 1998; MILLAN *et al.,* 2000). The enzyme-linked immunosorbent assay (ELISA) methods developed for determination of *Fasciola* coproantigens in stool samples from animals and humans provide an alternative to coprological examination (ESPINO & FINLAY, 1994; ABDEL-RAHMAN *et al.,* 1998). One of these methods is the MM3 capture ELISA (MM3-COPRO) test for fascioliasis diagnosis detection of fecal excretory-secretory antigens (ESAs) by using the monoclonal antibody (mAb), whose usefulness for detection of *F. hepatica* and *F. gigantica* coproantigens in experimental and natural *Fasciola* infections of sheep and cattle has already been demonstrated (MEZO *et al.,* 2004; VALERO *et al.,* 2009b). This test proved to be highly sensitive (confirmed by necropsy) and specific (no cross reaction was observed with antigens from other helminths), and allowed

for the detection of *Fasciola* infections 1–5 weeks before patency in cattle. Furthermore, other researchers recently tested a commercial version of the test, and its appropriateness for the detection of *F. hepatica* infections in cattle was confirmed under field conditions (CHARLIER *et al.,* 2008). The suitability of the MM3-COPRO method for detection of *Fasciola* coproantigens in both fresh and preserved stools from hospital patients has been demonstrated (UBEIRA *et al.,* 2009), but its application for detection of *F. hepatica* infections in humans under field conditions has not been proved.

Counting with an efficient coproantigen test for human fascioliasis diagnosis could represent a valuable tool to facilitate population screening and post-treatment surveillance to be implemented within control campaigns, above all in communities were people do not accept to furnish stool samples due to ethnic/religious aspects.

1.2. OBJECTIVES

The overall aim of the present research is the phenotypic characterization of faciolids adults and eggs from human endemic areas. Moreover, new techniques and their potential use as immunological diagnostic tests in those areas are evaluated.

To reach the first target, the following specific objectives are addressed:

- characterization of eggs of both *F. hepatica* and *F. gigantica* for their differential diagnosis;
- to analyse the possible relationship between the liver fluke uterus area and egg-shedding;
- to phenotypically characterise *F. hepatica* in Andean human endemic areas.

To reach the latter target:

- to validate the DRG *F. hepatica* IgG ELISA test as a serological diagnostic test in human fascioliasis;
- to validate MM3-COPRO ELISA test as new coprological human fascioliasis test.

MATERIAL Y METODOS

MATERIAL AND METHODS

2. MATERIAL Y METODOS

2.1. MATERIAL PARASITOLOGICO PARA EL ESTUDIO MORFOMETRICO

2.1.1. RECOLECCION HUEVOS DE *FASCIOLA*

La fijación ejerce una influencia decisiva sobre el tamaño y la forma de los huevos de helmintos. Por este motivo, en el presente estudio, los huevos utilizados no estaban fijados si no que estaban suspendidos en agua y guardados en oscuridad a 4 ºC hasta su utilización. Tras su obtención, los huevos se midieron dentro de la siguiente quincena.

2.1.1.1. HUEVOS PROCEDENTES DE HUMANOS

Se analizaron un total de 1049 huevos procedentes de humanos. El material de Bolivia, Perú y Egipto se obtuvo de individuos de una escuela, el material de Georgia procedió de individuos dentro de un estudio de población total y el material de Vietnam se obtuvo de pacientes adultos del Hospital Quy Nhon. Se analizó el siguiente material:

- 154 huevos procedente de 5 niños (6-12 años de edad) de Huacullani (en el Altiplano norte de Bolivia)
- 167 huevos procedente de 4 niños (7-14 años de edad) de Santa Rosa de Chaquil (en el Valle de Cajamarca, Perú)
- 53 huevos procedente de 3 individuos (10-40 años de edad) de Akhali Terjola e Itkhvisi (en la región de Kutaisi, Georgia)
- 608 huevos procedente de 13 niños (8-11 años de edad) de Abis, Ola Garbea, Hosh Essa y El Waquad (en el Delta del Nilo, Egipto)
- 67 huevos procedente de 2 adultos (17, 27 años de edad) de Quy Nhon (Vietnam)

Los individuos fueron escogidos al azar y participaron en el estudio con su consentimiento. A todos los individuos se les proporcionó un envase de plástico limpio de boca ancha, numerado y con tapa a presión, de 30 mL de capacidad, el cual debería ser devuelto en el menor tiempo posible con sus heces. Las muestras se recolectaron de todos los individuos que dieron su consentimiento, anotándose los datos personales en el envase de entrega. Todas las muestras de heces se transportaron al laboratorio en las siguientes 5 horas. De cada muestra se realizó

una lámina de Kato-Katz (helm-Test®, AK test, AK Industria e Comèrcio Ltda, Belo Horizonte, Brasil). De las muestras que dieron positivo se recuperaron los huevos del trematodo mediante filtrado.

2.1.1.2. HUEVOS PROCEDENTES DEL GANADO

Los datos del estudio corresponden a huevos de fasciólidos de animales domésticos de las mismas áreas, o alrededores, de procedencia de las muestras humanas. Para la comparación de los huevos de muestras humanas del Altiplano norte de Bolivia y del valle de Cajamarca de Perú se utilizaron datos de huevos de *F. hepatica* procedentes de los reservorios más comunes del área hiperendémica humana del Altiplano norte de Bolivia (104 huevos de ovino, 168 de ganado bovino, 186 de porcino y 161 de asno) (VALERO *et al.*, 2001a). Se incluyó un total de 117 huevos de *F. hepatica* procedentes de 3 bovinos de la región de Kutaisi, Georgia. De la región del Delta del Nilo, se analizaron 35 huevos de *F. hepatica* y 73 de *F. gigantica* obtenidos de tres y cuatro individuos de bovino respectivamente. Se analizaron un total de 101 huevos de *F. gigantica* de tres bovinos del área de Qhy Nhon, Vietnam. Para la comparación con *F. gigantica*, se usaron datos de una población conocida estándar, concretamente 142 huevos de bovinos del área Bobo Dioulasso, Burkina Faso, donde *F. hepatica* está ausente y *Radix natalensis* es el único molusco vector presente (PERIAGO *et al.*, 2006).

2.1.1.3. CRITERIO PARA LA CLASIFICACION ESPECIFICA DE HUEVOS PROCEDENTES DE HUMANOS Y ANIMALES

En Bolivia y Perú, *F. hepatica* es la única especie presente del género *Fasciola* infectando tanto humanos como ganado en el Altiplano norte de Bolivia (MAS-COMA *et al.*, 1999a) y en el Valle de Cajamarca (ESPINOZA *et al.*, 2007).

En Georgia, Egipto y Vietnam, sin embargo, se tomaron precauciones especiales ya que la distribución de ambas especies de fasciólidos se solapa en cada una de las respectivas áreas endémicas de donde proceden las muestras de huevos estudiadas. Los adultos fasciólidos tienden a cruzarse, a pesar de ser hermafroditas (HURTREZ-BOUSSES *et al.*, 2004). Así, el problema en estas áreas de solapamiento es que los adultos de ambas especies de fasciólidos se hibridan y encontramos vermes adultos que muestran características morfológicas de formas intermedias (ASHRAFI *et al.*, 2006a; PERIAGO *et al.*, 2008; LE *et al.*, 2008). Los huevos en estas áreas endémicas pueden diferir fenotípicamente de los huevos típicos de las especies estándar. Por tanto, la única forma para asegurar la clasificación específica de los huevos es la obtención de los huevos directamente del adulto y después

procesar este adulto para su clasificación específica. Desafortunadamente, esto sólo es posible para las muestras de animales y no para las muestras humanas. Por lo tanto, los adultos fasciólidos se obtuvieron de los hígados del ganado bovino en mataderos próximos a las áreas humanas estudiadas. Los adultos se clasificaron como *F. hepatica* o *F. gigantica* siguiendo el criterio de PERIAGO *et al.* (2006, 2008). Para evitar coger huevos inmaduros del útero, los cuales pueden diferir fenotípicamente de los huevos maduros, se obtuvieron directamente a partir de la parte final del útero de adultos grávidos. Cabe recordar que los huevos en la parte final del útero están completamente formados, maduros y viables. Para la obtención de huevos maduros se aplicó al Trematodo adulto una ligera presión en la zona del acetábulo y así se forzó a los huevos maduros a salir por el poro genital. Otro procedimiento para obtener huevos maduros es rasgar el parénquima en la parte superior del acetábulo con dos agujas enmangadas. Hay que tener en cuenta que en las especies de *Fasciola* el útero no sólo se considera un órgano en donde maduran los huevos sino que también actúa como un órgano de almacenaje (VALERO *et al.*, 2001b). La ausencia de diferencia en tamaño, madurez y viabilidad entre los huevos de la parte final del útero y los encontrados en la bilis y las heces fue verificada en diferentes individuos animales. Obtener los huevos de esta forma resulta fácil ya que los adultos fasciólidos maduros son muy grandes, además la mayor parte de su útero es post-acetabular pero termina pre-acetabularmente. Los huevos obtenidos por esta técnica son del mismo tamaño y forma que los encontrados en la bilis y las heces. Los resultados muestran que en Georgia sólo está presente *F. hepatica*, en Egipto están presentes ambas especies de fasciólidos mientras que en los animales analizados en Vietnam solo habían presente formas de *F. gigantica*.

2.1.2. RECOLECCION DE ADULTOS DE *FASCIOLA*

2.1.2.1. OVINOS INFECTADOS NATURALMENTE

Después del sacrificio de los individuos ovinos (*Ovis aries*) se analizaron los hígados. Los fasciólidos presentan en el hospedador definitivo una migración desde el parénquima hepático hasta su ubicación final en el canal biliar. VALERO *et al.*, (2006b) a través de un modelo logístico caracterizó el crecimiento corporal de los adultos fasciólidos en dos fases: a) la parte "exponencial" del crecimiento logístico correspondiente al desarrollo corporal durante la migración en la cavidad abdominal y en el parénquima hepático; b) su desarrollo y maduración sexual en el sistema de conductos biliares hasta el inicio de la producción de huevos. A partir de este momento, el desarrollo del adulto fasciólido sigue la parte "saturada" del modelo

logístico de crecimiento, es decir, la entrada en el conducto biliar induce la maduración y la producción de huevos. En este sentido, sólo se analizaron los adultos de *F. hepatica* que se encontraron dentro de los canales biliares. En el estudio morfológico de la presente Tesis doctoral únicamente se han incluido fasciólidos con huevos en el útero (n=542). Las muestras analizadas incluían la mayor variabilidad de vermes posible (diferentes estados de maduración, tamaño corporal y gravidez uterina). Los vermes adultos fueron fijados en solución de Bouin entre porta y cubre pero sin ejercer presión para evitar la distorsión. Los especímenes fueron teñidos con Carmín Borácico de Grenacher, diferenciados, deshidratados y montados en bálsamo de Canadá.

Todos los ovinos analizados eran adultos originarios de zonas cercanas al matadero donde se obtuvo el material. Pasaremos a detallar ahora el material analizado especificando zona geográfica, número de parásitos adultos analizados, número de hospedadores y matadero de procedencia:

a) Material procedente del valle de Cajamarca (Perú): 130 vermes adultos de *F. hepatica* procedentes de 8 ovinos de la raza Merino (2, 2, 12, 15, 18, 21, 28 y 32 adultos por ovino, respectivamente), del matadero de Rodicio (altitud aproximada de 2663 m sobre el nivel del mar)

b) Material procedente del valle del Mantaro (Perú): 47 vermes adultos de *F. hepatica* procedentes de 5 ovinos autóctonos de la raza Junin (3, 4, 8, 10 y 21, vermes por ovino, respectivamente), de los mataderos de Huayucachi, Pachacayo y Huancayo (altitud aproximada de 3271 m sobre el nivel del mar)

c) Material procedente del Altiplano norte (Bolivia): 201 vermes adultos de *F. hepatica* procedentes de 12 ovejas de la raza Merino (6, 8, 9, 12, 15, 18, 19, 22, 22, 26 y 30, vermes adultos por ovino, respectivamente), del matadero de Batallas (altitud aproximada de 4000 m sobre el nivel del mar)

d) Material procedente de la costa Mediterránea (España): 37 vermes adultos de *F. hepatica* procedentes de 5 ovejas de la raza Merino (2, 7, 8, 10 y 10, vermes adultos por ovino, respectivamente), del matadero de Massamagrell (a una altitud cercana al nivel del mar)

2.1.2.2. *OVINOS INFECTADOS* EXPERIMENTALMENTE

Las metacercarias de *F. hepatica* se obtuvieron experimentalmente, tal y como describe MAS-COMA *et al.* (2001). Tanto el aislado de *F. hepatica* como su caracol hospedador intermediario son originarios de Bialowieza, Polonia y de la Albufera de Valencia, España, respectivamente. Los procesos de recolección como transporte, mantenimiento y cría de los caracoles Lymnaeidos, embrionación de huevos del parásito, infección de los caracoles y obtención de las metacercarias experimentales fueron llevados a cabo por el equipo de la Prof. Dra. M. D. Bargues. El grupo experimental incluyo 4 ovejas de entre 4 y 5 semanas de edad (raza autóctona Guirra). Los animales se criaron y mantuvieron en la granja de ovinos experimental de la Universidad Politécnica de Valencia (al nivel del mar). Los animales fueron infectados *per os* con 200 metacercarias de *F. hepatica*/ovino. La comida y el agua se proporcionaron *ad libitum*. Las experiencias fueron llevadas a cabo con el correspondiente permiso para la investigación de Comité de Ética Animal para la Investigación de la Universidad Politécnica de Valencia. La necropsia se llevó a cabo a las 40 semanas post-infección. Se recuperaron un total de 127 vermes adultos de *F. hepatica* (20, 28, 34 y 45 vermes por oveja).

2.1.2.3. RATAS WISTAR INFECTADAS EXPERIMENTALMENTE

Sólo se utilizaron especímenes *F. hepatica* y *Galba truncatula* procedentes del Altiplano norte de Bolivia. Los caracoles emisores de las cercarias que posteriormente dieron origen a las metacercarias, son cepas mantenidas en el laboratorio (en cámaras climáticas Heraeus-Vöstsch HPS 1500 y HPS 500; condiciones experimentales: 20 ºC de temperatura, fotoperiodo de 12/12 h luz/oscuridad; humedad relativa 90%). Estos caracoles fueron a su vez infectados monomiracidialmente con huevos de *F. hepatica* que se encontraron en heces de ovinos, bovinos, porcinos y humanos infectados naturalmente (MAS-COMA *et al.*, 2001). Se obtuvieron cuatro aislados de metacercarias (aislado bovino, aislado ovino, aislado porcino y aislado humano). Las metacercarias se almacenaron en agua mineral a 4 ºC en oscuridad. Se infestaron un total de 73 ratas Wistar macho (Iffa Credo, Barcelona, España) con una edad de entre 4-5 semanas, utilizándose la técnica de infección oral mediante una jeringa orogástrica. Las ratas fueron alojadas en jaulas aisladas (Iffa Credo, Barcelona, España), en un recinto estéril libre de patógenos, con calefacción controlada y con un ciclo de 12/12 h de luz/oscuridad (condiciones en cumplimiento del acuerdo Europeo de Estrasburgo, 18 de Marzo 1986). La comida y el agua fueron provistas *ad libitum*. Las ratas Wistar se dividieron en cuatro grupos de acuerdo a cada tipo de aislado: bovino (12

ratas), ovino (24), porcino (19) y humano (18), y se infectaron con una dosis de 20 metacercarias por rata. La infección se confirmó mediante la disección del animal y por la detección de huevos en heces. La disección se llevó a cabo a distintos días post-infección (dpi) (entre 40 y 300 dpi) con el fin de obtener vermes en diferentes estados de madurez, tamaño corporal y gravidez del útero. El parásito se localizó en el conducto biliar a partir de los 40 dpi. La detección de huevos emitidos en heces se llevó a cabo mediante la técnica de Kato-Katz (Helm-Test®, AK test, AK Industria e Comercio Ltda, Belo Horizonte, Brasil). La intensidad de la infección se caracterizó por el número de adultos que se encontraron en el hígado. Sólo se utilizó el material parasitario procedente de infecciones leves (≤4 adultos fasciólidos/rata) para evitar el posible efecto de la carga parasitaria en la producción de huevos (VALERO *et al.*, 2006b). Igualmente, para prevenir el posible efecto de la patología presente en el hospedador en la producción de huevos (VALERO *et al.*, 2000, 2006a) sólo se utilizó material procedente de ratas sin signos de litiasis.

Las 73 ratas fueron sacrificadas en tiempos de infección diferentes entre 40 y 300 dpi (ver Tabla 3.6). Se obtuvieron los siguientes adultos de fasciólido por aislado:

a) Aislado bovino: 14 adultos (50 dpi:1 en una rata, 1 en otra rata; 75dpi: 1,1; 100 dpi: 1,1; 150 dpi: 3,1; 200 dpi: 1,1; 225 dpi: 1,1; media: 1,2±0,6, rango: 1-3)
b) Aislado ovino: 51 adultos (40 dpi:1,1; 75dpi: 3,3,2,3; 100 dpi: 3,2,3,1; 150 dpi: 2,1,1,1; 225 dpi: 2,2,1; 300 dpi: 3,3,3,4,2,2,2; media: 2,1±0,9, rango: 1-4)
c) Aislado porcino: 22 adultos (40 dpi:1,1; 50 dpi: 1,1; 75dpi: 1,1; 100 dpi: 1,1; 150 dpi: 1,1; 175 dpi: 1,1,1; 200 dpi: 2,1; 225 dpi: 2,1; 261 dpi: 2,1; media: 1,2±0,4, rango: 1-2)
d) Aislado humano: 29 adultos (50 dpi:1,2; 75dpi: 1,2; 100 dpi: 1,2; 150 dpi: 1,2; 175 dpi: 2,2,2,2,3; 225 dpi: 1,2; 300 dpi: 1,1,1; media: 1,6±0,6, rango: 1-3)

Los especímenes adultos de *F. hepatica* se fijaron en solución de Bouin entre portaobjetos y cubreobjetos, teñidos con Carmín Borácico de Grenacher y montados en Bálsamo de Canadá (Panreac, Barcelona, España).

2.1.2.4. ESTUDIO DE SEGUMIENTO DE EMISION DE HUEVOS

Se recogieron muestras fecales frescas a las 9:00 a.m. todos los días de las 73 ratas, y se almacenaron en placas Petri cerradas para evitar la desecación de las

muestras antes de su examen. La detección de huevos se llevó a cabo mediante la lectura de una lámina de Kato-Katz por día y por rata, calculándose los huevos por gramo de heces (epg). Los análisis se llevaron a cabo diariamente a partir de 30 dpi hasta los 300 dpi en el caso de los aislados ovinos y de humanos, hasta el 225 dpi en el aislado bovino y hasta 261 dpi en el aislado porcino. Se asumió que no se produjo la desparasitación de las ratas hospedadoras, ya que el número de adultos de *F. hepatica*/rata no varió a lo largo del experimento en los distintos grupos de edad analizados. Esta premisa está apoyada por el hecho de que el número de huevos detectados en heces no disminuye de forma brusca en ninguna de las ratas analizadas. La producción de huevos por verme se calculó dividiendo los huevos por gramo de heces por el número de adultos recuperados en el conducto biliar. Se obtuvo la media de producción de huevos emitidos por gramo de heces por adulto y día entre los 10 días anteriores hasta el día correspondiente a la obtención de los datos del área uterina (UA). Este día corresponde al día de la necropsia del animal (40, 50, 75, 100, 150, 175, 200, 225, 261 y 300 dpi).

2.2. MATERIAL PARASITOLOGICO PARA EL ESTUDIO INMUNOLOGICO

2.2.1. MUESTRAS HUMANAS

El suero se obtuvo de la seroteca de la Unidad de Parasitología de la Facultad de Farmacia, Universidad de Valencia, España, WHO Collaborating Centre on Fascioliasis and Its Snail Vectors. Se analizó sueros obtenidos entre los años 2004-2008. Se realizaron alícuotas y se almacenaron a -80 ºC hasta su uso. Los sueros humanos utilizados se clasificaron en tres grupos:

a) 54 sueros humanos de individuos con fascioliasis procedentes de tres áreas geográficas con situaciones epidemiológicas diferentes. La positividad a la infección fue verificada a través tanto de la técnica ELISA CL1 como por la presencia de huevos en heces (técnica de Kato-Katz)

b) 168 sueros de individuos con otras enfermedades parasitas/fúngicas distintas a la fascioliasis (para evaluar potenciales reacciones cruzadas) obtenidos de casos confirmados clínicamente, serológicamente y a veces parasitológicamente

c) 89 sueros parasitológicamente negativos procedentes de colecciones de sueros almacenados en el Centro Nacional de Microbiología (ISCIII, Madrid, España) y el Servicio de Microbiología del Hospital Universitario y Politécnico "La Fe" (Valencia, España)

2.2.2. PROCEDIMIENTOS Y ANALISIS DE MUESTRAS FECALES

Los estudios coprológicos se realizaron en la localidad de Huacullani, la cual pertenece al municipio de Tiwanku, tercera sección de la provincia de Ingavi del Departamento de La Paz, Bolivia. Esta localidad se encuentra a 85 km de la capital, La Paz, en el extremo occidental del denominad corredor Tambillo-Aygachi en el Altiplano Norte de Bolivia (MAS-COMA *et al.,* 1999a). Según el censo de 2005 por parte del Instituto Nacional de Estadística Boliviano, Huacullani tiene un total de 2525 habitantes. La recolección de muestra se realizó en las escuelas de la localidad obteniéndose un total de 437 muestras procedentes de niños. Estudios anteriores en dicha localidad mostró altas prevalencias en niños del 38,2% en el año 1992, 31,2% en 1993, y 34,8% en 1996, y en el total de la comunidad (niños más adultos) del 18,4% en 1996 y 11,8% en 1997 (ESTEBAN *et al.,* 1999).

También se recolectaron muestras en el Departamento de Cajamarca, Perú, el cual ocupa un área de alrededor de 35.400 km^2 en la parte Norte Andina de Perú y está habitada por 1.416.000 personas. Este departamento comprende 13 provincias y en el caso de la provincia de Cajamarca incluye 12 distritos (GONZALEZ *et al.,* 2011). Un total de 362 muestras fecales fueron recolectadas de niños procedentes de las siguientes escuelas: Escuela de Varones del Distrito (Distrito de Jesús), Santa Rosa de Chaquil (distrito de La Encañada), y Andrés Avelino Caceres (distrito de Baños del Inca). Estudios previos mostraron prevalencias muy altas en esta área endémica, con una media del 24,4% con una prevalencia máxima del 47,7% en Santa Rosa de Chaquil, la mayor prevalencia local detectada en Perú (GONZALEZ *et al.,* 2011).

En Huacullani, se obtuvieron muestras fecales de 437 niños seleccionados al azar (220 varones y 217 mujeres) de entre 5 a 16 años de edad (media ± SD = 8,8 ± 2,3). A cada participante en el estudio se le dio un bote de plástico, limpio y de boca ancha. A todos los participantes se les pidió que llenaran el bote con sus propias heces y lo devolvieran inmediatamente. Se recogió una muestra por sujeto y se anotaron los datos personales (nombre, sexo y edad) en el bote. Las muestras fueron transportadas al laboratorio de parasitología en la Facultad de Farmacia, Universidad Mayor de San Andrés (UMSA), La Paz, entre 1 y 3 horas después de la recolección. Una de las alícuotas se utilizó para llevar a cabo el test MM3-COPRO ELISA y otra fue preservada a 4 ºC para realizar las láminas de Kato-Katz. Las láminas de Kato-Katz se realizaron en la Facultad de Medicina, UMSA, y fueron analizados inicialmente en menos de 1 h desde su preparación para evitar la sobre clarificación de algunos huevos de helmintos.

En Cajamarca, las muestras fecales se obtuvieron al azar de 362 niños (264 varones y 98 mujeres) de entre 7 y 15 años de edad (media ± SD = 9,9 ± 2,2) por medio de procedimientos similares. Las muestras fueron transportadas a la ciudad de Cajamarca entre 1 y 3 horas después de la recolección y almacenada a 4º C hasta su posterior envió al Laboratorio de Parasitología en el Instituto de Medicina Tropical Alexander von Humboldt, Lima, donde se llevó a cabo el análisis coproparasitológico. Se realizaron tanto las láminas de Kato-Katz como el ELISA a dos alícuotas de la muestra, mientras que una tercera alícuota fue preservada en una solución al 10% de formalina (1:3) para la detección de huevos mediante la técnica de sedimentación rápida (LUMBRERAS *et al.*, 1962).

2.3. TECNICAS PARASITOLOGICAS

Las técnicas utilizadas en la presente Tesis se incluyen en cuatro grandes bloques:

- Técnicas mastozoológicas: van a proporcionar información sobre el tratamiento de los hospedadores definitivos utilizados para poder recuperar los diferentes estadios (adultos y huevos) de los digénidos.
- Técnicas helmintológicas: se utilizan para la preparación y estudio de los digénidos adultos, para su posterior estudio morfométrico y morfoanatómico.
- Técnicas inmunológicas: se utilizan para el estudio de la respuesta inmune que los adultos digénidos producen en el hospedador definitivo.
- Métodos matemáticos: los cuales, finalmente, se aplicaran al resultado de los estudios de los materiales parasitológicos antes nombrados.

2.3.1. TECNICAS MASTOZOOLOGICAS

2.3.1.1. ESTUDIO DEL HIGADO Y CANALES BILIARES

Dado que en *F. hepatica* el microhábitat de parasitación específico del adulto es el hígado, se realizó un minucioso estudio de este órgano. Después de la apertura ventral del animal, al visualizar el hígado *in situ* se observa si existen lesiones en el parénquima hepático y si los canales biliares están engrosados, lo que nos indicara la presencia de adultos en su interior. El hígado se extrae cortando el canal biliar principal a la altura de su unión con el intestino y se pesa. Asimismo se efectúan las medidas morfométricas del canal biliar y se fotografía las lesiones

macroscópicas si las hubiera. El parénquima hepático se examina a la lupa binocular raspando con un bisturí, especialmente donde se observan lesiones, pudiéndose encontrar en ellos individuos jóvenes de *F. hepatica* que aún no han completado su migración hasta el canal biliar principal (fase de invasión). Los canales biliares aumentan mucho su diámetro en la fase de estado, siendo fácil detectar si van a existir adultos de *F. hepatica* en ellos. Al abrir el canal biliar con unas tijeras, los digénidos adultos salen vivos, procediéndose a su recolección y fijación. Posteriormente se efectúa un enjuagado con agua tanto de la cavidad abdominal, como de los distintos órganos para tener la seguridad de la total recogida de los parásitos (PANOVA, 2002).

2.3.2. TECNICAS HELMINTOLOGICAS

2.3.2.1. TECNICAS MACROSCOPICAS GENERALES

Estas técnicas incluyen los procesos realizados desde la recogida de los vermes hasta su estudio en el microscopio, es decir, la fijación de los Trematodos, su conservación, coloración, diferenciación, deshidratación y montaje.

2.3.2.1.1. FIJACION

Para que la visualización de la anatomía interna del Trematodo sea óptima, el verme se debe fijar *in vivo* y sometido a una ligera presión. El proceso de fijación fue el siguiente:

En ejemplares pequeños: se coloca el Trematodo vivo con un pincel sobre un portaobjetos en una gota de agua. Se añade una gota de líquido de Bouin en la cara inferior de un cubreobjetos y se deja caer sobre el Trematodo, cuidando que éste no se encuentre ladeado, en cuyo caso se enderezará con ayuda de una aguja enmangada. El verme debe permanecer entre porta y cubreobjetos unos 20 minutos. Luego se levanta y se traslada el Trematodo con un pincel a una placa Petri con fijador de Bouin, en el que permanecerá unos 30 minutos (MARCOS, 1993).

En ejemplares grandes: los Trematodos fueron fijados con Bouin entre dos placas Petri. Se introduce líquido de Bouin en una de las placas y con un pincel se coloca el verme vivo sobre dicha placa. Se deja caer la otra placa Petri sobre el Trematodo por su cara inferior, procurando que quede lo más plano posible, pero sin presionar demasiado. El Trematodo debe quedar unos 30 minutos entre las dos placas y luego, con uno o dos pinceles, se pasan a otra placa Petri con líquido de Bouin unos 40 minutos (MARCOS, 1993).

En ambos casos, transcurrido el tiempo establecido, se llevan a alcohol de 70º, cambiando este diariamente hasta que el verme pierda la coloración amarilla dada por el fijador; en nuestro caso oscila entre 30 a 90 días dependido del tamaño del Trematodo.

El líquido de Bouin se considera uno de los mejores fijadores topográficos. Su composición por cada 100 ml es la siguiente:

- Solución acuosa de ácido pícrico 75 ml
- Formol (solución comercial al 40%) 25 ml
- Ácido acético 5 ml

Se prepara de la siguiente manera: en un matraz, se pone un litro de agua y se calienta hasta que esté templada. Se coloca el matraz sobre un agitador y se le va añadiendo poco a poco ácido pícrico hasta conseguir una solución saturada del mismo, es decir, hasta que el ácido pícrico no se disuelva en el agua.

Una vez conseguido se deja reposar 24 horas (solución madre). Para utilizar el fijador se añade a pequeñas fracciones de la mezcla la cantidad correspondiente de ácido acético (5 partes de ácido acético por cada 100 partes de solución) (PANOVA, 2002).

El líquido de Bouin fija de forma homogénea y penetra rápidamente. La retracción en el momento de la fijación es más débil que con otras buenas mezclas fijadoras (BARGUES, 1986).

2.3.2.1.2. CONSERVACION

La conservación se realiza en viales con alcohol de 70º, en el cual los Digénidos pueden permanecer hasta el momento de su tinción (PANOVA, 2002).

2.3.2.1.3. COLORACION

Las tinciones utilizadas dan lugar a preparaciones permanentes, imprescindibles en el estudio llevado a cabo. El colorante utilizado ha sido el Carmín Borácico de Grenacher, cuya fórmula es la siguiente:

- Solución acuosa de bórax al 4% (8 gramos de bórax en 200 cc de agua destilada)
- Carmín (casa Merck)

El protocolo de preparación del carmín Borácico de Grenacher es el siguiente: en un matraz redondo se pone la solución acuosa anterior y se le añaden 5 gramos de carmín. Se calienta en un recipiente de reflujo, en el cual tenemos un cazo con tierra y el matraz esférico, con un refrigerador por el que entra y sale agua. Se deja hervir suavemente durante 30 minutos y se le añaden 200 cc de alcohol de 70º. Se deja reposar 24 horas y se filtra (PANOVA, 2002).

Los Digénidos obtenidos deben permanecer en el colorante un tiempo determinado que depende de su grosor y su capacidad de tomar el colorante. Los adultos de *F. hepatica* y *F. gigantica* deben teñirse de forma suficiente pero no excesiva para facilitar su posterior diferenciación. Si los ejemplares se tiñen en exceso deben estar más tiempo en el líquido diferenciador, lo que puede alterar el inicial de la tinción e incluso los tejidos del verme. En líneas generales diremos que los adultos de *F. hepatica* de mayor grosor y los adultos de *F. gigantica* se han teñido durante 24 horas en la mezcla de carmín Borácico de Grenacher. La tinción óptima se controla periódicamente bajo la lupa binocular.

2.3.2.1.4. DIFERENCIACION

Una vez que el Trematodo ha sido teñido efectuamos la diferenciación en alcohol clorhídrico. Con un pincel bien seco extraemos los vermes del colorante y los colocamos, de uno en uno, en una placa Petri añadiendo, gota a gota, ácido clorhídrico comercial (35%). El trematodo va alcanzando una tonalidad rosada con luz superior de la lupa, debiéndose observar con la inferior todas las estructuras del parásito por transparencia. La duración de esta operación depende del tamaño y grosor del Trematodo (PANOVA, 2002).

2.3.2.1.5. DESHIDRATACION

Tras la diferenciación colocamos una serie de placas Petri con distintos alcoholes en los que, con ayuda de un pincel, iremos pasando sucesivamente los parásitos. En la primera placa, con etanol de 70º, el digénido debe estar 10 minutos. Después lo llevamos a otra placa con alcohol de 96º, 15 minutos. De ésta pasa a alcohol de 100º 15 minutos más, otros 15 minutos en alcohol butílico y finalmente 15 minutos en xilol, terminando con este paso la cadena de deshidratación.

2.3.2.1.6. MONTAJE

El montaje se realiza con un portaobjetos, un cubreobjetos y bálsamo de Canadá, resina comercial que solidifica rápidamente y proporciona preparaciones de

larga vida. Sin embargo, para poderla emplear es necesaria una previa deshidratación del material, ya que no es soluble en agua.

Para realizar el montaje se coloca sobre el portaobjetos una gota de bálsamo de Canadá y sobre ella el Trematodo en posición ventral, es decir, con la ventosa o acetábulo ventral de cara al cubreobjetos. Con un pincel mojado en xilol eliminamos las posibles burbujas que se hayan podido formar en el bálsamo antes de colocar el cubreobjetos sobre él.

Si una vez montado, el parásito no presenta suficiente bálsamo, o si éste se retrae, se puede añadir más pincelando con xilol los bordes entre porta y cubre, añadiendo una gota de bálsamo de Canadá. que entra en la preparación por capilaridad (MARCOS, 1993).

Por último, se introducen las preparaciones en la estufa a 20º C de temperatura de 4 a 24 horas hasta que estén secas. Si el bálsamo se retrae por el calor, se repite la operación anteriormente descrita de pincelado con xilol y bálsamo por capilaridad (PANOVA, 2002).

2.3.2.2. TECNICAS COPROLOGICAS

La técnica de Kato-Katz es utilizada para la búsqueda, hallazgo y recuento de los huevos de helmintos en heces, además resulta ser una técnica muy útil y práctica para conseguir un diagnóstico rápido de las parasitosis producidas por Digénidos, Cestodos y Nematodos. Para la realización de la técnica se siguió las recomendaciones de la OMS (ASH *et al.,* 1994) usando el kit Helm-Test, AK test, AK industria e Comércio Ltda, Belo Horizonte, Brasil. Para realizar este examen se precisa de: una placa de plástico con un orificio central que admite un volumen de heces de 41,7 mg (o sea 1/24 g), un tamiz de nailon con una apertura de malla de entre 200 y 300 µm, una espátula o cuchara de una casa comercial, un portaobjetos y un papel de celofán empapado en un líquido colorante. El líquido colorante del papel de celofán se compone de: solución al 50% en agua destilada de solución glicerina/verde malaquita (preparación de la solución de glicerina/verde malaquita: 100 ml de agua destilada + 10 ml de glicerina + 1 ml de solución de verde malaquita al 3%). En esta solución se introducen el papel celofán en tiras y en 24 horas podrán ser utilizadas ya que estarán bien impregnadas de la misma.

Para la realización de la técnica procederemos como sigue (ORDOÑEZ, 2010):

1. Depositamos una muestra de heces en un trozo de papel satinado, con el propósito de que el papel no absorba la humedad de las heces (por ejemplo, la hoja de una revista).

2. Colocamos la malla sobre las heces y presionamos suavemente. De esta forma dejamos en el filtro o malla las partículas grandes que puedan impedir una óptima visualización microscópica de los huevos de *Fasciola* sp.

3. Con la ayuda de una espátula se toma el filtrado y se deposita en un molde situado en un portaobjetos etiquetado con los datos del hospedador definitivo utilizado.

4. A continuación se enrasa la muestra con ayuda de la espátula hasta colmar el orificio. Posteriormente retiramos la placa con precaución y tenemos un flan de 41,7 mg de heces.

5. Por último, se coloca sobre la muestra un papel de celofán previamente recortado y embebido en el líquido colorante. Se da la vuelta al porta-objetos y se presiona suavemente sobre un papel absorbente para recoger el líquido sobrante. Conviene esperar al menos 30 minutos para realizar la primera observación al microscopio, aunque se recomienda una espera de 24 horas para optimizar la visualización de los huevos. Por su alto contenido en glicerina, es posible el mantener estas preparaciones durante varios meses sin deterioro. Posteriormente, se realiza la observación microscópica de la preparación, realizando un barrido completo y anotando el número total de huevos de *F. hepatica* que aparecen en la misma. Ya que la cantidad de heces analizadas es de 1/24 g y como el dato que a nosotros nos interesa es el de huevos por gramo de heces (hgh), lo único que tenemos que hacer es multiplicar el resultado por 24.

En el caso del seguimiento de la cinética de emisión de huevos de *F. hepatica* en el modelo murino, se utilizó exclusivamente la técnica de Kato-Katz. En el caso de la validación de la técnica MM3-COPRO ELISA en muestras humanas se utilizó tanto la sedimentación rápida como la técnica de Kato-Katz. Estas técnicas coprológicas clásicas para la detección de huevos se utilizaron como pruebas estándares para el diagnóstico cualitativo (sedimentación rápida y Kato-Katz) y cuantitativo (Kato-Katz). Para la técnica de Kato-Katz los huevos fueron detectados en muestras frescas después del análisis de tres láminas de Kato-Katz por muestra, dependiendo de la concentración de huevos de *F. hepatica.* La cantidad media de

huevos de las tres láminas se calculó como huevos por gramo de heces (epg). La inspección parasitológica fue realizada mediante microscopia por un parasitólogo entrenado. La intensidad de la infección, medida como huevos por gramo de heces (epg), se utilizó como indicador de la carga de *F. hepatica* en humanos infectados.

2.3.3. ESTUDIO MORFOMETRICO CIAS

Para la obtención de imágenes y para el cálculo de las diferentes medidas morfométricas con un analizador de imágenes, tanto unidimensionales como bidimensionales y para la obtención de imágenes se han utilizado los siguientes programas:

- Image-Pro® Plus, versión 5.1 para Windows (Media Cybernetics Inc., Silver Spring, USA): con dicho programa comercializado se han creado macros para el cálculo de las dimensiones en imágenes digitalizadas mediante cámara digital Nikon Coolpix 5400 y Leica V-LUX 1 (para medidas de adultos) y mediante un microscopio Nikon modelo SE equipado con un revolver de 4 objetivos (4x, 10x, 40x, 100x y 2 oculares de 10x), conectado con videocámara de color 3CCD (Sony DXC-930P) (para medidas de ventosas y faringes).

- Microsoft Excel: programa comercializado para análisis y cálculos de datos. Los datos obtenidos a través de Image-Pro® Plus se exportaban a los archivos de dicho programa para poder operar después con ellos.

La estandarización de la metodología para la realización de las medidas es fundamental en cualquier estudio morfométrico (VALERO & MAS-COMA, 1985; VALERO, 1986; VALERO *et al.*, 1987; VALERO *et al.*, 1996, 2005; PERIAGO *et al.*, 2006, 2008). Los trabajos de VALERO *et al.* (1996, 2005) y PERIAGO *et al.* (2006, 2008) proponen la metodología Computer Image Analysis System (CIAS). para el estudio morfométrico de los adultos de *F. hepatica* y *F. gigantica*. Se han realizado mediciones de adultos de fasciólidos en material fijado y montado *in toto* en preparaciones permanentes. La medida del útero de los fasciólidos adultos se realizó de acuerdo al método propuesto por VALERO *et al.* (2001b), calculando el área delimitada por el perímetro exterior de la imagen uterina. Las medidas del área uterina de los adultos fasciólidos (UA) vienen expresadas en mm^2.

Los parámetros morfométricas analizados en el huevo de *Fasciola* son los siguientes:

Características biométricas lineales: EL = Longitud del huevo; EW = anchura del huevo; EP = perímetro del huevo; ER = circularidad del huevo ($ER=EP^2/4\pi EW$).

Superficies: EA = área del huevo.

Relaciones: EL/EW = relación entre longitud del huevo y anchura del huevo.

Los parámetros morfométricas analizados en el adulto son los siguientes (ver Fig. 2.1.):

Características biométricas lineares: BL= longitud corporal; BW= anchura corporal; BWOv= anchura corporal a nivel del ovario; BP= perímetro corporal; BR= circularidad corporal ($BR=BP^2/4\pi BW$); CL= longitud del cono; CW= anchura de cono; OSMax= diámetro máximo de la ventosa oral; OSmin= diámetro mínimo de la ventosa oral; VSMax= diámetro máximo de la ventosa ventral; VSmin= diámetro mínimo de la ventosa ventral; A-VS= distancia entre el extremo anterior del cuerpo y la ventosa ventral; OS-VS= distancia entre la ventosa oral y ventosa ventral; VS-Vit= distancia entre la ventosa ventral hasta el final de las glándulas vitelógenas; Vit-P= distancia entre las glándulas vitelógenas hasta la parte posterior corporal; VS-P= distancia entre la ventosa ventral y la parte posterior corporal; PhL= longitud de la faringe; PhW: anchura de la faringe; TL= longitud testicular; TW= anchura testicular; TP= perímetro testicular; TR= circularidad testicular ($TR=TP^2/4\pi TW$).

Superficies: BA= superficie corporal; OSA= superficie de la ventosa oral; VSA= superficie de la ventosa ventral; PhA= superficie de la faringe; TA= superficie testicular.

Relaciones: BL/BW= relación entre longitud corporal y anchura corporal; BWOv/CW= relación entre la anchura corporal a nivel de ovario y anchura de cono; BL/VS-P= relación entre la longitud corporal y la distancia entre la ventosa ventral y el final corporal; OS/VS= relación entre área de la ventosa oral y área de la ventosa ventral.

La medida de la circularidad se utilizó para cuantificar su forma. Esta medida sirve para conocer como de circular es un objeto. Un objeto circular tendrá una circularidad de 1.0, cuanto más irregular es el objeto más grande son los valores (PERIAGO *et al.*, 2008).

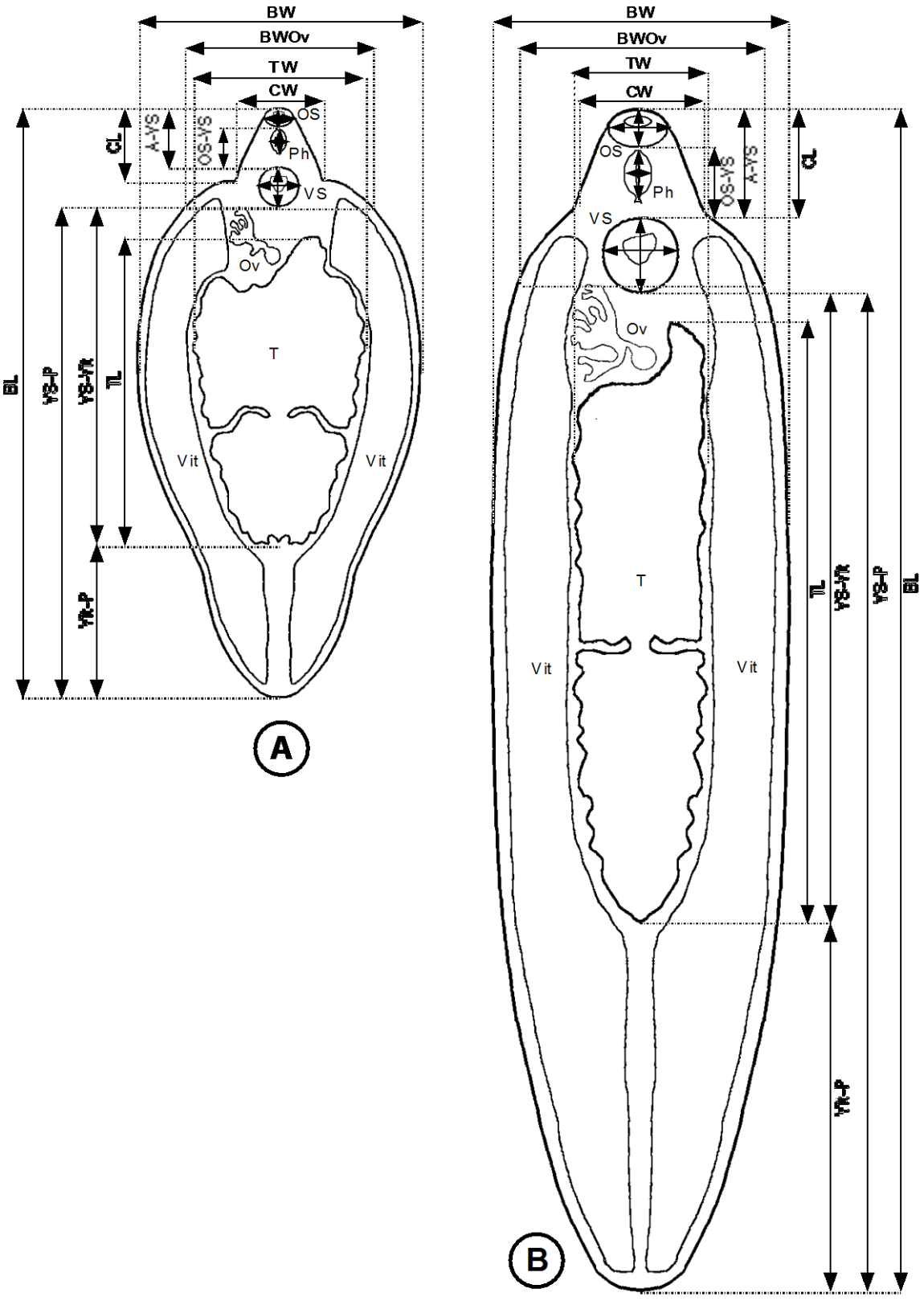

Figura 2.1. Medidas estandarizadas en adulto de: A) *F. hepatica* y B) *F. gigantica.* (PERIAGO *et al.,* 2006).

2.3.4. METODOS INMUNOLOGICOS

2.3.4.1. ELISA

2.3.4.1.1. ELISA DE CATEPSINA L1

La catepsina L1 (CL1) fue obtenida del Laboratorio de Parasitología Molecular, Escuela de Biotecnología, Facultad de Salud y Ciencia, Dublin City University, Dublin, Irlanda. La aplicación del ELISA de catepsina CL1 fue llevado a cabo y descrito previamente (O´NEILL *et al.,* 1999). Se incubaron "overnight" 100 µl de antígeno CL1 (5 µg/mL) a 37 ºC en placas de microtitulación (Nuclon, Kamstrup, Roskilde, Denmark). Los pocillos se bloquearon con una solución de albumina de suero bovino al 2%/ 0,1% de Tween 20 en un tampón salino fosfatado durante 30 minutos a 37º C , posteriormente se añadió el suero humano (dilución 1:100) en los pocillos por duplicado. Los anticuerpos humanos específicos se detectaron usando IgG4 biotin-conjugada anti-humano (dilución 1:2000), fosfatasa alcalina avidin-conjugada y el sustrato 2, 2-azino-bis (ácido 3-etilbenzotiazolina-6-sulfonico) (todos los productos procedentes de Sigma Chemical Co., Poole, Dorset, UK). Después de 20 minutos las placas se leyeron a una absorbancia de 405 nm en un lector de placas de ELISA (Bio-Rad, Modelo 680, Hércules, CA, USA). El "cut-off" se estableció en 3,09 y la desviación estándar de la media del suero control, el cual fue 0,098.

2.3.4.1.2. ELISA DRG

Todos los kits comerciales usados en el presente capitulo fueron enviados por DRG Instruments GmbH, Alemania. El test está basado en la detección de antígenos de excreción/secreción de *F. hepatica* que contienen predominantemente proteasas de cisteína del verme (SALIMI-BEJESTANI *et al.,* 2005). Todos los pasos se llevaron de acuerdo al protocolo facilitado por el fabricante. El suero humano, los controles positivos y negativos así como el control del "cut-off" (CO) (dilución 1:100) se añadieron por triplicado en los pocillos. Las placas se leyeron en un lector de ELISA a una absorbancia de 450 nm (Bio-Rad, Modelo 680, Hércules, CA, USA). El test se consideró valido cuando se cumplieron las siguientes condiciones, el substrato blanco tuvo una absorbancia por debajo de 0,100, el control negativo por debajo de 0,200, el control CO entre 0,250 y 0,750 y el control positivo superior a 0,600. Los resultados están expresados como la media de la densidad óptica (OD) obtenida en las muestras por triplicado, expresadas como un porcentaje del CO usando la siguiente formula:

Porcentaje positivo (PP)= (Media OD de la muestra/ Media OD del Control+)*100.

Un suero era considerado positivo cuando el valor de la media de su absorbancia era superior al 10% del CO.

Los resultados mostrados en unidades DRG (DU) se calcularon de acuerdo a la siguiente formula:

DU= (valor medio de la absorbancia de la muestra*10)/CO.

Los resultados eran considerados negativos cuando DU<9, y positivos cuando DU>11.

Para evitar cualquier prejuicio, todos los sueros fueron procesados sin el conocimiento de su clasificación en los tres grupos anteriormente mencionados (positivos para fascioliasis, otras parasitosis o sueros negativos).

2.3.4.1.3. ELISA MM3-COPROANTIGENO

Los kits de MM3-COPRO ELISA fueron proporcionados por el Dr. F.M. Ubeira de la Universidad de Santiago de Compostela (España), fueron preparados y testados como ha sido descrito anteriormente (MEZO *et al.,* 2004, VALERO *et al.,* 2009b; UBEIRA *et al.,* 2009). Existe una versión comercial del MM3-COPRO ELISA llamada BIO K 201, Bio-X Diagnostics, La Jemelle, Belgium. En resumen, se incubaron placas de microtitulación de poliestireno de tiras 1 x 8 F (Greiner Bio-One GmbH, Frickenhausen, Alemania) durante toda la noche con 100 µL/pocillo de una solución que contenía 10 µg/mL de un anticuerpo de conejo policlonal IgG anti-*Fasciola* en una solución tampón salina fosfato (PBS) (pocillos de filas impares), o con 100 µL/pocillo de una solución que contenía 10 µg/mL de anticuerpos policlonales IgG procedentes de conejos no inmunizados (pocillos de filas pares). Los sitios sin revestir se bloquearon con caseinato sódico al 1,5% en PBS durante 1 hora a temperatura ambiente. Cada sobrenadante fecal (100 µL) se añadió por cuadruplicado (2 pocillos de filas impares más 2 pocillos de filas pares), y se incubo durante toda la noche a 4º C. Después de lavar 6 veces con PBS al 0,2% Tween-20 (PBS-T), se añadió a cada pocillo 100 µL de una solución que contenía 0,3 µg de anticuerpos biotinados con MM3 en PBS-T más 1% albumina bovina de suero (PBS-T-BSA) y se incubo durante 1 h a 37º C. Después de lavar, tal y como hemos comentado anteriormente, la unión con el anticuerpo MM3 se detectó mediante incubación, primero con neutravidin conjugada con peroxidasa (Pierce, Rockford, Illinois; dilución 1:2000 en PBS-T-BSA) durante 1 h a 37º C , y después con el substrato (tampón H_2O_2 y o-fenilendiamina, OPD, Sigma-Aldrich, Madrid, España).

Después de una incubación de 20 minutos a temperatura ambiente, la reacción se paró mediante la adición de H_2SO_4 3N. Finalmente, la DO se midió a 492 nm. El valor de la DO se calculó como DO_1-DO_2, donde DO_1 es la media de los pocillos de filas impares (recubiertas con anticuerpos policlonales anti-*Fasciola*), y DO_2 es la media de los pocillos de filas pares (recubiertos con anticuerpos policlonales inespecificos). El valor de DO para cada muestra se calculó restando la DO de los pocillos pares a la DO de los pocillos impares. El valor de corte usado fue 0,097 (UBEIRA *et al.*, 2009).

2.3.5. ANALISIS MATEMATICO

2.3.5.1. TRAYECTORIAS ONTOGENICAS

Las nubes de dispersión de puntos obtenidas de las medidas morfométricas frente a la edad (*t*) ofrecen trayectorias ontogenéticas empíricas para los adultos de *F. hepatica* (VALERO *et al.*, 2006b). En el presente estudio, la trayectoria ontogénica describe el cambio del UA como una función respecto al tiempo en días. Las trayectorias ontogénicas son el resultado de ecuaciones diferenciales que expresan las leyes de crecimiento de variables morfológicas (ALBERCH *et al.*, 1979; DE RENZI, 1988). Un análisis preliminar de los resultados mostro que el modelo "amortiguado" ofrece la mejor representación de los datos. Para las trayectorias ontogénicas del UA, se ha verificado dos modelos "amortiguados": el modelo saturado (a) ($y=y_m[1-z_0^{(-kt)}]$) y el modelo logístico (b) ($y=y_m/[1+z_0^{(-kt)}]$), donde y_m es el valor máximo alcanzado por el UA, y z_0 y k son constantes o parámetros de las trayectorias; z_0 está relacionada con el valor inicial del área uterina (y), es decir y_0 cuando t=0, y exhibe una expresión diferente en cada caso. El parámetro k es una tasa de crecimiento en ambos casos. Para ajustar los modelos se utilizó el paquete estadístico McCurve Fit. El procedimiento consiste en seleccionar la función que minimiza la suma de los mínimos cuadrados residuales (sse), es decir $(yi-ÿi)^2$, con los datos brutos de yi y sus estimaciones ÿi. El ajuste de las funciones de curvas no lineales se testó usando r^2 y sse. El software GRAPHPAD PRISM 5.0 se utilizó para representar las trayectorias ontogénicas. Se han considerado resultados estadísticamente significativos cuando P<0,05. Se utilizó el software SPSS Statistics versión 19 para el cálculo del test chi-cuadrado, del test de Fisher y de la correlación de Pearson. Se han considerado resultados estadísticamente significativos cuando P<0,05.

2.3.5.2. ANALISIS DE COMPONENTES PRINCIPALES (ACP)

El análisis de los componentes principales (ACP), permite explorar la

variación individual y reconocer eventualmente los agrupamientos particulares sobre un "mapa factorial", que generalmente se trata de un "plot" de los dos primeros componentes principales (ROHLF & MARCUS, 1993; KLINGERBERG, 1996; DUJARDIN *et al.*, 2002; DUJARDIN & LE PONT, 2004). Este análisis no toma en cuenta la pertenencia de cada individuo a grupos distintos. Es un análisis ciego y frecuentemente puede detectar estos grupos a partir de los valores individuales (DUJARDIN *et al.*,2002). En resumen, la función del análisis en componentes principales es doble. Por una parte, permite reconocer una estructuración en particular de los individuos, correspondiente o no a su clasificación inicial, así que también puede ser utilizado como instrumento de clasificación. Por otra parte, el análisis en componentes principales presenta el interés adicional de proporcionar una "variable de tamaño", en general el primer componente principal (CPI), variable que será utilizada después para eliminar el "efecto tamaño" de las poblaciones en comparación (DUJARDIN *et al.*, 2002). Así, el análisis en componentes principales brinda una variable de tamaño (CPI), que es la combinación lineal que representa la varianza máxima. Además, geométricamente CPI se corresponde con la dirección del eje más largo a través de la nube de puntos. Las subsiguientes variables (CPII, CPIII, etc…), matemáticamente independientes, alcanzan la varianza máxima, siendo un eje ortogonal al resto de los componentes (KLINGERBERG, 1996). Además esta clase de análisis permite separar la influencia del tamaño de la influencia de la forma sobre la estructuración observada (DUJARDIN *et al.*, 2002).

En esta tesis hemos utilizado un análisis discriminante canónico en ausencia de tamaño, en la matriz de covarianza de logaritmos transformados. Esta técnica consiste en la regresión de cada carácter por separado dentro del grupo del primer componente principal (CP1), que es una estimación multivariada del tamaño (BOOKSTEIN, 1989; DOS REIS *et al.*, 1990). El análisis se llevó a cabo usando el software BAC v.2 (DUJARDIN, 2002). El método estadístico usado para las medidas tanto de los huevos como para los adultos de *F. hepatica* es muy similar a la utilizada por PERIAGO *et al.,* (2008) en especies adultos de *F. hepatica* y *F. gigantica* de la misma zona geográfica.

Para realizar de manera óptima estos dos objetivos del análisis en componentes principales, los datos iniciales deben transformarse en logaritmos en base 10. Esta transformación presenta numerosas ventajas estadísticas (normalización de las distribuciones, igualación de las varianzas, etc...) y permite comparar las diferencias relativas más que las absolutas (DUJARDIN *et al.*,2002).

2.3.5.3. ANALISIS DISCRIMINANTE CANONICO

Se realizó un estudio de inclusión por pasos usando la λ de Wilk´s (SPSS versión 12.0 y versión 15.0) de las poblaciones de fasciólidos por un análisis discriminante de medias usando el origen del área geográfica y la especie hospedadora como criterio de agrupación. Las variables canónicas se presentan como combinaciones lineales de las variables originales y se expresan por una función discriminante.

2.3.5.4. ANALISIS ESTADISTICO DE COMPARACION DE MEDIAS

La comparación de las medias sè lleva.on a cabo usando los siguientes test: a) ANOVA de una vía, b) ANOVA de tres vías, c) test post hoc Bonferroni y d) test t de student (SPSS v 19.0). El efecto del tamaño de la muestra se controla por el POWER (NORUSIS, 1994). Los valores se consideraron significativamente estadísticos cuando $P < 0,05$.

2.3.5.5. CORRELACIONES

Para el calculó de la correlación entre dos variables se utilizó la correlación de Pearson. Se han considerado resultados estadísticamente significativos cuando $P<0,05$.

2.3.5.6. CALCULO DE LA SENSIBILIDAD, ESPECIFICIDAD, VALOR PREDICTIVO POSITIVO (PPV) Y VALOR PREDICTIVO NEGATIVO (NPV)

En los test inmunológicos, se ha calculado los valores de sensibilidad y especificidad (FLETCHER & FLETCHER 2005) con un intervalo de confianza del 95% (95% CI; EPIINFO). El valor predictivo positivo (PPV) se calculó usando la siguiente formula:

*PPV= (sensibilidad*prevalencia) / (sensibilidad*prevalencia) + ((1-especificidad)*(1-prevalencia)).*

El valor predictivo negativo (NPV) se calculó usando la siguiente formula:

NPV= (especificidad(1-prevalencia)) / (((1-sensibilidad)*prevalencia) + ((especificidad)*(1-prevalencia))).*

El test del t-Student se calculó usando el software del SPSS v 19.0.

FENOTIPAJE DE FASCIOLIDOS

PHENOTYPING OF FASCIOLIDS

3. FENOTIPAJE DE FASCIOLIDOS

3.1. LAS CACTERISTICAS MORFOLOGICAS DE LOS HUEVOS DE LOS FASCIOLIDOS COMO HERRAMIENTAS EN EL DIAGNOSTICO DE LA FASCIOLIASIS HUMANA Y ANIMAL CAUSADA POR *F. HEPATICA* Y *F. GIGANTICA*

El objetivo del presente apartado es validar la identificación de *F. hepatica* y *F. gigantica* basándose en el tamaño y forma de los huevos emitidos por humanos y animales infectados, caracterizando sus rasgos morfométricos mediante un analizador de imágenes. Se ha analizado la influencia tanto del lugar de procedencia de la muestra (Sur América, Europa, África y Asia) como de la especie hospedadora (humano y diferentes especies de animales domésticos).

Para ilustrar la situación dentro de un área local endémica, hemos comparado las medidas de los huevos del parásito emitidos por humanos con huevos emitidos por el ganado procedente de la misma área geográfica. Este mismo esquema de trabajo lo hemos efectuado en distintas áreas geográficas consideradas importantes áreas endémicas de fascioliasis humana. En el nuevo mundo, hemos utilizado material del Altiplano Norte de Bolivia (MAS-COMA *et al.,* 1999a) y el valle de Cajamarca, Perú (ESPINOZA *et al.,* 2007), áreas caracterizadas por sus altas prevalencias e intensidades en humanos causada únicamente por *F. hepatica*.

En Europa, hemos utilizado material de la región de Kutaisi en Georgia, área donde está ampliamente distribuida *F. hepatica*, pero en donde sólo se ha detectado esporádicamente *F. gigantica* hace varias décadas (GIGITASHVILI, 1969) y en donde la fascioliasis humana está emergiendo actualmente (ZENAISHVILI *et al.,* 2004).

En África, el material procede de la región del Delta del Nilo de Egipto, en donde ambas especies, *F. hepatica* y *F. gigantica* co-existen y además los humanos presentan altas prevalencias e intensidades (ESTEBAN *et al.,* 2003; CURTALE *et al.,* 2007).

En Asia, el análisis se llevó a cabo en Vietnam, donde la enfermedad ha aparecido en humanos en los últimos años a lo largo de todo el país (DE *et al.,* 2003; LE *et al.,* 2008).

3.1.1. MATERIAL Y METODOS

Para la realización del presente apartado se utilizó el material que viene detallado en el punto 2.1.1. del capítulo de Material y Métodos (ver tabla 3.1, tabla 3.2 y tabla 3.3), incluyendo un aislado de Bolivia, uno de Perú, dos de Georgia, tres de Egipto y dos de Vietnam. Cuatro aislados de animales descritos previamente (VALERO *et al.*, 2001a) fueron incluidos para su comparación.

Las medidas morfométricas analizadas y la técnica de medición a través del analizador de imágenes utilizada en los huevos de Fasciólidos estudiadas vienen detallados en el punto 2.3.3. del capítulo de Material y Métodos (ver tabla 3.1, tabla 3.2 y tabla 3.3). Para la comparación de medias se ha utilizado el test t de student y un test post-hoc (Bonferroni) (véase apartado 2.3.5.4.).

Se ha utilizado un análisis de los componentes principales (véase apartado 2.3.5.2. del capítulo de Material y Métodos) incluyendo sólo cinco de las medidas realizadas (LH, AH, PH, ArH, LH/AH). Asimismo, se ha realizado un análisis discriminante canónico (véase apartado 2.3.5.3. del capítulo de Material y Métodos).

Aislado	LH(µm)	AH(µm)	PH(µm)	CH(µms)	ArH(µm^2)	LH/AH
humano, Bolivia (n=154)	119,8-159,5 (139,7±8,2)	67,6-102,1 (76,9±5,2)	321,2-429,5 (366,5±33,4)	1,1-1,4 (1,3±0,0)	6623,3-12166,4 (8356,4±847,6)	1,4-2,2 (1,8±0,1)
F. hepatica bovino, Bolivia (n=168)	105,3-155,9 (132,0±10,5)*	61,7-82,5 (71,14±4,3)	270,6-422,9 (340,0±33,4)*	-	5286,5-9676,8 (7170,2±802,5)*	1,6-2,3 (1,8±0,2)
F. hepatica ovino, Bolivia (n=104)	114,8-151,1 (130,8±7,1)*	65,5-81,4 (72,6±3,9)*	294,2-368,2 (327,6±15,0)*	-	5998,2-8608,5 (7238,0±532,8)*	1,5-2,1 (1,8±0,1)
F. hepatica porcino, Bolivia (n=186)	73,8-148,6 (123,8±11,3)*	58,1-82,6 (71,8±4,4)*	47,8-360,0 (313,7±20,9)*	-	3988,7-8626,9 (6837,0±820,3)*	1,1-2,0 (1,7±0,2)*
F. hepatica asno, Bolivia (n=161)	96,4-140,8 (125,4±8,3)*	63,3-84,7 (75,0±3,7)*	272,1-350,4 (318,8±16,3)*	-	5562,6-8686,2 (7177,4±646,1)*	1,3-2,0 (1,7±0,1)*
humano, Perú (n=167)	100,6-161,0 (138,4±9,9)	65,9-104,6 (80,2±6,6)	296,4-441,2 (369,0±23,3)	1,2-1,4 (1,3±0,1)	5779,3-12433,8 (8554,1±1068,3)	1,3-2,1 (1,7±0,2)

Tabla 3.1- Comparación de datos morfométricos de huevos de *Fasciola* procedentes de humanos y animales domésticos de regiones Andinas Sudamericanas: Altiplano Norte (Bolivia), valle de Cajamarca (Perú). Todos los valores se muestran como rango con la media ± la desviación estándar entre paréntesis. n= tamaño de la muestra.

Aislado	LH(µm)	AH(µm)	PH(µm)	CH(µms)	ArH(µm²)	LH/AH
humano, Georgia (n=53)	104,5-162,2 (140,1±11,8)	72,1-96,6 (82,1±4,7)	310,1-394,1 (366,4±17,5)	1,1-1,3 (1,2±0,0)	6594,6-10876,5 (8833,9±1012,1)	1,3-2,0 (1,7±0,1)
F. hepatica bovino, Georgia (n=117)	116,3-156,8 (140,2±10,1)	72,2-98,1 (83,4±6,9)	337,6-415,4 (373,0±16,2)$^{£}$	1,1-1,4 (1,2±0,1)	7191,2-11315,0 (8883,4±721,8)	1,3-2,0 (1,7±0,2)

Tabla 3.2- Comparación de datos morfométricos de huevos de *Fasciola* procedentes de humanos y bovinos de regiones europeas: región de Kutaisi (Georgia). Todos los valores se muestran como rango con la media ± la desviación estándar entre paréntesis. n= tamaño de la muestra.

Aislado	LH(µm)	AH(µm)	PH(µm)	CH(µms)	ArH(µm²)	LH/AH
humano, Egipto (n=608)	106,5-171,5 (139,4±11,0)	63,9-95,4 (76,7±5,4)	276,5-404,0 (346,0±20,5)	1,1-1,3 (1,1±0,0)	5540,9-11483,6 (8308,7±857,2)	1,4-2,5 (1,8±0,2)
F. hepatica bovino, Egipto (n=35)	120,6-163,9 (146,7±14,2)^{v}	69,2-93,8 (80,3±6,4)^{v}	317,9-419,8 (380,9±33,6)^{v}	1,2-1,4 (1,3±0,1)^{v}	6547,5-10957,8 (9093,7±1386,9)	1,6-2,1 (1,8±0,1)
F. gigantica bovino, Egipto (n=73)	130,3-175,0 (149,4±8,3)^{v}	74,0-123,6 (94,6±6,8)^{v}	358,6-456,7 (408,9±19,8)^{v}	1,1-1,4 (1,2±0,1)^{v}	7893,6-15603,4 (10996,0±1067,9)^{v}	1,3-2,0 (1,6±0,1)^{v}
humano, Vietnam (n=67)	150,9-189,2 (165,3±6,2)	85,1-106,2 (95,9±4,4)	394,8-453,3 (416,9±11,8)	1,1-1,2 (1,1±0,0)	11065,4-14537,8 (12152,0±671,5)	1,5-2,0 (1,7±0,1)
F. gigantica bovino, Vietnam (n=101)	156,2-182,8 (165,3±6,2)	90,6-114,9 (104,1±5,7)	405,2-487,3 (454,9±15,6)^{œ}	1,1-1,3 (1,2±0,0)^{œ}	11217,4-15518,2 (13487,4±924,8)^{œ}	1,4-1,9 (1,6±0,1)^{œ}
F. gigantica bovino, Burkina Faso (n=35)	129,6-204,5 (156,8±1,1)	61,6-112,5 (89,4±0,7)	335,5-417,8 (390,1±2,2)	1,0-1,3 (1,1±0,0)	7846,3-15890,7 (11144,1±124,3)	1,3-2,6 (1,8±0,0)

Tabla 3.3- Comparación de datos morfométricos de huevos de *Fasciola* procedentes de humanos y bovinos de regiones de Asia y África: Delta del Nilo (Egipto), Quy Nhon (Vietnam) y Burkina Faso. Todos los valores se muestran como rango con la media ± la desviación estándar entre paréntesis. n= tamaño de la muestra.

Las diferencias significativas encontradas cuando comparamos cada medida del huevo de las muestras humanas con los distintos hospedadores animales de la misma área se realizaron mediante un test *post hoc* (Bonferroni) en Bolivia (*) y Egipto (^{v}) y mediante el estudio de t de student en Georgia ($^{£}$) y Vietnam (*œ*) ($p<0,05$) tal y como se muestra en tabla 3.1, tabla 3.2 y tabla 3.3. Los datos procedentes de individuos de *F. gigantica* "estándar" procedentes de Burkina Faso se incluyeron en la comparación.

3.1.2. RESULTADOS

En las muestras de los huevos de las especies de *Fasciola* emitidas por humanos, el extremo opuesto al opérculo de la cubierta del huevo es normalmente irregular o arrugado (más o menos pronunciado) (Fig. 3.1). Aunque a veces, esta irregularidad está localizada lateralmente. El análisis morfológico de los huevos muestra que esta irregularidad en la superficie de la cubierta es muy común en Bolivia (99 casos con una protuberancia de 154 huevos: 64,3%) igual que en Perú (121 casos de 167: 72,5%). Esta irregularidad es menos frecuente en las muestras humanas de Egipto (30,1%). Además, es muy infrecuente en las muestras humanas de Georgia (solo fue detectado un huevo de 53: 1,9%) y Vietnam (solo en 8 huevos de 67: 11,9%).

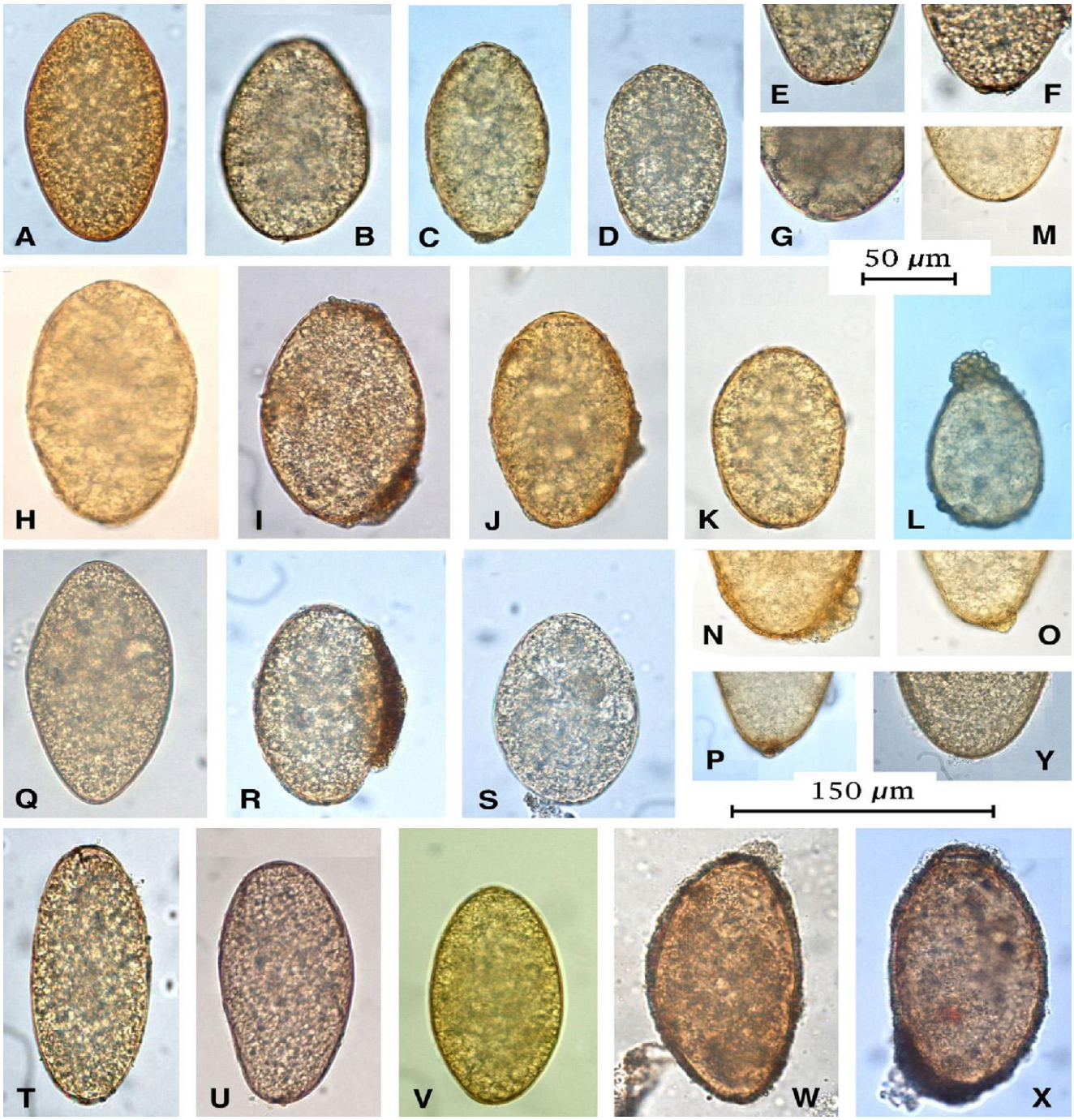

Figura 3.1- Variabilidad de la forma y tamaño de huevos emitidos por adultos fasciolidos en muestras de humanos: (A–G) *F. hepatica* del Altiplano Norte de Bolivia; (H–P) *F. hepatica* del valle de Cajamarca, Perú; (Q–S) *F. hepatica* de la región de Kutaisi, Georgia; (T–V) huevos de fasciolidos (principalmente fenotipos de *F. hepatica*) de la región del Delta del Nilo, Egipto; (W–Y) *F. gigantica* del área de Quy Nhon, Vietnam. Nótese la presencia de irregularidades laterales en la superficie de la cubierta de los huevos en I, J y R, presencia de las mismas irregularidades de la cubierta en el extremo opuesto al opérculo en C, E–G, N–P y X, y su ausencia en M e Y. Escalas: A–D, H–L, Q–X= 150µm; E–G, M–P, Y = 50µm (VALERO *et al.,* 2009).

3.1.2.1. ESTUDIO DE LA INFLUENCIA DE LA LOCALIZACION GEOGRAFICA

El tamaño de los huevos de *F. hepatica* obtenidos tanto de muestras animales como humanas se muestran en la tabla 3.1, tabla 3.2 y tabla 3.3. El estudio de la influencia de la localización geográfica en el tamaño de los huevos fasciólidos emitidos por humanos fue llevado a cabo mediante el análisis del CP1 y un análisis discriminante canónico. Un estudio similar fue llevado a cabo también en los huevos obtenidos en infecciones de bovinos:

A. Huevos emitidos por humanos: Las variables de los huevos de fasciolidos procedentes de muestras humanas de Bolivia, Perú, Georgia, Egipto y Vietnam se correlacionan significativamente en CP1, el cual contribuyo en un 78% a la variación total. El mapa factorial resultante (Fig. 3.2) ilustra claramente las diferencias globales en las poblaciones analizadas. Se pueden diferenciar dos zonas: una zona formada por Bolivia, Perú, Georgia y Egipto, mientras que la otra zona está formada sólo por Vietnam y se solapa con la zona de las muestras bolivianas y peruanas. Los resultados muestran que los datos de Georgia y Egipto se incluyen en el tamaño global de *F. hepatica.* Las funciones canónicas discriminantes tienen valores de λ de Wilks estadísticamente significantes ($P < 0,001$). Las funciones y_1 e y_2 incluyen 99,4% de la varianza acumulada y son las siguientes:

$$y_1 = 0,908LH + 0,696AH – 5,366PH + 3,668AH + 1,693\ LH/AH$$

$$y_2 = 0,825LH + 1,225AH – 0,401PH – 2,139AH + 0,684\ LH/AH$$

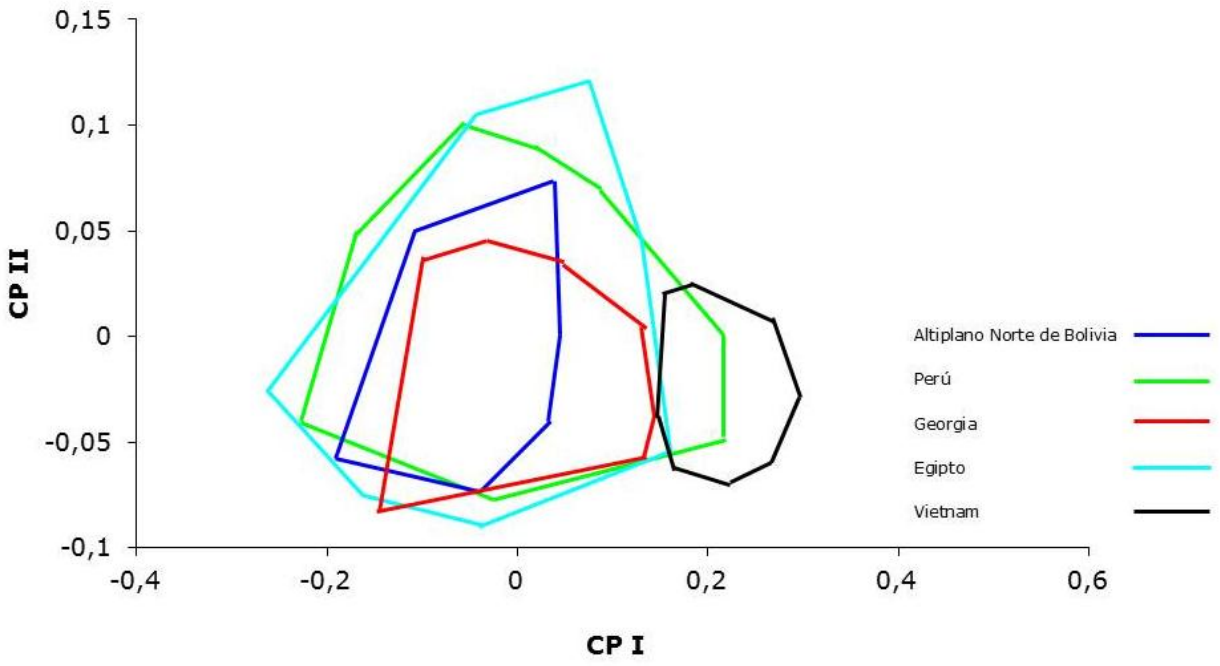

Figura 3.2- Mapa factorial correspondiente a huevos de *Fasciola* emitidos por humanos procedentes de Altiplano Norte de Bolivia, Perú, Georgia, Egipto y Vietnam. Las muestras son proyectadas sobre el primer (CPI, 74%) y el segundo (CPII, 19%) componente principal. Cada grupo está representado por su perímetro.

Las funciones lineares discriminantes (ejes 1, y_1 y 2, y_2) diferencian claramente los huevos de *F. hepatica* procedentes de Bolivia y Perú de los huevos de *F. gigantica* procedentes de Vietnam. Los resultados de acuerdo a la clasificación de predicción de grupos obtenidos con estas funciones discriminantes se incluyen en la tabla 3.4. Los especímenes procedentes de Georgia se solapan con algunos especímenes procedentes de Bolivia, Perú, Egipto y Vietnam. Aun así, los especímenes procedentes de Egipto forman un grupo diferente a los formados por los especímenes estándar de *F. hepatica* y *F. gigantica* y sólo se solapan con algunos especímenes de Vietnam. La media de los datos obtenidos en las muestras humanas muestra valores muy similares en todas las medidas de los huevos procedentes de Bolivia, Perú, Georgia y Egipto.

En resumen, el rango de la longitud y anchura de los huevos de *F. hepatica* de los humanos en zonas Andinas de altitud, donde solo *F. hepatica* está presente, es de 100,6-161,0/65,9-104,5 µm, valores que están muy cerca de los valores obtenidos en las muestras humanas procedentes de Georgia (104,5-162,0/72,1-96,6 µm) y Egipto (106,5-171,5/63,9-95,4 µm), donde *F. hepatica* y *F. gigantica* co-existen. Las

muestras humanas procedentes de Vietnam tienen los valores más altos, 150,9-182,2/85,1-106,2 µm, solapándose con los obtenidos en las otras muestras humanas analizadas.

		F, hepatica humano, Bolivia	*F, hepatica* humano, Perú	*F, hepatica* humano, Georgia	*Fasciola sp,* humano, Egipto	*F, gigantica* humano, Vietnam	Número Total
Original	*F, hepatica* humano, Bolivia	103	36	11	3	1	154
	F, hepatica humano, Perú	61	92	12	2	0	167
	F, hepatica humano, Georgia	7	25	7	15	2	56
	Fasciola sp, humano, Egipto	0	0	3	596	9	608
	F, gigantica humano, Vietnam	0	0	0	0	67	67
%	*F, hepatica* humano, Bolivia	66,9	23,4	7,1	1,9	0,6	100,0
	F, hepatica humano, Perú	36,5	55,1	7,2	1,2	0,0	100,0
	F, hepatica humano, Georgia	12,5	44,6	12,5	26,8	3,6	100,0
	Fasciola sp, humano, Egipto	0,0	0,0	0,5	98,0	1,5	100,0
	F, gigantica humano, Vietnam	0,0	0,0	0,0	0,0	100,0	100,0

Tabla 3.4- Resultados de la clasificación de predicción de grupos.

B. Huevos procedentes de bovino: Las variables de los huevos de fasciolidos procedentes de bovino de Bolivia, Georgia, Egipto y Vietnam se correlacionan significativamente en CP1, el cual contribuye el 94% a la variación total. El mapa factorial resultante (Fig. 3.3) ilustra claramente las diferencias globales del tamaño en las poblaciones analizadas. Este mapa factorial muestra que, aunque el tamaño de los huevos de *F. hepatica* y *F. gigantica* procedente de bovino no se solapa cuando se comparan poblaciones alopátricas de zonas geográficas distantes, como por ejemplo Bolivia y Vietnam, el tamaño de los huevos de *F. hepatica* y *F. gigantica* se solapan cuando se comparan poblaciones simpátricas, como por ejemplo

en Egipto. Las funciones canónicas discriminantes tienen valores significativos de λ de Wilks (P<0,001). Las funciones y_1 e y_2, incluyen el 97,7% de la variación acumulada y son las siguientes:

$$y_1 = 1{,}082LH + 2{,}228AH - 0{,}103PH - 1{,}645AH + 2{,}299LH/AH$$

$$y_2 = -3{,}469LH - 0{,}053AH - 0{,}300PH + 2{,}911AH + 3{,}089LH/AH$$

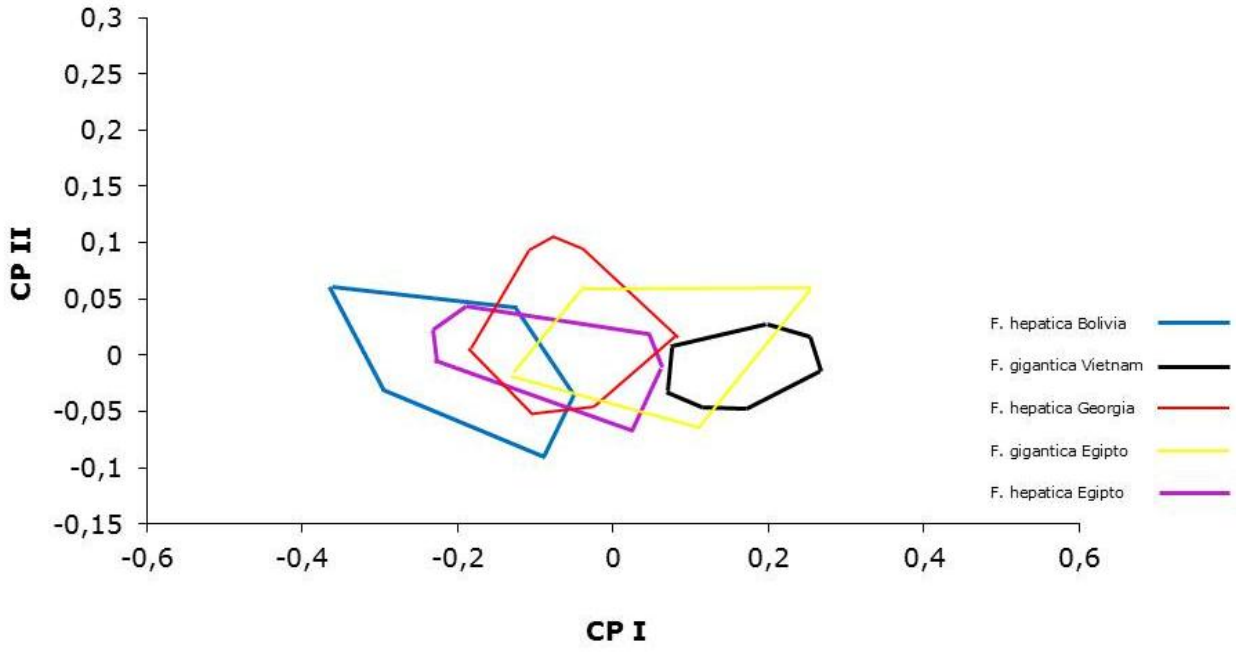

Figura 3.3- Mapa factorial correspondiente a huevos de *Fasciola* procedentes de ganado bovino infectado naturalmente de Bolivia, Georgia, Egipto y Vietnam. Las muestras se proyectan sobre el primer (PC1, 94%) y el segundo (PC2, 5%) componente principal. Cada grupo es representado por su perímetro.

Las funciones lineares discriminantes (ejes 1, y_1 y 2, y_2) claramente diferencian los huevos de *F. hepatica* de Bolivia de los huevos de *F. gigantica* procedente de Vietnam. Los resultados de la clasificación de acuerdo a la predicción de grupos obtenidos con esta función discriminante se incluyen en la tabla 3.5. Los especímenes de Georgia se solapan con algunos especímenes de Bolivia y Egipto. Aun así, los especímenes de *F. gigantica* procedente de Egipto forman un grupo diferente pero se solapan con algunos especímenes de *F. hepatica* procedentes de Bolivia, Georgia y Egipto y algunos especímenes de *F. gigantica* procedentes de Vietnam.

		F, hepatica bovino, Bolivia	*F, gigantica* bovino, Vietnam	*F, hepatica* bovino, Georgia	Adultos similares a *F, gigantica* bovino, Egipto	Adultos similares a *F, hepatica* bovino, Egipto	Número Total
Original	*F, hepatica* bovino, Bolivia	57	0	9	1	1	68
	F, gigantica bovino, Vietnam	0	92	0	9	0	101
	F, hepatica bovino, Georgia	15	0	56	6	40	117
	Adultos similares a *F, gigantica* bovino, Egipto	1	7	2	60	3	73
	Adultos similares a *F, hepatica* bovino, Egipto	8	0	4	3	20	35
%	*F, hepatica* bovino, Bolivia	83,8	0,0	13,2	1,5	1,5	100,0
	F, gigantica bovino, Vietnam	0,0	91,1	0,0	8,9	0,0	100,0
	F, hepatica bovino, Georgia	12,8	0,0	47,9	5,1	34,2	100,0
	Adultos similares a *F, gigantica* bovino, Egipto	1,4	9,6	2,7	82,2	4,1	100,0
	Adultos similares a *F, hepatica* bovino, Egipto	22,9	0,0	11,4	8,6	57,1	100,0

Tabla 3.5- Resultados de la clasificación de predicción de grupos.

3.1.2.2. ESTUDIO DE LA INFLUENCIA DE LA ESPECIE HOSPEDADORA

El estudio de la influencia de la especie hospedadora sobre el tamaño del huevo se llevó a cabo mediante un análisis de CP1 en dos áreas geográficas, Bolivia y Vietnam. Las variables morfométricas de los huevos de *F. hepatica* del Altiplano Norte de Bolivia procedente de humanos y animales domésticos (ovino, bovino, porcino y asno) se correlacionaron significativamente en CP1, el cual contribuyo el 86% a la variación total. El mapa factorial resultante (Fig.3.4) ilustra claramente las diferencias globales de tamaño en la población humana analizada frente a las especies hospedadoras animales domésticas, incluyendo un mayor tamaño en los huevos procedentes de humanos. Las funciones canónicas discriminantes tienen valores significativos de λ de Wilks (P<0,001). Las funciones y_1 e y_2, incluyen 95,0% de la varianza acumulada y son las siguientes:

$$y_1 = 0{,}074LH + 1{,}930AH - 1{,}349PH - 1{,}224AH + 1{,}518LH/AH$$

$$y_2 = -3{,}915LH + 3{,}853AH + 0{,}867PH - 1{,}563AH + 5{,}366LH/AH$$

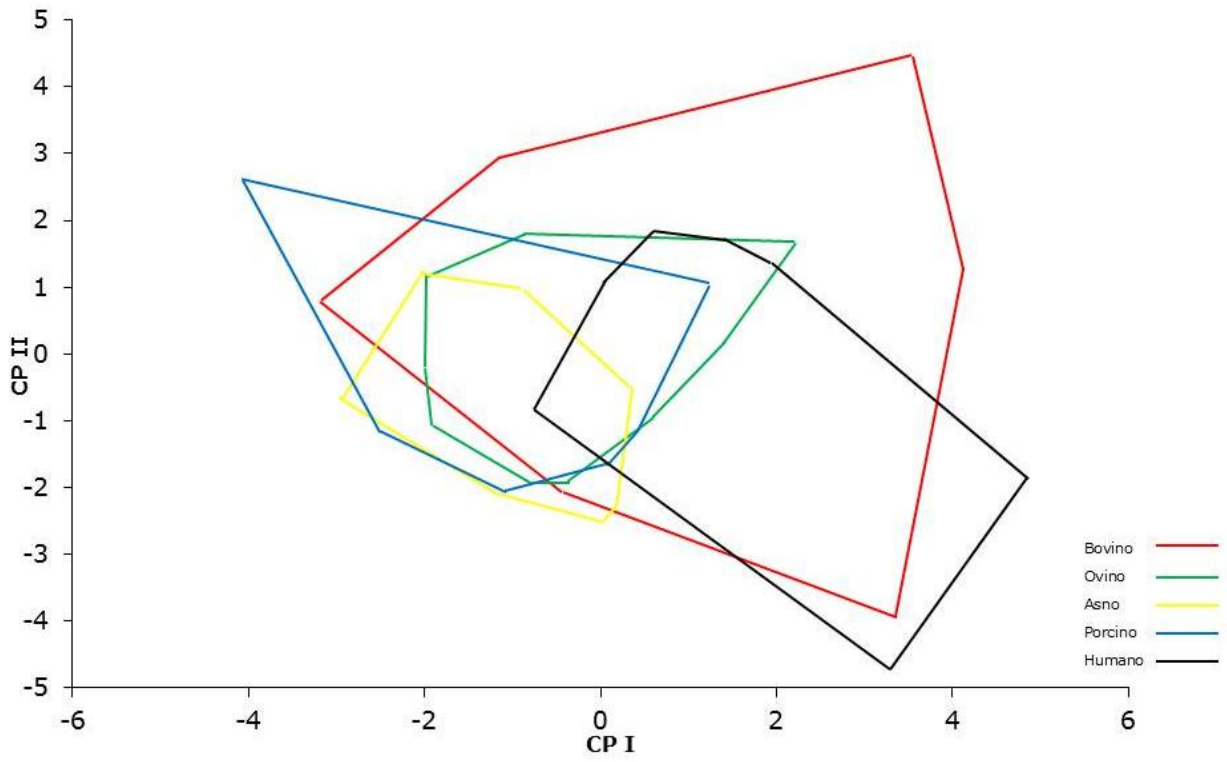

Figura 3.4- Mapa factorial correspondiente a huevos de *Fasciola* procedentes de humanos y ganado (bovino, ovino, porcino y asno) de la misma zona endémica en el Altiplano Norte de Bolivia. Las muestras se proyectan sobre el primer (CP1, 86%) y el segundo (CP2, 11%) componente principal. Cada grupo está representado por su perímetro.

Las funciones lineares discriminantes (ejes 1, y_1 y 2 y_2) diferencian claramente la población de huevos de *F. hepatica* procedente de humanos y la población de huevos de *F. hepatica* de otras especies hospedadoras. Los resultados de la clasificación de acuerdo a la predicción de grupos obtenido con estas funciones discriminantes se muestran en la tabla 3.6. Los especímenes procedentes de humanos forman un grupo diferente pero se solapan con algunos especímenes procedentes de ovino, bovino, porcino o asno. El análisis de la media de las medidas absolutas de los huevos obtenidos del Altiplano Norte de Bolivia muestra que los valores de los huevos de *F. hepatica* de las muestras humanas son mayores que la media de los valores de huevos procedentes de las especies hospedadoras. Las diferencias significativas obtenidas cuando se comparan cada par de datos de medidas de los huevos procedentes tanto de muestras de humanos como de animales usando el test Bonferroni se muestran en la tabla 3.1, tabla 3.2 y tabla 3.3. Los resultados confirman que el tamaño de los huevos de *F. hepatica* en las muestras humanas poseen su propia identidad morfológica.

Las medidas de los huevos de *F. gigantica* procedente de muestras humanas y bovino están correlacionadas significativamente con el CP1, que contribuye al 74% de la variación total. El mapa factorial resultante (Fig. 3.5) ilustra claramente las diferencias globales de tamaño en la población humana analizada, incluyendo un menor tamaño global de los huevos de *F. gigantica* de humanos que los huevos procedentes de bovino.

		F, hepatica bovino, Bolivia	*F, hepatica* ovino, Bolivia	*F, hepatica* asno, Bolivia	*F, hepatica* porcino, Bolivia	*F, hepatica* humano, Bolivia	Número Total
Original	*F, hepatica* bovino, Bolivia	19	22	7	12	8	68
	F, hepatica ovino, Bolivia	16	45	26	13	4	104
	F, hepatica asno, Bolivia	0	13	36	12	0	61
	F, hepatica porcino, Bolivia	3	18	23	41	1	86
	F, hepatica humano, Bolivia	20	2	5	1	126	154
%	*F, hepatica* bovino, Bolivia	27,9	32,4	10,3	17,6	11,8	100,0
	F, hepatica ovino, Bolivia	15,4	43,3	25,0	12,5	3,8	100,0
	F, hepatica asno, Bolivia	0,0	21,3	59,0	19,7	0,0	100,0
	F, hepatica porcino, Bolivia	3,5	20,9	26,7	47,7	1,2	100,0
	F, hepatica humano, Bolivia	13,0	1,3	3,2	0,6	81,8	100,0

Tabla 3.6- Resultados de la clasificación de predicción de grupos.

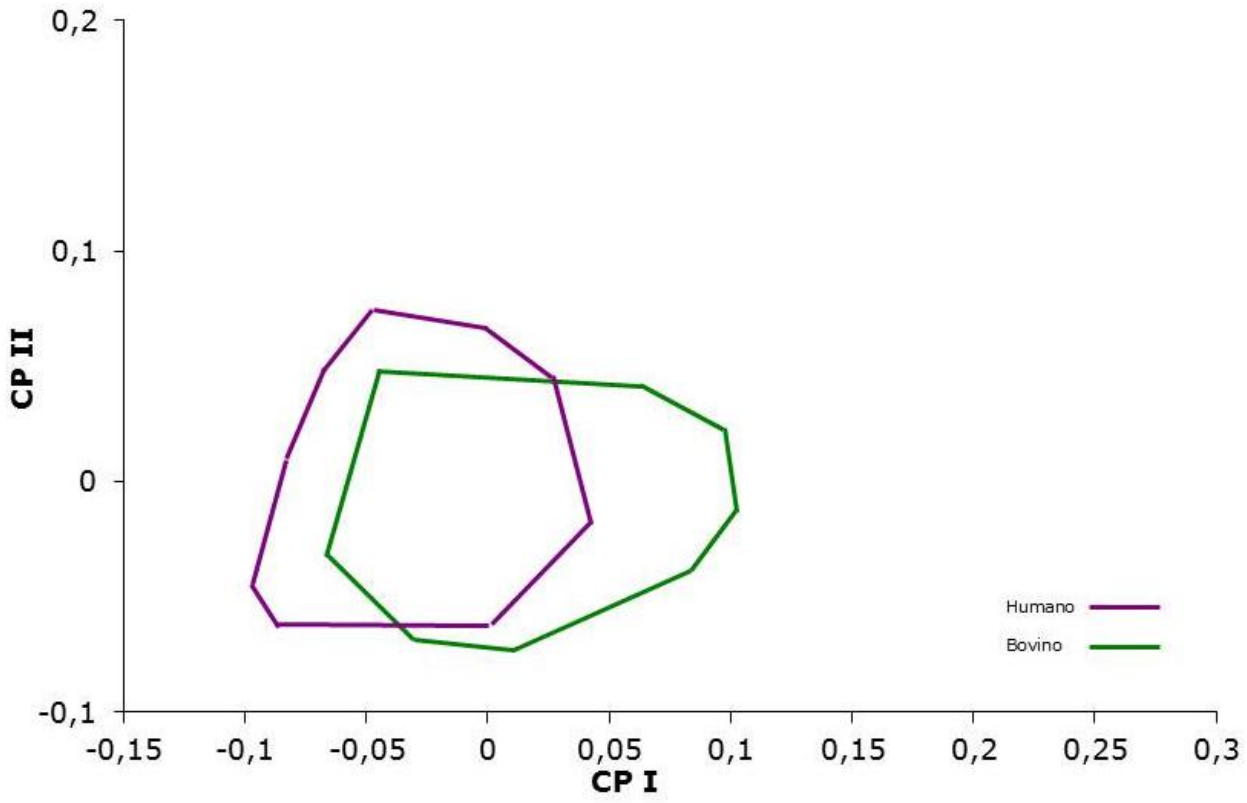

Figura 3.5- Mapa factorial correspondiente a huevos de *Fasciola* procedentes de humanos y bovinos de la misma zona endémica en Vietnam. Las muestras se proyectan sobre el primer (PC1, 70%) y el segundo (PC2, 13%) componente principal. Cada grupo está representado por su perímetro.

Las funciones discriminantes canónicas tienen valores de λ de Wilks estadísticamente significativa (P<0,001). Los resultados de la clasificación de acuerdo a la predicción de grupos, obtenidos con estas funciones discriminantes se muestran en la tabla 3.7. Las diferencias significativas obtenidas cuando se comparan los pares de puntos de cada media de los huevos procedentes de muestras humanas y animales usando un test t student se muestran en la tabla 3.1, tabla 3.2 y tabla 3.3. Los resultados confirman que el tamaño de los huevos de *F. gigantica* en las muestras humanas posee su propia identidad, y estos huevos de *F. gigantica* procedentes de muestras humanas tienen valores medios más pequeños que los valores medios de los huevos procedentes del bovino.

		F, gigantica humano, Vietnam	*F, gigantica* bovino, Vietnam	Número Total
Original	*F, gigantica* humano, Vietnam	67	0	67
	F, gigantica bovino, Vietnam	6	95	101
%	*F, gigantica* humano, Vietnam	100,0	0,0	100,0
	F, gigantica bovino, Vietnam	5,9	94,1	100,0

Tabla 3.7- Resultados de la clasificación de predicción de grupos.

3.1.3. DISCUSION

Hasta el final de 1990, la fascioliasis humana fue considerada como una enfermedad adquirida raramente en zonas endémicas animales. Así, la mayoría de los estudios publicados se limitaban solo a casos individuales o citas de grupos familiares. En estas situaciones, los huevos encontrados eran escasos y se presentaban en intervalos irregulares. Siendo a veces necesaria la repetición del examen coproparasitológico de las muestras para detectar los huevos (ARJONA *et al.*, 1995). No obstante en la actualidad se han descrito zonas hiperendémicas humanas, en las que se han detectado intensidades y prevalencias de parasitación pronunciadamente altas, especialmente en los niños (MAS-COMA *et al.*, 2005). Desafortunadamente, los análisis realizados en estas áreas, sólo incluían estudios epidemiológicos humanos a gran escala, y hasta el presente estudio, no se había efectuado un análisis exhaustivo de la morfometría de los huevos de fasciolidos procedentes de parasitaciones humanas. Los huevos de las especies de *Fasciola* son operculados, largos, amarillentos parduzcos, ovalados y no embrionados en el momento de la puesta, y su anchura y longitud están dentro de un rango especifico, que es uno de los criterios utilizados para el diagnóstico de la fascioliasis humana.

Clásicamente, se ha considerado que en el extremo opuesto al opérculo, la superficie de los huevos de *Fasciola* es a menudo rugosa y esta zona irregular no se observa en los huevos de *Fasciolopsis* (ASH & ORIHEL, 1997). Cabe destacar que nuestro estudio muestra que la frecuencia de esta característica en *F. hepatica* es población dependiente, y por tanto no es un criterio patognomónico en el diagnóstico. No obstante, no podemos analizar ni establecer si la irregularidad en la superficie del huevo en el extremo opuesto al opérculo y/o lateral observada en este estudio está relacionada con el origen geográfico o es debida a las anormalidades de la producción de huevos por el tratamiento antiparasitario administrado a los humanos (como por ejemplo, el uso habitual de Albendazol en campañas de tratamiento de los países Andinos).

Tradicionalmente, la Fascioliasis se ha considerado un problema veterinario en todo el mundo, y por lo tanto el rango de tamaño de los huevos que podemos encontrar en la literatura aplicado al diagnóstico humano en realidad se ha obtenido a través del análisis de muestras parasitas procedentes de hospedadores animales domésticos: *F. hepatica* 130-148/63-90 µm (BORAY, 1982), 130-145/70-90 µm (WHO, 1991) o 130-150/63-90 µm (MAS-COMA & BARGUES, 1997), y *F. gigantica* 150-196/90-100 µm (BORAY, 1982; MAS-COMA & BARGUES, 1997). Por lo tanto, tradicionalmente los extremos que permitían la diferenciación de ambas especies, se consideraba que eran 150 µm de longitud y 90 µm de anchura, donde los valores menores representaban a individuos de *F. hepatica* y los mayores a *F. gigantica*.

Nuestro estudio revela que los humanos, en comparación con otras especies hospedadoras, tienen una influencia decisiva en el tamaño de los huevos de *F. hepatica* y *F. gigantica*, mostrando una gran variación con el rango de tamaño mencionado anteriormente. Así, en humanos el tamaño de los huevos de *F. hepatica* es mayor y el tamaño de los huevos de *F. gigantica* es más pequeño que el tamaño clásicamente indicado en la literatura. Aun así, estas variables de tamaño intraespecíficas se solapan cuando ambas especies se comparan. Esta variabilidad en el tamaño de los huevos de *Fasciola* también es similar al tamaño de los huevos de otras especies de Trematodos que pueden infectar al hombre y que presentan una morfología similar, como por ejemplo las especies de los géneros *Fasciolopsis*, *Gastrodiscoides* (MAS-COMA *et al.*, 2005) y *Echinostoma* (WHO, 1991) y pueden provocar una confusión en la clasificación de huevos cuando esta es llevada a cabo por personal sin experiencia, si no se considera ningún otro criterio diagnostico (ver, por ejemplo, BELISARIO *et al.*, 2007).

El solapamiento de la distribución geográfica de *F. hepatica* y *F. gigantica* también ha provocado una gran controversia en la identificación taxonómica de las especies de *Fasciola* encontradas en países del Sud-Este Asiático, en donde algunos ejemplares se parecen a *F. hepatica*, mientras que otros son similares a *F. gigantica* y también hay presentes formas morfológicamente intermedias. Cabe añadir también la existencia de un fenómeno anormal de gametogénesis, partenogénesis diploide, triploide y mixoploide, y procesos de hibridación entre genotipos diferentes (MAS-COMA & BARGUES, 1997). Hoy en día, el camino más fácil para confirmar genéticamente que un espécimen recolectado en la naturaleza es un híbrido es detectando una introgresión (DOWLING & SECOR, 1997; MAS-COMA & BARGUES, 2009). En el caso de *Fasciola*, ha sido confirmada la existencia de formas hibridas cuando se mostró que los fasciólidos procedentes de animales en Japón presentaban secuencias de DNA casi idénticas a una de las especies de fasciolidos y secuencias mitocondriales de DNA casi idénticas a las obtenidas en la otra especie (ITAGAKI & TSUTSUMI, 1998; ITAGAKI *et al.*, 1998). Por otra parte, en áreas asiáticas simpátricas de ambas especies de *Fasciola* se ha detectado un gran solapamiento en las medidas de los huevos emitidos (WATANABE, 1962; SAHBA *et al.*, 1972; KIMURA *et al.*, 1984; SRIMUZIPO *et al.*, 2000). En Vietnam, los vermes adultos encontrados en animales y pacientes humanos se dividían en dos categorías morfológicas, una típica de *F. gigantica* y otra muy similar a *F. hepatica* (LE *et al.*, 2008). LE *et al.* (2008) público datos que informaban que la hibridación ocurría en las poblaciones de *Fasciola* en Vietnam y éstas poblaciones parásitas estaban implicadas en la infección humana. Los huevos analizados en este capítulo muestran que el tamaño de los huevos emitidos por humanos en Vietnam sólo se corresponde con la morfología de los emitidos por *F. gigantica*.

F. gigantica es el principal fasciólido presente en África. Los valores morfométricos de los huevos de fasciólidos procedentes de bovinos europeos y africanos fueron analizados por PERIAGO *et al.* (2006) usando CIAS. Los resultados mostraron que el rango de las medidas de los huevos analizados se solapa con poblaciones alopátricas de *F. hepatica* y *F. gigantica* (LH: 107,3-152,7 µm en *F. hepatica* procedente de España, 100,2-155,6 µm en *F. hepatica* procedente de la isla francesa de Córcega, y 129,6-204,5 µm en *F. gigantica* de Burkina Faso; AH: 52,4-89,1 µm en *F. hepatica* procedente de España, 55,0-87,7 en *F. hepatica* procedente de Córcega, y 61,6-112,5 µm en *F. gigantica* de Burkina Faso). En Egipto, tanto *F. hepatica* como *F. gigantica* están presentes, pero normalmente se utiliza la terminología de *Fasciola sp*. para la fascioliasis humana en el Delta del Nilo (ESTEBAN *et al.*, 2003; CURTALE *et al.*, 2007). En Egipto, en áreas endémicas humanas, se ha descrito en bovino formas fenotípicas de vermes adultos

intermedias de *F. hepatica*/*F. gigantica*, siendo la morfología de *F. hepatica* más frecuente que la morfología típica de *F. gigantica* (PERIAGO *et al.*, 2008). Estos datos concuerdan con los obtenidos en nuestro estudio, en donde el tamaño de los huevos emitidos por humanos corresponden morfológicamente con *F. hepatica*.

Cabe resumir que las medidas de los huevos de *F. hepatica* y *F. gigantica* que pueden obtenerse a partir de un microscopio como son LH, AH y LH/AH, tanto de origen animal como humano, en parte se solapan en áreas donde ambas especies de fasciólidos coexisten, como es el caso de Egipto, por lo que se debe tener precaución cuando se utiliza el tamaño de los huevos como único criterio de diagnóstico diferencial (tabla 3.1, tabla 3.2 y tabla 3.3). Se alcanza una conclusión similar cuando se comparan otras medidas obtenidas mediante el CIAS como son PH, CH y ArH (tabla 3.1, tabla 3.2 y tabla 3.3). En este sentido, nuestros hallazgos deberían ayudar a los clínicos de estas áreas ya que el uso del rango de tamaño clásico de los huevos de *Fasciola* puede conducir a un diagnóstico erróneo.

Las medidas de los huevos de fasciólidos emitidos en las muestras de un paciente japonés (INOUE *et al.*, 2007) constituye un buen ejemplo para ilustrar el problema expuesto. En este paciente, los adultos fasciólidos fueron obtenidos vivos mediante endoscopia y mostraron características morfométricas similares al patrón *F. hepatica*. Las secuencias de DNA ribosomales ITS-2 e ITS-1 (fragmentos completos de 327 y 351 pb y secuencias espaciadoras de 364 y 432 pb, respectivamente) obtenidas de una mezcla de huevos procedentes de las heces del paciente coincidían con esta especie de fasciólido. La secuencia mitocondrial COI obtenida (desafortunadamente sólo se pudo obtener un fragmento corto de 493-pb y no la secuencia genética completa de 1533-pb) era también cercana a *F. hepatica*. Aunque todas estas características indicaban que se trataba de *F. hepatica*, el tamaño de los huevos 153-175/75-95 µm (media 164,7/83,7 µm) (INOUE *et al.*, 2007) no se ajustaba a las características de tamaño tradicionales de *F. hepatica* (130-150/63-90 µm), sin embargo sí que se ajusta a las de *F. gigantica* (150-196/90-100 µm) (MAS-COMA & BARGUES, 1997). Cuando se comparan con los datos de huevos obtenidos de muestras humanas en el presente estudio, el tamaño los huevos procedentes de Japón se ajusta bien con el tamaño de los huevos de *F. hepatica* observados en zonas donde ambas especies de fasciolidos se solapan, excepto por la longitud del huevo (175 µm), la cual sobrepasa ligeramente la detectada en humanos en Egipto (171,5 µm). La media de la anchura de huevo de los fasciólidos procedentes de Japón (83,7 µm) es muy parecida a la obtenida en los huevos de *F. hepatica* procedentes de Perú (80,2 µm) o Georgia (82,1 µm). Además, la anchura mínima de los huevos procedentes de Japón (75 µm) es mucho

menor que la anchura mínima obtenida en los adultos de *F. gigantica* procedentes de Vietnam (85,1 µm). Esta comparación sugiere que los fasciólidos Japoneses se originaron probablemente a partir de una introgresión en el linaje a largo plazo y que dicha introgresión no fue detectada en el corto fragmento de la secuencia COI obtenida en el artículo. Hay que tener en cuenta que la introgresión puede suponer sólo la substitución de una parte del genoma mitocondrial, y no necesariamente de todos los genes del mtDNA, por lo que en un área de solapamiento de ambas especies, se puede esperar una acumulación de muchas formas hibridas de introgresión genéticamente diferentes como resultado de un cruzamiento (MAS-COMA & BARGUES, 2009).

3.2. CORRELACION ENTRE LA EMISION DE HUEVOS Y EL DESARROLLO UTERINO EN AISLADOS HUMANOS Y ANIMALES DE *F. HEPATICA*

El objetivo del presente apartado es estudiar en el modelo murino Wistar, bajo unas condiciones de dosis infectivas estandarizadas, la relación entre el crecimiento uterino vs la emisión de huevos en adultos de *F. hepatica* obtenidos experimentalmente a partir de aislados procedentes de áreas hiperendémicas humanas como es el caso del Altiplano Norte de Bolivia. Hemos realizado un seguimiento a largo plazo de más de 300 días post-infección, incluyendo así el estado de cronicidad avanzada. Este es el primer estudio que relaciona el tamaño del útero y el número de huevos por gramo de heces (epg) emitidos, cuantificados mediante la técnica de Kato-Katz, la herramienta más común en el diagnóstico para infecciones por helmintos en humanos. En este apartado se ha estudiado la evolución del área uterina de *F. hepatica* vs el tiempo y se ha analizado la influencia de diferentes factores, como son el tipo de aislado y el estado de cronicidad avanzado, sobre el desarrollo uterino.

3.2.1. MATERIAL Y METODOS

Para la realización del presente apartado se utilizó el material que viene detallado en el punto 2.1.2.3. y 2.1.2.4. del capítulo de Material y Métodos.

En la tabla 3.8 se especifica la evolución del área uterina y epg de los distintos aislados a lo largo de toda la infección.

Aislado		40 dpi (N)	50 dpi (N)	75 dpi (N)	100 dpi (N)	150 dpi (N)	175 dpi (N)	200 dpi (N)	225 dpi (N)	261 dpi (N)	300 dpi (N)
Humano	UA±SD	-	1,8±1,2(3)	9,0±2,4(3)	8,6±2,7(3)	12,7±1,2(3)	7,0±3,6(11)	-	10,0±2,7(3)	-	7,3±0,7(4)
	epg±SD	-	21,4±16,8	193,8±225,6	272,5±192,4	331,3±272,4	301,7±164,3	-	457,1±174,4	-	527,9±573,8
Bovino	UA±SD	-	2,0±0,1(2)	4,2±1,1(2)	9,5±1,3(2)	9,1±3,5(4)	-	11,5±1,3(2)	9,7±1,5(2)	-	-
	epg±SD	-	42,1±11,9	77,5±14,8	268,8±128,6	215,2±146,7	-	223,2±185,8	120,0±196,6	-	-
Porcino	UA±SD	1,8±0,2(2)	3,9±1,2(2)	5,72±1,2(2)	7,1±1,0(2)	4,4±0,1(2)	8,8±0,3(3)	9,0±0,9(3)	7,4±2,8(3)	3,02±0,9(3)	-
	epg±SD	32,0±18,3	58,2±33,2	114,9±31,1	247,2±58,5	96±104,6	242,7±33,2	227,2±109,0	125,6±63,1	143,0±119,7	-
Ovino	UA±SD	1,8±0,1(2)	-	5,3±1,8(11)	6,8±2,9(9)	7,6±1,0(5)	6,6±0,8(7)	-	9,2±2,7(5)	-	6,2±2,2(19)
	epg±SD	2,5±3,1	-	251,7±73,4	163,9±47,1	260,6±160,8	188,9±66,6	-	230,1±61,7	-	302,9±122,4

Tabla 3.8- Comparación entre el desarrollo del área del útero (UA) en mm^2 y los datos de emisión de huevos a lo largo del tiempo en el modelo murino de rata Wistar infectadas experimentalmente con *F. hepatica*. Epg, media de emisión de huevos por gramo de heces/adulto entre 10 días antes hasta el día correspondiente a la obtención de los datos de UA (dpi, días post infección). Todos los valores se muestran como media ± desviación estándar; N, numero de adultos.

Para la realización del presente apartado se utilizó técnica de Kato-Katz detallada en el punto 2.3.2.2 del capítulo de Material y Métodos. Las medidas morfométricas y la técnica de medición utilizada en los úteros de *Fasciola*, vienen detallados en el punto 2.3.3 del capítulo de Material y Métodos. Por último, en el punto 2.3.5.1 del citado capítulo se detalla el método matemático que se utilizó en el cálculo de las trayectorias ontogenéticas. Para evaluar el posible efecto del origen del aislado de las metacercarias experimentales, se ha utilizado un ANOVA de tres vías, con cada valor individual de UA en cada grupo de edad.

3.2.2. RESULTADOS

3.2.2.1. SEGUIMIENTO DE LOS HUEVOS Y EL AREA UTERINA (40-300 dpi)

En la Fig. 3.6 se representa la evolución de la media epg/adulto/día obtenido para cada aislado frente al tiempo. Los resultados del recuento de huevos en heces muestran que existe una fuerte oscilación de un día para otro en todos los aislados. Se ha realizado el análisis de la correlación (ANOVA) de la media de UA vs la media de ln (epg), incluyendo: (a) la media de epg emitidos entre los 10 días antes de la obtención del valor correspondiente de UA; (b) la media de epg emitidos entre los 25 días antes de la obtención del valor correspondiente de UA. La correlación obtenida en el ANOVA sólo fue estadísticamente significativa cuando se correlacionó UA frente a la media de los últimos 10 días del ln (epg) (bovino: F=10.223, P<0,033, power: 42%; ovino: 11.215, 0,020, 99%; porcino: 11.215, 0,020, 52,2%; y humano: 7.444, 0,041, 25,5%). La evolución de la UA de acuerdo con los dpi en las ratas inoculadas con los cuatro aislados y la evolución de la emisión de huevos en heces 10 días antes de la obtención del correspondiente valor de UA obtenido se muestran en la tabla 3.8 y Fig. 3.7, respectivamente.

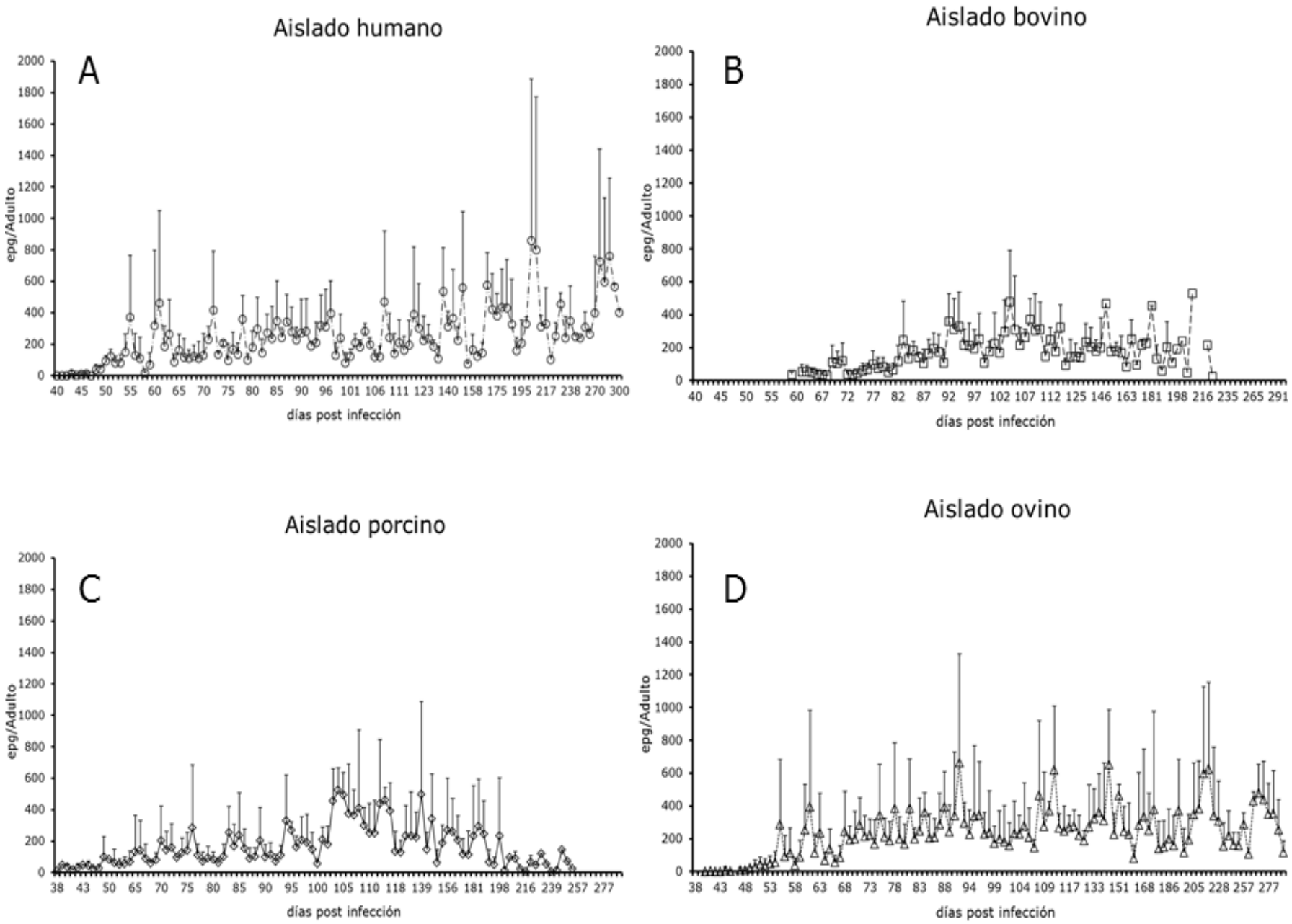

Figura 3.6- Huevos por gramo de heces (epg)/adulto/día obtenidos a lo largo de la infección en los cuatro aislados de *F. hepatica* analizados: (A) aislado humano (○); (B) aislado bovino (□); (C) aislado porcino (◇) y (D) aislado ovino (Δ). Cada símbolo representa la media por cada día analizado. Las barras representan la desviación estándar.

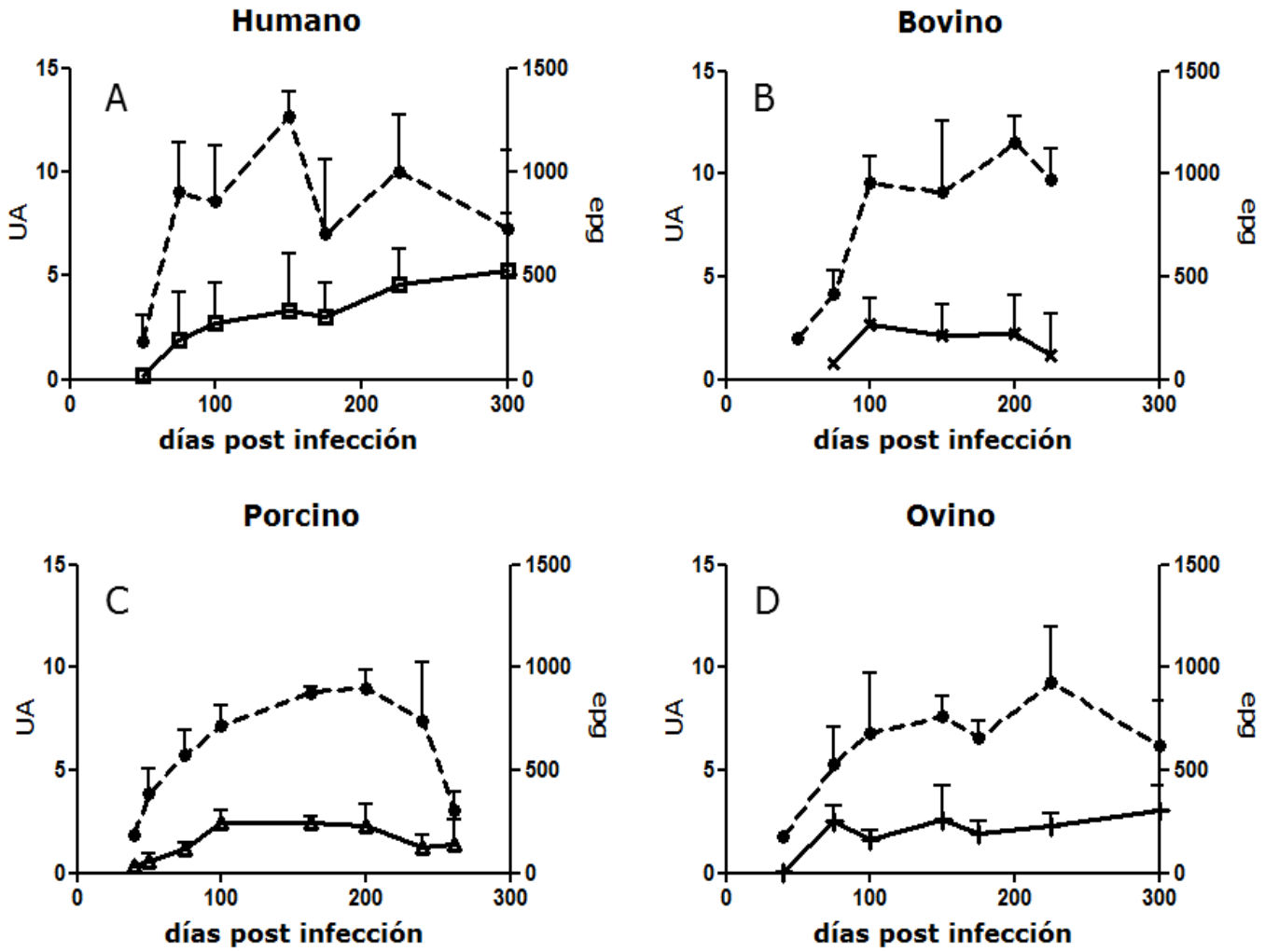

Figura 3.7- Evolución del área del útero de fasciolidos (UA) y huevos por gramo de heces (epg)/adulto a lo largo de la infección en los cuatro aislados de *F. hepatica* analizados: (A) aislado humano (□); (B) aislado bovino (X); (C) aislado porcino (Δ) y (D) aislado ovino (+). Cada símbolo representa la media por cada día analizado. UA: cada símbolo representa la media de UA de cada grupo de fasciolidos a diferentes días. Epg: cada símbolo representa la media de epg emitidos entre 10 días antes y el día cuando se obtuvo el dato de UA; --- = UA; __ = epg/adulto.

3.2.2.2. CRECIMIENTO DEL AREA UTERINA

El modelo de regresión múltiple muestra que UA es dependiente de los dpi ($P<0,001$) y del tipo de aislado ($P<0,001$). El análisis general de todos los parásitos correspondientes a los 225-300 dpi muestra una elevada dispersión del UA, que por lo general tiende a disminuir hasta los 225 dpi. Es interesante apuntar que se observó la completa ausencia de huevos en el útero en algunos adultos parásitos a los 300 dpi (8%), lo cual corresponde con el cese de emisión de huevos en la fase crónica avanzada. Por ello, se analizó el desarrollo medio de UA en adultos fasciólidos de cada aislado exclusivamente entre los 40 hasta 225 dpi. El UA sigue

un modelo de crecimiento asintótico hasta los 225 dpi y después disminuye. Se han estudiado las trayectorias ontogénicas de las medidas de UA en adultos fasciólidos. Los resultados obtenidos están reflejados en la Fig. 3.8.

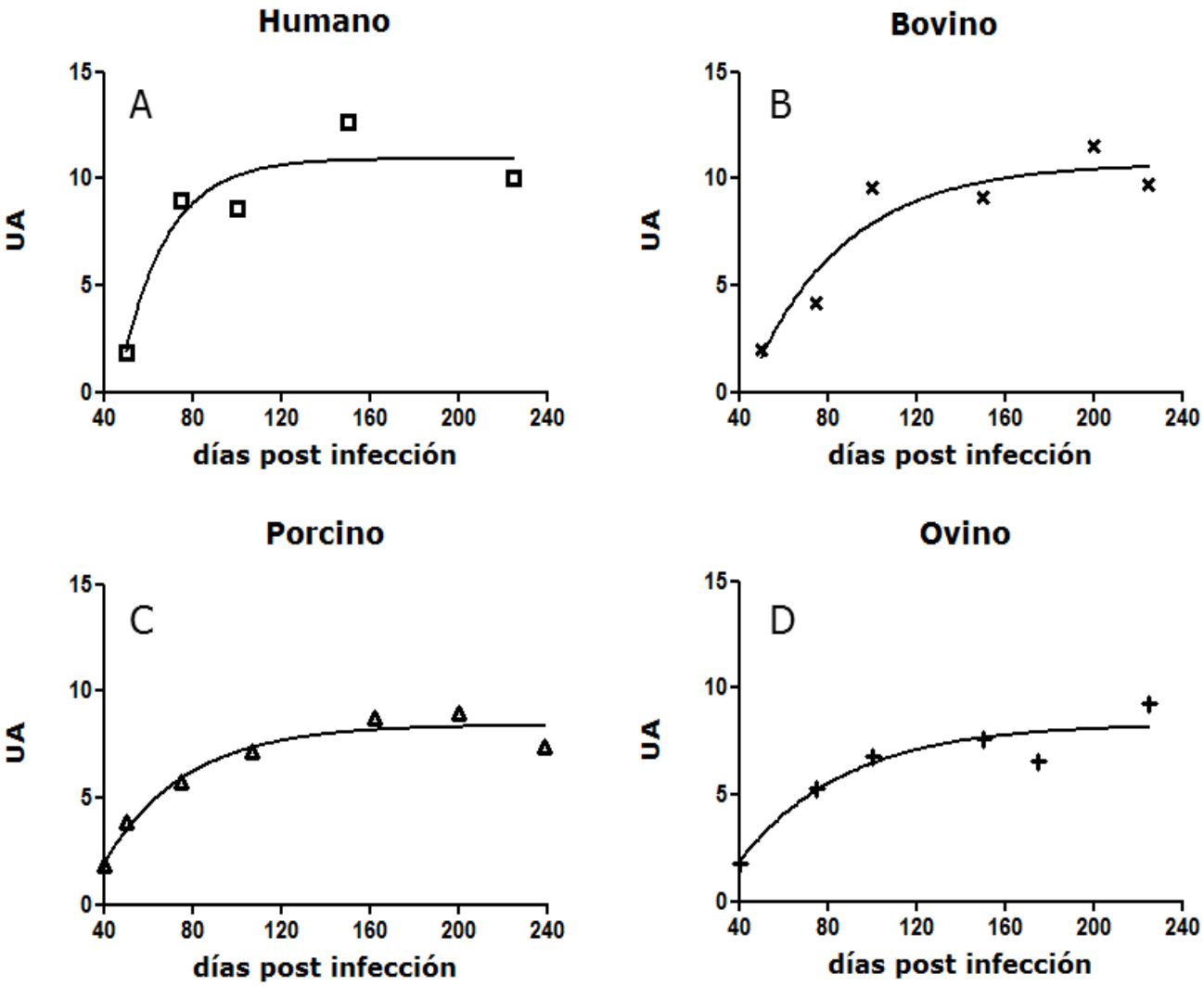

Figura 3.8- Trayectoria ontogénica del área del útero (UA) (en mm^2) en función del tiempo (días) con el modelo de crecimiento saturado $y=y_m[(1-z_o^{(-kt)}]$ en adultos obtenidos de ratas Wistar infectadas experimentalmente con los cuatro aislados de *F. hepatica* analizados: (A) aislado humano (□); (B) aislado bovino (X); (C) aislado porcino (Δ) y (D) aislado ovino (+). Cada símbolo representa la media de UA en el día correspondiente.

3.2.3. DISCUSION

En ensayos experimentales, la determinación de los epg emitidos por helmintos se realiza de forma aproximada. Así, la eficacia del tratamiento antihelmíntico experimental se determina por la disminución del recuento de huevos en heces (McCONVILLE *et al.*, 2009). Además de reducir la carga de helmintos adultos, algunas vacunas pueden inducir una reducción en la producción

de huevos de los parásitos (HILLYER, 2005; PIEDRAFITA *et al.*, 2010). Las técnicas microscópicas comúnmente empleadas en el diagnóstico cuantitativo de huevos de helmintos son muy específicas pero sin embargo son poco sensibles. Las cantidades de epg en materia fecal normalmente son muy variables. Además, la técnica de recuento de epg aplicada es de particular importancia (EBRAHIM *et al.*, 1997). La técnica de Kato-Katz se emplea rutinariamente para el diagnóstico y la cuantificación de la carga parasitaria (KATZ *et al.*, 1972) en la mayoría de las helmintiasis humanas. Tanto la simplicidad del test como su fácil realización en condiciones de trabajo de campo han asegurado su aplicación universal. Sin embargo, la sensibilidad de un único examen puede ser muy baja debido a diversos factores como pueden ser: (a) pequeña cantidad de muestra analizada; (b) variación en la distribución de los huevos en una sola muestra de heces; (c) diferentes cargas parasitarias; (d) las fluctuaciones diarias en un individuo debido a la producción y consistencia fecal; (e) las fluctuaciones diarias de un individuo debido a los patrones de ovoposición del parasito (HALL, 1982; DE VLAS *et al.*, 1992; ENGELS *et al.*, 1997; UTZINGER *et al.*, 2001). Además, la consistencia de la materia fecal también depende de la carga parasitaria y hay que tener en cuenta que las infecciones parasitarias con cargas tanto moderadas como altas pueden causar diarrea (TEESDALE *et al.*, 1985; FELDMEIER & POGGENSEE, 1993). También se debe tener en cuenta las cantidades de fibra o grasa presentes en la dieta del hospedador. Todos estos factores limitan la validez de la aplicación de las técnicas cuantitativas cuando se calculan los epg, lo cual provoca cierta imprecisión poniendo en peligro los ensayos experimentales (EBRAHIM *et al.*, 1997; UTZINGER *et al.*, 2001). Por consiguiente, a una sola cuantificación de epg no da la suficiente confianza, por lo que se requiere de numerosas muestras para la detección de diferencias entre grupos de hospedadores o parásitos. En este sentido, dado que el recuento de huevos emitidos en materia fecal es esencial, los estudios aplicados podrían beneficiarse de una mayor precisión si se dispusiera de un marcador poco variable.

En los últimos años se ha producido un dramático resurgimiento de la fascioliasis. Este fenómeno ha sido atribuido, entre otros factores, al cambio climático (MAS-COMA *et al.*, 2009b). Además, se conoce desde hace unos años la resistencia de *F. hepatica* a los fasciolicidas comerciales disponibles, como es el caso del Triclabendazol (Fasinex) y el Nitroxynil (Trodax) (OVEREND & BOWEN, 1995; FAIRWETHER & BORAY, 1999; GAASENBEEK *et al.*, 2001). Por ello, en la actualidad, se ha incrementado el número de estudios encaminados a encontrar nuevos posibles fármacos o vacunas eficaces. En estos estudios, la eficacia del fármaco o la de la vacuna normalmente se determina usando como biomarcador el

descenso de la producción de huevos. Sin embargo, tal y como hemos comentado, la emisión de huevos de *F. hepatica* en heces está normalmente sujeto a oscilaciones, tanto en el caso de animales así como en el hombre (VALERO *et al.*, 2002; EL-MORSHEDY *et al.*, 2002). Para evitar estas inexactitudes, algunos autores han cuantificado el estado de desarrollo de órganos sexuales (testículos, glándulas vitelógenas, ovario y útero) de acuerdo con un sistema de puntuación (HANNA *et al.*, 2006; McCONVILLE *et al.*, 2009).

En este estudio se muestra que existe una relación entre el UA del adulto fasciólido y la producción de huevos, lo cual coincide con los resultados obtenidos en otras helmintiasis. En el caso de Nematodos de animales, se ha descrito una correlación positiva entre el tamaño del verme y la producción de huevos (SKORPING *et al.*, 1991; STEAR *et al.*, 1997; STEAR & BISHOP, 1999; RICHARDS & LEWIS, 2001; IRVINE *et al.*, 2001; DEZFULI *et al.*, 2002; UGLAND *et al.*, 2004). Una correlación positiva similar también existe en el caso de infecciones humanas por *Ascaris lumbricoides* (SINNIAH & SUBRAMANIAM, 1991; WALKER *et al.*, 2009), detectándose una relación entre la producción de huevos y peso, longitud y diámetro del verme (SINNIAH & SUBRAMANIAM, 1991). Cabe remarcar que antes de que el adulto hembra de *A. lumbricoides* alcance su crecimiento corporal máximo, existe un descenso en la producción de huevos, tal y como ocurre con *F. hepatica*. Los adultos fasciólidos siguen un proceso de desarrollo establecido en su hospedador definitivo (VALERO *et al.*, 2001a, 2006b; PERIAGO *et al.*, 2008). En el caso de *F. hepatica*, la longitud del útero en sus diferentes estadios de desarrollo se correlaciona con el tamaño corporal (NEUHAUS, 1978). En poblaciones de bovino, ovino y porcino infectadas en la naturaleza del Altiplano Norte de Bolivia, el UA vs el área corporal sigue un modelo de crecimiento asintótico (VALERO *et al.*, 2001b). En el estudio experimental de PANOVA (2002) se demostró que el desarrollo del UA de *F. hepatica* con respecto el tiempo se ajusta a un modelo saturado, mientras que el crecimiento corporal sigue un modelo logístico caracterizado por dos fases (VALERO *et al.*, 1998): la parte "exponencial" del crecimiento logístico corresponde al desarrollo corporal durante la migración intraorgánica a través de la cavidad abdominal y el parénquima hepático, así como el desarrollo y la maduración sexual en los conductos biliares hasta el inicio de la producción de huevos. Desde este momento en adelante, el desarrollo corporal sigue un crecimiento logístico "saturado" con una persistencia considerable del crecimiento corporal después de la maduración sexual. La ovoposición es el punto de inflexión del crecimiento logístico el cual marca el final del periodo "exponencial" y el principio del periodo "saturado". Es decir, el comienzo de la emisión de huevos al medio externo constituye el factor biológico que marca el punto de inflexión (VALERO *et al.*, 2006b). PANOVA (2002)

muestra que el UA presenta un modelo saturado frente al tiempo y que el área corporal y el UA de *F. hepatica* siguen un modelo de crecimiento sincronizado después de comenzar la emisión de huevos. Estudios previos efectuados por otros autores demuestran la existencia de una variabilidad inherente en distintos aislados de *F. hepatica* y la relación entre esas diferencias y la fecundidad del parasito (WALKER *et al.*, 2006). Estos autores comparan la producción de huevos *in vitro* de dos aislados distintos, llamados Oberon y Fairhurst, y muestran que el segundo producía cuatro veces más huevos. Nuestro estudio también detecta diferencias en la producción de huevos entre los aislados analizados.

3.3. CARACTERIZACION FENOTIPICA DE *F. HEPATICA* EN AREAS ANDINAS HUMANAS ENDEMICAS: ANALISIS DEL PATRON VALLE VERSUS PATRON ALTIPLANICO DE FASCIOLIDOS PROCEDENTES DE OVINOS

El objetivo del presente apartado es la caracterización fenotípica de poblaciones de *F. hepatica* de áreas andinas hiperendémicas humanas de alta altitud. Dichas poblaciones se han comparado con poblaciones estándar europeas de baja altitud. La caracterización fenotípica es un paso más en la caracterización de la fascioliasis en áreas hiperendémicas humanas andinas, proporcionando información que permitirá aplicar las medidas apropiadas para el control de la enfermedad en los distintos países Sudamericanos. Aunque la infectividad de las metacercarias no parece diferir en los distintos aislados de ganado (VALERO & MAS-COMA, 2000), la especie de hospedador definitivo afecta de manera esencial al fenotipo tanto del adulto fasciólido como de los huevos emitidos en las heces, debido principalmente a la diferencia en el tamaño del microhábitat, el canal biliar (VALERO *et al.,* 2001a, b, 2002,2009).

3.3.1. MATERIAL Y METODOS

Para la realización del presente apartado se utilizó material de adultos grávidos de *F. hepatica* obtenidos de ovino, una especie hospedadora típica de este parásito. Como poblaciones andinas de altitud se utilizó material procedente del Altiplano norte de Bolivia (4000 m de altitud) y de los valles peruanos de Mantaro (3271 m de altitud) y Cajamarca (2663 m de altitud). Como material estándar europeo de baja altitud se utilizó parásitos procedentes de infecciones naturales de *F. hepatica* de Valencia (España) y adultos experimentales procedentes de un aislado de Polonia (ambas poblaciones de zonas geográficas próximas al nivel del mar). Este material que viene detallado en el punto 2.1.2.1. y 2.1.2.2. del capítulo de Material y Métodos. Todas las medidas estandarizadas realizadas a los adultos de *F. hepatica* fueron acordes a los métodos propuestos por VALERO *et al.* (2005) y

PERIAGO *et al.* (2006, 2008) (ver Fig. 2.1) y están descritas detalladamente en el punto 2.3.3.1. del capítulo de Material y Métodos. Los datos morfométricos del adulto se estudiaron mediante un análisis de los componentes principales, detallado en el punto 2.3.5.2. del capítulo de Material y Métodos.

3.3.2. RESULTADOS

Los valores morfométricos de los adultos de *F. hepatica* procedente de zonas de alta altitud del valle de Cajamarca, valle del Mantaro y del Altiplano Norte de Bolivia, así como adultos estándar europeos de *F. hepatica* de zonas situadas a nivel de mar se indican en la tabla 3.9 (características biométricas lineares), tabla 3.10 (superficies) y tabla 3.11 (relaciones entre medidas). Estos resultados son la primera descripción fenotípica de los adultos de *F. hepatica* procedentes de Perú. El análisis de la circularidad corporal (BR) de las poblaciones de *F. hepatica* muestra un solapamiento general entre ellas, a pesar de su diferente área geográfica de origen. El tamaño de los adultos fasciólidos se analizó mediante un análisis multivariante. La Fig. 3.9 muestra el mapa factorial de los dos primeros componentes principales (CP). Cabe recordar que el primer componente principal (CP1) se interpreta como una medida del tamaño total. El análisis de la proyección en el CP1 de las 5 poblaciones de *F. hepatica* estudiadas indica que las poblaciones procedentes de los valles de Cajamarca y Mantaro (Perú), así como las poblaciones estándar de España y la población experimental, presentan un tamaño corporal máximo y mínimo similar. Por otra parte, la población procedente del Altiplano Norte de Bolivia comparte el mismo valor máximo con las poblaciones anteriormente mencionadas pero presenta un tamaño mínimo más bajo (Fig. 3.9). Estos resultados indican que los fasciólidos procedentes de Perú y Europa (tanto los de infecciones naturales como experimentales) poseen un tamaño mínimo común en el cual los adultos parásitos son grávidos, mientras que, este tamaño mínimo es más bajo en los adultos fasciólidos procedentes de Bolivia.

Medidas del adulto (mm) Características biométricas lineares	Cajamarca Perú n=130	Mantaro Perú n=47	Altiplano N, Bolivia n=201	Valencia España n=37	Bialowieza Polonia n=127
Longitud corporal, BL	13,48-30,97 18,86± 0,31	13,41-27,75 19,70± 0,40	9,64-31,04 18,08± 0,31	14,21-31,17 20,82± 0,64	12,45-26,68 18,52±0,29
Anchura corporal, BW	5,06-14,23 10,25± 0,14	7,60-13,93 10,88± 0,23	4,23-13,41 8,26± 0,14	7,49-12,76 9,75± 0,16	6,88-12,74 10,19±0,10
BW a nivel del ovario, BWOv	4,23-11,97 8,30± 0,11	5,48-11,31 8,50± 0,19	3,51-11,71 6,71±0,12	6,45-10,57 8,13±0,17	5,69-10,17 7,98±0,08
Perímetro corporal, BP	30,20-71,11 45,70± 0,66	33,71-64,51 48,15± 0,98	26,89-68,35 47,51± 0,72	39,85-71,41 52,90± 1,33	33,22-66,06 47,91±0,64
Circularidad corporal, BR	1,09-1,94 1,25± 0,01	1,13-1,48 1,26± 0,01	1,11-2,12 1,53±0,01	1,31-1,76 1,46±0,02	1,23-1,73 1,43±0,01
Longitud de cono, CL	1,32-2,98 2,11± 0,02	1,36-2,59 1,89± 0,04	1,32-3,04 2,12± 0,02	1,55-2,98 2,10± 0,06	1,10-3,07 2,01±0,03
Anchura de cono, CW	2,12-4,41 3,17± 0,04	2,30-4,21 3,04± 0,06	1,78-3,92 2,65± 0,03	2,46-4,12 3,25± 0,06	2,08-4,19 3,27±0,03
Diámetro máximo de la ventosa oral, OSmax	0,63-1,14 0,84±0,01	0,61-1,02 0,81±0,01	0,53-1,06 0,76±0,01	0,63-1,00 0,84±0,01	0,70-1,01 0,86±0,01
Diámetro mínimo de la ventosa oral, OSmin	0,36-0,85 0,66±0,01	0,29-0,90 0,62±0,02	0,42-0,86 0,63±0,01	0,52-0,86 0,67±0,01	0,33-0,74 0,57±0,1
Diámetro máximo de la ventosa ventral, VSmax	0,89-1,29 1,12±0,01	0,83-1,31 1,08±0,01	0,75-1,25 1,00±0,01	0,92-1,40 1,19±0,02	0,89-1,40 1,19±0,01
Diámetro mínimo de la ventosa ventral, VSmin	0,77-1,21 0,99±0,01	0,72-1,22 0,95±0,01	0,67-1,29 0,98±0,01	0,68-1,48 1,06±0,03	0,79-1,23 1,04±0,01
Distancia entre la parte anterior corporal y la ventosa ventral, A-VS	1,61-3,26 2,48± 0,02	1,75-2,88 2,29± 0,04	1,52-3,35 2,24± 0,02	1,66-3,15 2,32± 0,05	1,16-3,09 2,13±0,03
Distancia entre la ventosa oral y la ventosa ventral, OS-VS	1,19-2,40 1,81±0,02	1,16-2,15 1,66±0,03	0,87-2,56 1,60±0,02	1,13-2,49 1,65±0,05	0,64-2,50 1,56±0,03
Distancia entre la ventosa ventral y el punto de unión de las glándulas vitelógenas, VS-Vit	6,78-20,60 11,56± 0,21	8,11-17,36 12,30± 0,30	4,49-19,82 11,32±0,22	9,65-22,97 13,84±0,47	7,71-19,75 12,33±0,23
Distancia entre el punto de unión de las glándulas vitelógenas y la parte posterior corporal, Vit-P	2,69-9,47 4,91± 0,12	2,64-8,09 5,20± 0,19	0,49-9,83 3,76±0,14	1,12-6,89 3,45±0,20	1,99-7,02 4,35±0,09
Distancia entre la ventosa ventral y la parte posterior corporal, VS-P	11,39-28,37 16,47± 0,30	11,27-25,36 17,50± 0,43	7,11-27,39 15,07± 0,31	11,21-26,84 17,29± 0,58	10,43-24,66 16,68±0,29
Longitud de la faringe, PhL	0,55-0,96 0,78±0,01	0,58-0,91 0,75±0,01	0,37-0,93 0,68±0,01	0,59-0,89 0,74±0,01	0,51-1,07 0,77±0,01
Anchura de la faringe, PhW	0,24-0,55 0,36±0,005	0,25-0,48 0,36±0,01	0,18-0,50 0,34±0,004	0,30-0,51 0,40±0,01	0,26-0,56 0,36±0,004
Longitud testicular, TL	4,54-23,53 8,86±0,20	5,56-12,70 9,05±0,26	3,12-14,62 8,12±0,17	6,03-14,53 9,39±0,32	5,33-14,50 8,92±0,17
Anchura testicular, TW	3,12-9,07 6,84±0,09	4,84-9,87 7,01±0,16	2,84-8,97 5,69±0,10	5,08-9,38 6,42±0,16	4,66-9,16 7,37±0,07
Perímetro testicular, TP	16,76-48,75 28,71±0,44	20,08-41,44 30,68±0,75	13,06-48,53 26,94±0,56	20,81-44,36 30,93±0,82	22,88-47,93 33,13±0,49

Tabla 3.9- Comparación de los resultados morfométricos relativo a las características biométricas lineares (valores máximos y mínimos, media y desviación estándar) de adultos de *F. hepatica* procedentes de ovinos infectados de forma natural en zonas de alta altitud del valle de Cajamarca, valle del Mantaro y el Altiplano Norte de Bolvia, y adultos de *F. hepatica* procedentes de ovinos infectados tanto de forma natural como experimental de zonas europeas de baja altitud (n = número de adultos).

Medidas del adulto (mm) **Superficies**	**Cajamarca** **Perú** **n=130**	**Mantaro** **Perú** **n=47**	**Altiplano N,** **Bolivia** **n=201**	**Valencia** **España** **n=37**	**Bialowieza** **Polonia** **n=127**
Área corporal, BA	47,34-283,95 135,87± 3,73	79,89-250,58 149,30± 5,64	31,11-236,14 106,39± 3,35	75,09-239,13 142,75± 6,22	68,09-227,11 129,78±3,17
Área ventosa oral, OSA	0,20-0,67 0,46±0,01	0,19-0,72 0,42±0,01	0,21-0,66 0,38±0,01	0,33-0,66 0,44±0,01	0,24-0,53 0,39±0,01
Área ventosa ventral, VSA	0,53-1,22 0,89±0,01	0,49-1,26 0,82±0,02	0,44-1,23 0,78±0,01	0,95-1,35 1,13±0,01	0,56-1,31 0,98±0,01
Área de la faringe, PhA	0,12-0,35 0,21±0,004	0,13-0,31 0,20±0,01	0,05-0,34 0,17±0,003	0,15-0,31 0,22±0,01	0,11-0,34 0,20±0,003
Área testicular, TA	13,10-112,85 46,25±1,32	22,54-71,71 48,33±1,92	9,31-89,48 37,43±1,35	29,90-87,37 46,27±2,35	24,12-89,10 50,99±1,29

Tabla 3.10- Comparación de los resultados morfométricos relativo a las superficies (valores máximos y mínimos, media y desviación estándar) de adultos de *F. hepatica* procedentes de ovinos infectados de forma natural en zonas de alta altitud del valle de Cajamarca, valle del Mantaro y el Altiplano Norte de Bolivia, y adultos de *F. hepatica* procedentes de ovinos infectados tanto de forma natural como experimental de zonas europeas de baja altitud (n = número de adultos).

Medidas del adulto (mm) Relaciones entre medidas	Cajamarca Perú n=130	Mantaro Perú n=47	Altiplano N, Bolivia n=201	Valencia España n=37	Bialowieza Polonia n=127
Ratio BL/BW	1,31-3,73 1,86± 0,03	1,30-2,46 1,83± 0,04	1,41-3,74 2,22± 0,03	1,70-2,89 2,14± 0,05	1,30-2,62 1,82±0,02
Ratio BWOv/CW	1,79-3,72 2,64± 0,03	1,86-3,94 2,82± 0,07	1,53-3,83 2,53±0,03	2,03-3,90 2,51±0-05	1,86-3,81 2,46±0,03
OSA/VSA ratio	0,23-0,76 0,52±0,01	0,31-0,69 0,51±0,01	0,31-0,71 0,49±0,01	0,27-0,51 0,39±0,01	0,24-0,58 0,41±0,01
Ratio BL/VS-P	1,08-1,22 1,15± 0,003	1,09-1,19 1,13± 0,004	0,88-1,42 1,22± 0,01	1,15-1,27 1,21± 0,004	1,04-1,20 1,11±0,002

Tabla 3.11- Comparación de los resultados morfométricos relativo a las relaciones entre medidas (valores máximos y mínimos, media y desviación estándar) de adultos de *F. hepatica* procedentes de ovinos infectados de forma natural en zonas de alta altitud del valle de Cajamarca, valle del Mantaro y el Altiplano Norte de Bolivia, y adultos de *F. hepatica* procedentes de ovinos infectados tanto de forma natural como experimental de zonas europeas de baja altitud (n = número de adultos).

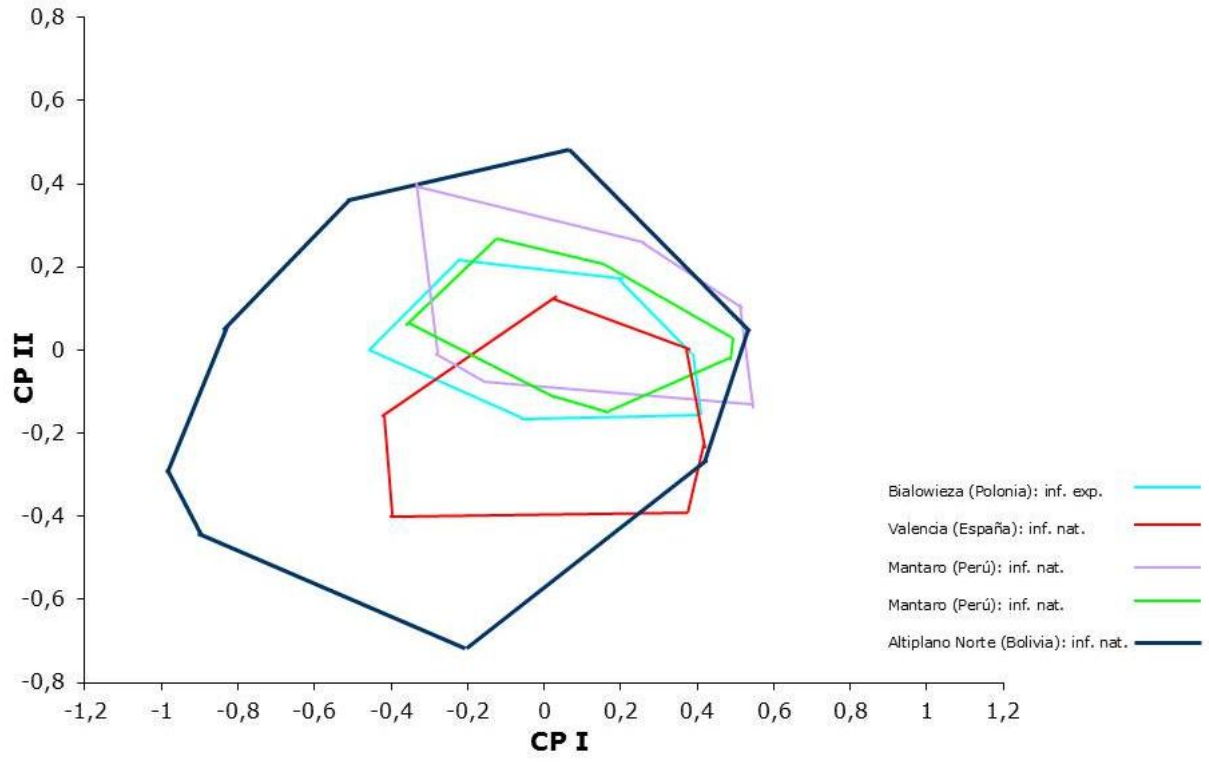

Figura 3.9- Mapa factorial correspondiente a especímenes de *F. hepatica* procedentes de ovejas infectadas de forma natural de los valles de Cajamarca y Mantaro (Perú), Altiplano Norte (Bolivia), Valencia (España) y ovinos infectados experimentalmente (*F. hepatica* = aislado procedente de Polonia). Los resultados se proyectan sobre el primer (CP1, 59%) y el segundo (CP2, 17%) componente principal. Cada grupo está representado por su perímetro.

No existe una relación consistente entre el patrón de variación "size-free" y la diferencia altitudinal entre poblaciones (Fig. 3.10). No existe tampoco una correlación significativa entre las distancias de Mahalanobis y las distancias geográficas (datos no mostrados), así como tampoco se detectó una correlación significativa entre la media del CP1 de los adultos fasciólidos y la carga parasitaria (datos no mostrados).

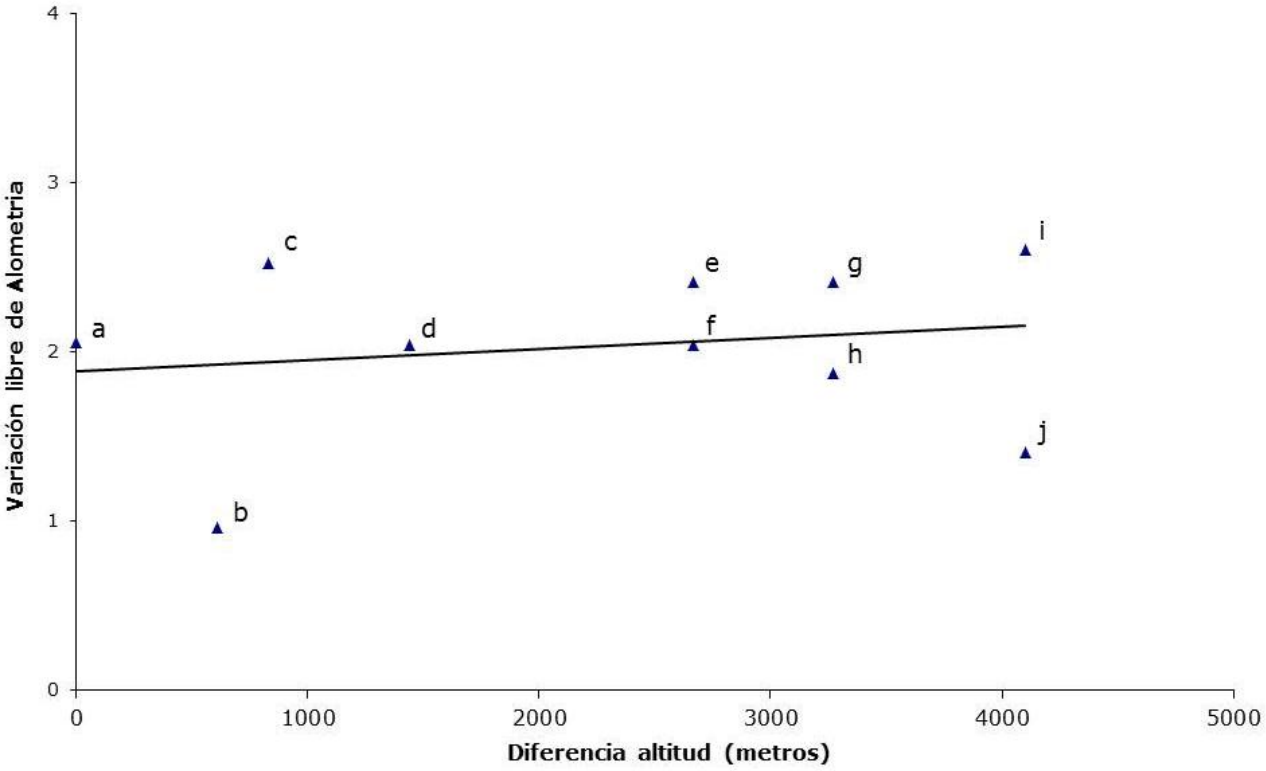

Figura 3.10- Grafica de las distancias de Mahalanobis procedentes de las variables libre de alometrías (eje vertical) frente a las diferencias altitudinales (eje horizontal en m). La predicción de la regresión linear viene representada por la línea recta. a= ovejas infectadas naturalmente procedentes de Valencia (España) – ovejas infectadas experimentalmente (aislado procedente de Polonia); b= ovejas infectadas naturalmente procedentes del valle de Cajamarca – valle del Mantaro (Perú); c= ovejas infectadas naturalmente procedentes del valle del Mantaro (Perú) – Altiplano Norte (Bolivia); d= ovejas infectadas naturalmente procedentes del valle de Cajamarca (Perú) – Altiplano Norte (Bolivia); e= ovejas infectadas naturalmente procedentes del valle de Cajamarca (Perú) – Valencia (España); f= ovejas infectadas naturalmente procedentes del valle de Cajamarca (Perú) - ovejas infectadas experimentalmente (aislado procedente de Polonia); g= ovejas infectadas naturalmente procedentes del valle del Mantaro (Perú) – Valencia (España); h= ovejas infectadas naturalmente procedentes del valle del Mantaro (Perú) - ovejas infectadas experimentalmente (aislado procedente de Polonia); i= ovejas infectadas naturalmente procedentes del Altiplano Norte (Bolivia) - ovejas infectadas experimentalmente (aislado procedente de Polonia); j= ovejas infectadas naturalmente procedentes del Altiplano Norte (Bolivia) – Valencia (España).

3.3.3. DISCUSION

La variación morfológica cuantitativa informa tanto de la variación genética como de las influencias externas (DUJARDIN *et al.*, 2009). En el caso de los endoparásitos, es necesario distinguir entre macrohábitat (características medioambientales externas relativas a la geografía) y microhábitat (órgano parasitado en el interior del hospedador). Las áreas geográficas analizadas en este estudio incluyen ambientes de zonas de alta y baja altitud. Vale la pena mencionar que el entorno en zonas de alta altitud, hay menos oxígeno y la densidad del aire

es menor, la temperatura y la humedad son bajas y hay un incremento de la radiación. Estos factores medioambientales pueden ejercer una influencia decisiva sobre los mamíferos y por lo tanto los animales que nacen y viven en zonas de alta altitud pueden mostrar características morfológicas y fisiológicas diferentes en comparación con aquellas características que muestran los mamíferos que habitan en zonas de baja altitud (JACKSON *et al.*, 1987; FRISANCHO & FRISANCHO, 1992).

Los resultados del presente estudio muestran que aparentemente no hay relación entre la forma del fasciólido adulto con respecto a la diferencia altitudinal o geográfica entre muestras de dos zonas geográficas diferentes. Los resultados obtenidos en el análisis de los componentes principales muestran que las poblaciones procedentes de los valles Andinos y las poblaciones tanto naturales como experimentales de *F. hepatica* de zonas europeas de baja altitud presentan una homogeneidad fenotípica. Los resultados muestran que el tamaño corporal mínimo para alcanzar la gravidez es similar en el material procedente tanto de los valles peruanos como en las zonas de baja altitud europeas. Por otra parte, la población procedente del Altiplano presenta un tamaño corporal mínimo más bajo para alcanzar la madurez en los adultos. Esto significa que las poblaciones de adultos de *F. hepatica* procedente del Altiplano alcanzan antes la madurez del útero en su crecimiento en el interior del hígado (Fig. 3.9).

Cabe mencionar que el tamaño del útero y el tamaño corporal son proporcionales (VALERO *et al.*, 2001b), por lo que se podría concluir que los adultos procedentes de los valles de Mantaro y Cajamarca presentan un tamaño de útero mayor al que presentan los adultos fasciólidos procedentes de poblaciones Altiplánicas.

En otros organismos como los insectos, la "forma libre de alometría" requiere cambios externos importantes para producir modificaciones significativas. Por ejemplo, DUJARDIN & LE PONT (2004) detectaron en Flebotómidos que la variación libre de alometría estaba estrechamente relacionada con diferencias altitudinales, indicando su posible relación con la temperatura y/o la vegetación. Estos resultados son contrarios a los obtenidos en nuestro estudio, en donde la forma libre de alometría aparece como un rasgo más estable que el tamaño en especies de fasciólidos en diferentes condiciones de altitud y latitud.

Muchos mamíferos pueden actuar como hospedadores definitivos para *F. hepatica* aunque hay una variación considerable en la susceptibilidad y patología de acuerdo a las distintas especies (MAS-COMA *et al.*, 2009a). Usando un modelo alométrico, VALERO *et al.*, (2001a) indica que la especie hospedadora definitiva

(ovino, bovino, porcino) ejerce una influencia decisiva en el tamaño de los adultos de *F. hepatica*, aunque esta influencia desaparece en un modelo experimental murino infectado con diferentes aislados. Además, se ha demostrado la existencia de ciertos rasgos morfológicos (diferencias alométricas entre BW vs. BL y BL vs. VS-P) que caracterizan a los adultos de *F. hepatica* en diferentes hospedadores definitivos (VALERO *et al.*, 2001a).

La carga parasitaria es otro de los factores medioambientales que afectan a la variación del tamaño en los fasciolidos. El *"crowding effect"* se refleja en los adultos de *F. hepatica* como una reducción de los valores máximos morfométricos (y_m) de las trayectorias ontogénicas del área corporal, BP, BL y BW debido a una reducción de la tasa de crecimiento (VALERO *et al.*, 2006b). Así, en roedores, el tamaño del verme es dependiente del nivel de infección, disminuyendo el tamaño cuando la carga parasitaria aumenta. Este fenómeno no está presente en infecciones ligeras en ovino, es decir con cargas parasitarias menores o iguales a 45 adultos/oveja.

En el caso de poblaciones de *F. hepatica* naturales, se ha detectado ligeras diferencias en modelos alométricos (BL, BW, P vs. BA o BL) entre los adultos de *F. hepatica* procedentes de ovinos infectados tanto en áreas de alta altitud bolivianas como áreas de baja altitud españolas (VALERO *et al.*, 1999). Sin embargo, comparando las mismas especies hospedadoras, las poblaciones de *F. hepatica* procedentes de ovinos y bovinos del Altiplano Norte de Bolivia presentan, con la misma área corporal, una menor área uterina que la de las poblaciones de *F. hepatica* europeas (VALERO *et al.*, 2001b).

El ciclo biológico de *F. hepatica* tiene lugar alternativamente entre un ecosistema acuáticos y otro terrestre. Los huevos del parásito los emite un hospedador definitivo terrestre. Sin embargo, el huevo se desarrolla en agua dulce y el desarrollo de la fase larvaria ocurre luego en caracoles Lymnaeidos acuáticos y anfibios (MAS-COMA, 2005). La emisión de los huevos de *F. hepatica* con las heces normalmente está sujeta a oscilaciones a lo largo del tiempo tanto en animales como en humanos. Un ejemplo de la adaptación de la cronobiología de la emisión de huevo a la estacionalidad podemos encontrarlo en el trabajo de VALERO *et al.* (2002). Así, la rata negra naturalmente parasitada con fasciólidos en la isla Mediterránea de Córcega muestra una emisión continua de huevos, con valores mínimos en verano, cuando el desarrollo del huevo es más difícil (en los meses de verano en el área Mediterránea, la evapotranspiración es mayor que las precipitaciones), mientras que es en los meses de primavera y otoño se alcanza los

picos máximos de emisión, cuando existe un nivel aceptable de precipitaciones y suficiente humedad ambiental (VALERO *et al.*, 2002). Las poblaciones de *G. truncatula* europeas son mayoritariamente anfibias (EUZEBY, 1971). Por el contrario, los especímenes de *G. truncatula* altiplánicos, son más acuáticos y sólo en raras ocasiones pueden encontrarse en el barro, fuera del agua. Suelen habitar principalmente en cuerpos de agua permanentes y nunca en cuerpos de agua temporales originados en la temporada de lluvia (Octubre-Marzo). Los cuerpos de agua originados por las precipitaciones no permanecen el tiempo suficiente para permitir la colonización por Lymnaeidos e incluso, en años muy lluviosos, los periodos de sequía son demasiado largos como para permitir la supervivencia de Lymnaeidos enterrados y en letargo. Esto está relacionado con las elevadas tasas de evapotranspiración propias de la alta altitud, así como con que las temporadas de sequía coincidan con las temperaturas mínimas del año (Abril-Septiembre). De esta forma, los Lymnaeidos dispersos en pastos inundados irregularmente, lo cual constituye el foco de transmisión más importante en Europa Central, no se encuentra casi nunca en el Altiplano. De la misma forma, la relación entre la fascioliasis y los periodos de lluvia, reconocida tanto en animales como en humanos en el hemisferio norte (CHEN & MOTT, 1990), no puede aplicarse al Altiplano Norte de Bolivia. En el Altiplano, la presencia a lo largo de todo el año de poblaciones de Lymnaeidos en los focos de transmisión, permite la transmisión del parásito durante todas las estaciones. En el caso del Altiplano Norte de Bolivia, las diferencias entre las medias de temperatura entre las estaciones es muy baja, solo de 5,0-6,5º C. Las mayores diferencias en la temperatura ocurren a lo largo del mismo día, desde 10,3 hasta 13,0º C (en las costas del lago Titicaca), y de 13,4 hasta 22,4º C (lejos del Lago). Además, la ausencia de árboles y arbustos implica la inexistencia de sombra y que por lo tanto hay una intensa incidencia de la radiación solar, lo cual incrementa la temperatura de los cuerpos de agua al mediodía (el rango de temperatura a lo largo del día en los cuerpos de agua poblados por los Lymnaeidos oscila entre 3,0-10,0º C por la noche y entre 15,0-28.9º C durante el día) (MAS-COMA *et al.*, 1999a; FUENTES *et al.*, 1999).

Estudios realizados en el Altiplano Norte de Bolivia muestran que en el ciclo biológico del parásito no existe una fase en la cual no parezca estar modificada de tal manera que la transmisión se reduzca. No obstante, hay ciertos aspectos que parecen favorecer la transmisión, como es un periodo de emisión cercariana largo y una gran producción cercariana. Estos dos aspectos parecen estar relacionados con la mayor supervivencia de los caracoles Lymnaeidos infectados. Cuando se compara con áreas de baja altitud, estas diferencias pueden ser interpretadas como estrategias de adaptación a las condiciones extremas en las que se encuentran las

poblaciones de áreas de alta altitud (MAS-COMA *et al.*, 2001). Nuestros resultados indican que las poblaciones de adultos de fasciólidos tanto de Perú como Europa (naturales y experimentales) presentan un tamaño mínimo común para alcanzar la madurez, mientras que este valor de tamaño mínimo es más bajo en las poblaciones procedentes de las zonas altiplánicas de Bolivia. Esta diferencia se puede atribuir a una variabilidad intraespecifica o puede estar relacionada con la influencia que ejerce la alta altitud. La divergencia detectada entre la población altiplánica y el resto de poblaciones analizadas es difícil de entender, además es difícil prever ventajas evolutivas. Se sabe que se requiere oxígeno para la producción de huevos en *F. hepatica* (MANSOUR, 1958; BJORKMAN & THORSELL, 1963; McGONIGLE & DALTON, 1995) (el paso de gran altitud a alta altitud normalmente se realiza a partir de los 3200 m). La hipoxia existente a gran altitud puede ser el origen de la menor producción de huevos en los adultos fasciólidos. Por otra parte, el útero en los Digénidos adultos tradicionalmente no se ha considerado un órgano de almacenaje si no que principalmente se ha considerado como un órgano adaptado para dar tiempo al desarrollo de los huevos (en los fasciolidos, los huevos se emiten sin embrionar y el miracidio solo comienza su desarrollo una vez llega a agua dulce). En el apartado 3.2. del presente capítulo, hemos demostrado la existencia de una relación directa entre el tamaño del útero y el número de huevos por gramo de heces emitido. En los patrones de transmisión de los valles y de zonas de baja altitud de Europa, la capacidad de almacenaje del útero es mucho más necesaria, debido a la transmisión estacional que se da en estas zonas, con una emisión continua de huevos pero con oscilaciones, con valores mínimos en aquellas estaciones desfavorables, y picos de máximos de emisión en las estaciones favorables. Como se ha mencionado antes, en el Altiplano Norte de Bolivia, las condiciones climáticas, las características del agua dulce y la ecología de los Lymnaeidos permiten que la transmisión de la fascioliasis tenga lugar a lo largo de todo el año (FUENTES *et al.*, 1999; MAS-COMA *et al.*, 1999a), la emisión de huevos es continua y la menor capacidad de almacenaje del útero puede estar relacionada con esta transmisión continua, así como con la disponibilidad todo el año de poblaciones del molusco hospedador.

ASSESSING THE VALIDITY OF NEW INMUNOLOGICAL TEST DIAGNOSTIC IN HUMAN FASCIOLISIS

VALIDACION DE NUEVOS TEST DIAGNOSTICOS INMUNOLOGICOS EN LA FASCIOLISIS HUMANA

4. ASSESSING THE VALIDITY OF NEW INMUNOLOGICAL TESTS IN THE DIAGNOSIS OF HUMAN FASCIOLIASIS

4.1. DRG *F. HEPATICA* IgG ELISA TEST AS A SEROLOGICAL DIAGNOSIS OF HUMAN FASCIOLIASIS IN DIFFERENT EPIDEMIOLOGICAL SITUATIONS

The present section evaluates a qualitative microtiter strip-based enzyme-linked immunosorbent assay (ELISA) for the detection of IgG class antibodies against *F. hepatica* in human serum, a solid phase enzyme immunoassay based on the sandwich principle, known as 'DRG *Fasciola hepatica* IgG (human) ELISA', in the following situations: (i) a hyperendemic *F. hepatica* area where humans usually shed large amounts of parasite eggs in faeces, (ii) an epidemic *F. hepatica* area where humans usually shed small amounts of parasite eggs and (iii) an overlap area of both *Fasciola* species where human shedding of parasite eggs in faeces is usually scarce or non-existent.

4.1.1. MATERIAL AND METHODS

The serum used in the next study is detailed in section 2.2.1. in the Materials & Methods section. The gender and age characteristics of patients used in the study are detailed on table 4.1.

Fascioliasis positivity of the samples used had been verified through egg detection (Kato–Katz technique) (see 2.3.2.2. in Materials & Methods) or the ELISA Cathepsin L1 (CL1) (see 2.3.4.1.1.) protease test. ELISA CL1 is used here as the serological gold standard method in samples from areas where humans usually shed small amounts of parasite eggs or where parasite eggs in faeces are usually scarce or non-existent. ELISA CL1 was previously successfully employed for the diagnosis of human fascioliasis in the Northern Bolivian Altiplano (O'NEILL *et al.,* 1998) and in Iran (ROKNI *et al.,* 2002), showing it to be highly specific (100%) and sensitive (100%).

In the following study we assessed the validity of the DRG Kit (commercial ELISA kit). DRG Instruments GmbH, Germany, provided all commercial kits used in this study. The protocol used is detailed in 2.3.4.1.2.

The diagnostic sensitivity and specificity values were calculated (Fletcher & Fletcher 2005) with its 95% confidence interval (95% CI; EPIINFO). The positive predictive value (PPV) and negative predictive value (PNV) were calculated using formulae detailed in section 2.3.5.6.

Student's t-test was calculated using SPSS statistics version 19 software.

	Total		Ind		Male		Female	
	N	(min-max) Age Average SD	N	(min-max) Age Average SD	N	(min-max) Age Average SD	N	(min-max) Age Average SD
Individuals parasitologically negative	89	(1-73) (40,28±0,81)	1	Unknown Unknown	55	(22-73) (42,68±0,68)	33	(1-73) (36,33±0,95)
Individuals with parasitic/fungal disease other than fascioliasis	168*	(1-89) (38,15±1,07)	3	(23-40) (31,50±0,67)	82	(1-89) (40,42±1,11)	83	(1-89) (36,05±1,03)
Individuals positive fascioliasis	54	(7-75) (36,30±1,09)	-	-	25	(7-74) (34,25±1,21)	29	(10-75) (38,00±1,00)

*Amoebiasis 12; leishmaniasis 3; malaria 12; toxoplasmosis 10; schistosomiasis 15; hydatidosis 15; hymenolepiasis 1; taeniasis / cysticercosis 15; trichinellosis 15; strongyloidiasis 18; ascariasis 1; toxocariasis 8; anisakiasis 25; loasis 2; gnathostomiasis 10; trichuriasis and ascariasis coinfection 1; malaria, trichuriasis and ascariasis coinfection 2; Entamoeba coli, trichuriasis, ascariasis and ancylostomiasis coinfection 1; trichuriasis, ancylostomiasis and enterobiasis coinfection 1; histoplasmosis 1.

Table 4.1- Gender and age characteristics of individuals whose serum samples were used in the validation.

4.1.2. RESULTS

The mean of DRG units (DU) values (see 2.3.4.1.2.) for all serum groups is shown in Figure 4.1 A, B. DU values were significantly higher in the proven fascioliasis group than in the groups of parasitic diseases other than fascioliasis and the healthy controls, respectively ($P < 0,001$).

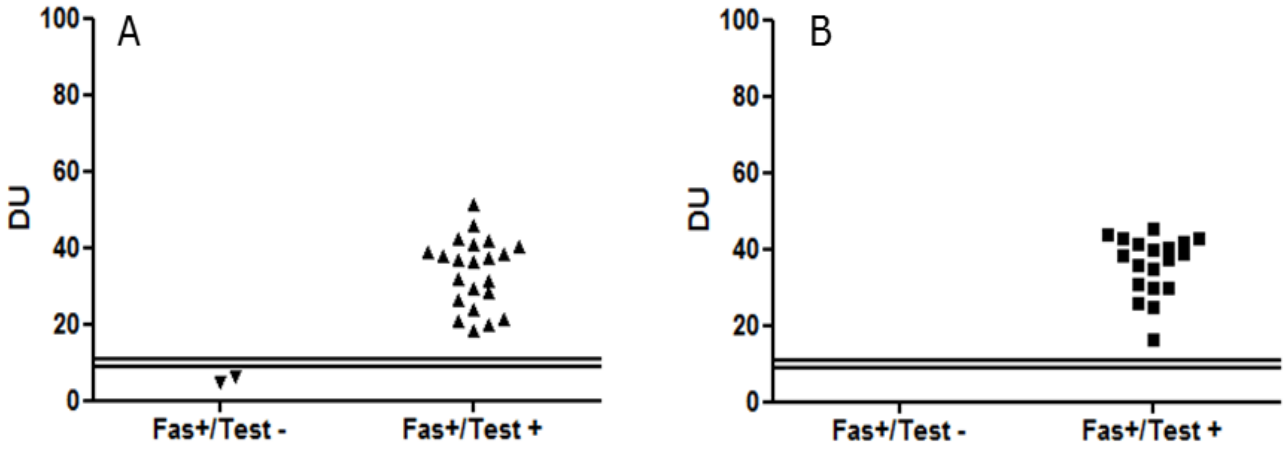

Figure 4.1- Mean DRG Units (DU) obtained with the DRG *F. hepatica* IgG ELISA test in fascioliasis patients from different epidemiological situations. (A) Endemic *F. hepatica* area where humans usually shed small amounts of parasite eggs; (B) an overlap area of both *Fasciola* species where human shedding of parasite eggs in faeces is usually scarce or non-existent. Data points are the mean optical density (OD) obtained from triplicate samples expressed as a percentage of the cut-off (CO), using the following formula: *Percent positive (PP) = ((Mean OD of test sample)/ Mean OD of C+)*100*. A serum is considered positive when its absorbance value is above 10% of CO. The results in DRG Units (DU) were calculated according to the following formula: DU= (sample (mean) absorbance value * 10)/ CO. The results were negative if DU <9, and positive if DU > 11 (black line).

The numbers of true positive and false negative test results in fascioliasis cases of humans with and without detection of eggs in their faecal samples are provided in table 4.2 and table 4.3.

	True Negative	False positive
Individuals with parasitic / fungal diseases other than fascioliasis	158	10
Individuals parasitologically negative	88	1
Total sera without fascioliasis	246	11

Table 4.2- Diagnostic characteristics of the *F. hepatica* IgG ELISA test applied to sera from patients with helminthiases other than fascioliasis and negative sera from healthy subjects.

	True positive	False negative
Hyperendemic *F. hepatica* area where humans usually shed a great deal of parasite eggs in faeces*	11	0
Epidemic *F. hepatica* area where humans usually shed small amounts of parasite eggs¥	22	2
Overlap area of both *Fasciola* species where human shedding of parasite eggs in faeces is usually scarce or non-existent¥	19	0
Total sera with fascioliasis	52	2

***** Gold standard determined according to the Kato–Katz technique.

¥ Gold standard determined according to the ELISA CL1 test.

Table 4.3- Diagnostic characteristics of the *F. hepatica* IgG ELISA test applied to fascioliasis patients sera from different disease endemic areas.

In the samples from a hyperendemic *F. hepatica* area egg detection in faeces through the Kato-Katz technique was used as reference standard, and the sensitivity of the *F. hepatica* IgG ELISA test was 100% (67,9–100%). No correlation between egg output - a measure of infection intensity - and the OD450 values of the *F. hepatica* IgG ELISA test was observed in this study area (Fig. 4.2).

In the samples from an epidemic *F. hepatica* area CL1 ELISA was used as reference standard. Sensitivity was 91,7% (95% confidence intervals, 71,5–98,5%), detecting 2 false negative cases of which one was egg positive in faeces, while the other was not.

CL1 ELISA was used as reference standard in the samples from an overlap area of both *Fasciola* species. Sensitivity was 100% (95,9–100%). Populations of the two latter endemic areas were grouped as result of the ELISA CL1 and the *F. hepatica* IgG ELISA test evaluation as follows: fascioliasis positive cases by CL1 (Fas+) / seropositive by *F. hepatica* IgG ELISA test (test+) and fascioliasis positive cases by CL1 (Fas+) / seronegative by *F. hepatica* IgG ELISA test (test-) (Fig. 4.1 A, B). Statistical differences between the results for sensitivity values from the two latter situations were not found, and true positive cases (41) and false negative

cases (2) for these two areas together were used for the calculation of sensitivity, presenting a value of 95,3% (82,9–99,2%).

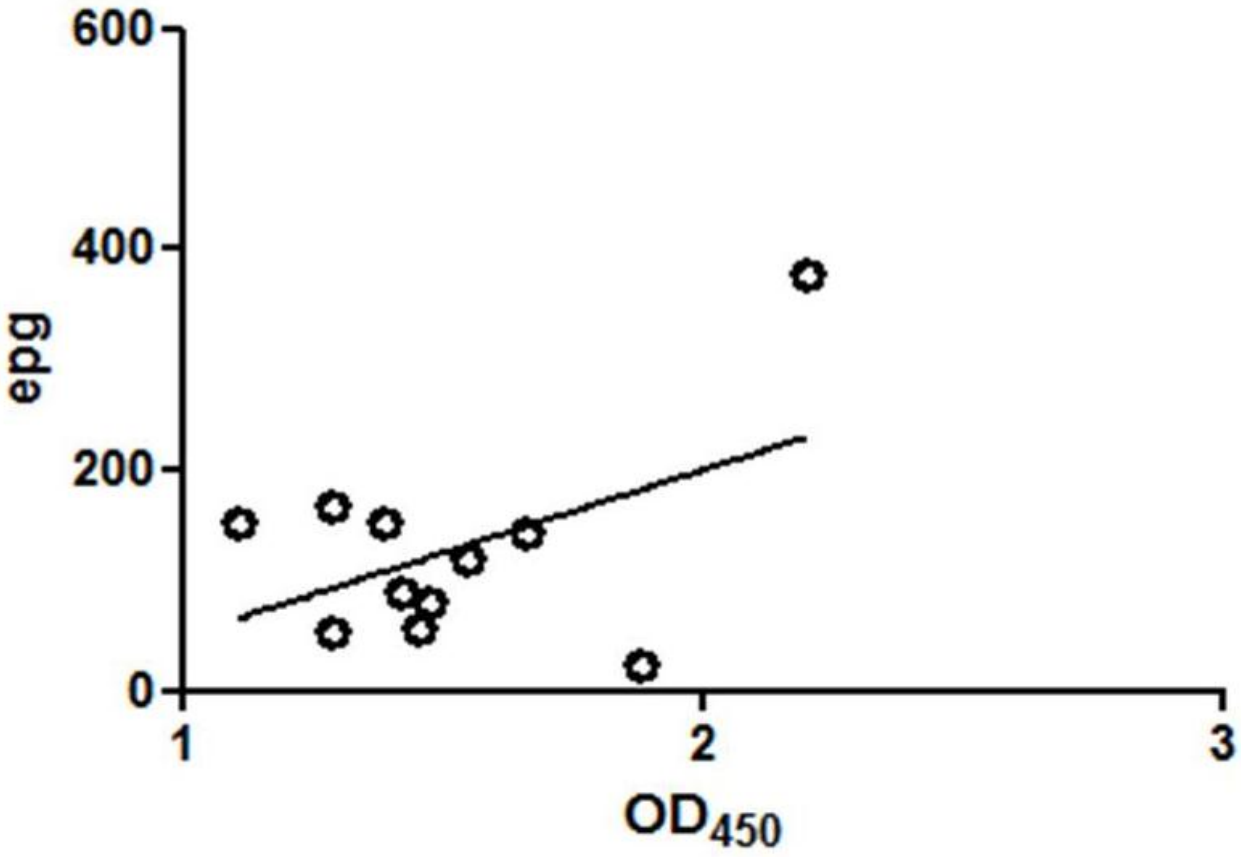

Figure 4.2- DRG *F. hepatica* IgG ELISA test and intensity of *F. hepatica* infection. Data points represent the mean absorbance at 450 nm from copropositive patients from a hyperendemic *F. hepatica* area where humans usually shed a great deal of parasite eggs in faeces. epg = egg count per gram of faeces.

Specificity was calculated from the results in table 4.2 and table 4.3 providing the numbers of false negative and true positive test results in infected sera other than fascioliasis and negative cases. These results can be grouped as follows: infected positive cases other than fascioliasis (infected+) / seropositive by *F. hepatica* IgG ELISA test (test+) and infected positive cases other than fascioliasis (infected+) / seronegative by *F. hepatica* IgG ELISA test (test-) (Figure 4.3 A). Table 4.2 also shows the total number of sera without fascioliasis, and these values were used to calculate specificity, presenting a value of 95,7% (92,3–97,5%). Ten serum samples from a heterologous infection cross-reacted with the kit, mainly originating from patients with helminthiases, namely schistosomiasis (4 samples), hydatidosis (2 samples), taeniasis / cysticercosis (1 sample), trichinosis (1 sample), strongyloidiasis (1 sample), and histoplasmosis (1 sample) (Figure 4.3 B).

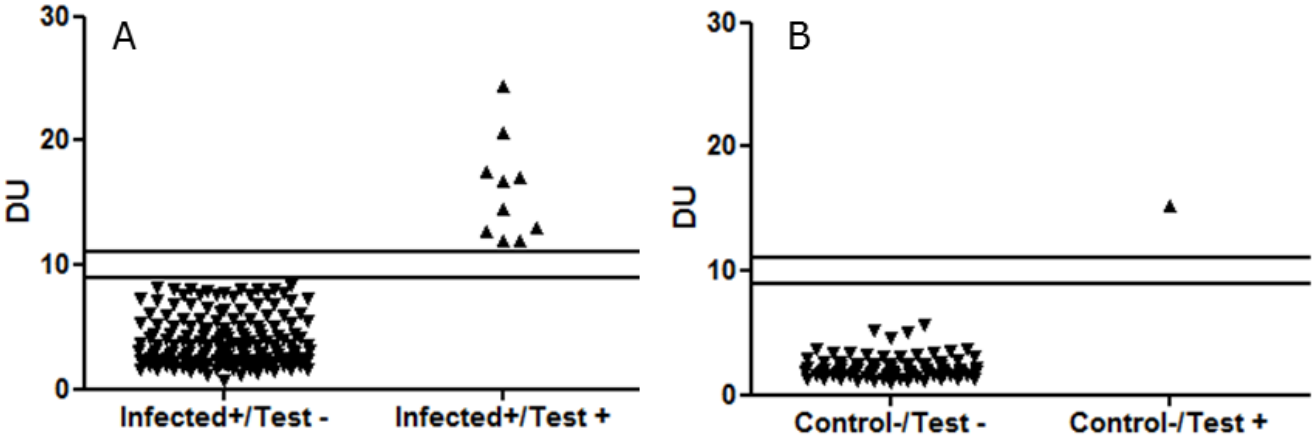

Figure 4.3- Mean DRG Units (DU) obtained with the DRG *F. hepatica* IgG ELISA test in (A) patients with parasitoses other than fascioliasis (B) and negative control sera. Data points represent the mean absorbance at 450 nm obtained from three replicates of each serum tested. Data points are the mean optical density (OD) obtained from triplicate samples expressed as a percentage of the cut-off (CO), using the following formula: *Percent positive (PP) = ((Mean OD of test sample)/ Mean OD of C+)*100*. A serum is considered positive when its absorbance value is above 10% of CO. The results in DRG Units (DU) were calculated according to the following formula: DU= (sample (mean) absorbance value * 10)/ CO. The results were negative if DU < 9, and positive if DU > 11 (black line).

Theoretical PPVs and NPVs vs fascioliasis prevalence are represented in Figure 4.4, showing the expected PPVs and NPVs depending on whether the test was used in low, medium or high prevalence scenarios.

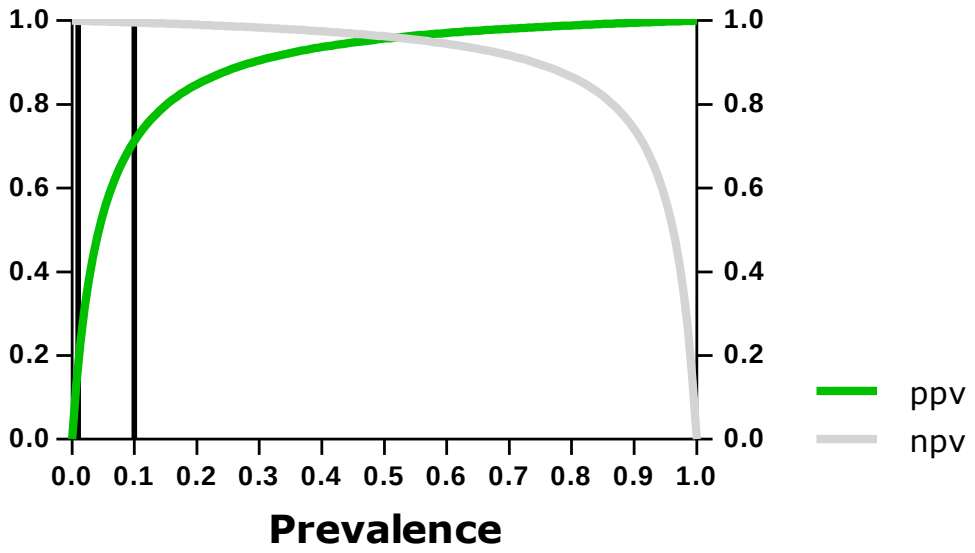

Figure 4.4- Theoretical PPVs and NPV values vs fascioliasis prevalence, showing the expected PPVs and NPV values if the test were used in low (below 1%), medium (between 1% and 10%) or high (above 10%) prevalence scenarios (expressed in vertical lines).

4.1.3. DISCUSSION

Coprological analysis, based on the identification of eggs found in stools, duodenal contents or bile analysis is still commonly employed to diagnose human fascioliasis (VALERO *et al.*, 2009a), despite the overwhelming consensus that this method is not wholly reliable (HILLYER 1999) for several reasons. Eggs are not detected until the latent period of infection when much of the liver damage has already occurred, and similarly patients may not shed eggs in faeces in ectopic cases known in both infections by *F. hepatica* and *F. gigantica* (LE *et al.*, 2007). Additionally, eggs are released sporadically from the bile ducts and hence stool samples of infected patients may not necessarily contain eggs (MAS-COMA *et al.*, 1999b).

However, coprological methods of egg detection are still usually considered the gold standard, although unfortunately they are not applicable in endemic areas where:

i. Humans do not shed eggs in faeces

ii. The shedding is often very low and difficult to detect

iii. In cases of communities that do not supply stool samples due to ethnic / cultural customs.

Hence, it is essential to have serological techniques also available when diagnosing the disease. Therefore, in this study, besides Kato–Katz, another serological test, the ELISA CL1 test, was also used as gold standard.

Serodiagnosis of fascioliasis in human and animal species has been successfully carried out employing several antigenic fractions of *Fasciola* (MEZO *et al.*, 2003; SANCHEZ-ANDRADE *et al.*, 2008; DEMERDASH *et al.*, 2011), purified antigens (O´NEILL *et al.*, 1998; ROKNI *et al.*, 2002), and recombinant antigens (O´NEILL *et al.*, 1999; CARNEVALE *et al.*, 2001). Cathepsins L are the most frequently used target antigens for detecting anti-*Fasciola* antibodies (CARNEVALE *et al.*, 2001; ROKNI *et al.*, 2002; MEZO *et al.*, 2004, 2007, 2010; INTAPAN *et al.*, 2005; WONGKHAM *et al.*, 2005; VALERO *et al.*, 2009b; MUIÑO *et al.*, 2011), as circulating antibodies to these molecules remain at high levels for long periods (VALERO *et al.*, 2009b).

The commercial DRG test was evaluated in cattle, obtaining a sensitivity and specificity of 98% (96–100%) and 96% (93–98%) respectively at a cut-off value of

15% positivity (SALIMI-BEJESTANI *et al.*, 2005). The sensitivity and specificity values of the DRG test here in agree with the results obtained by other authors for *F. hepatica* IgG ELISA in-house assays: 100% and 100% (O´NEILL *et al.*, 1998; ROKNI *et al.*, 2002), 92,4% and 83,6% (ESPINOZA *et al.*, 2007), 97,2% and 100% (RAHIMI *et al.*, 2011), 97% and 96,6% (CORNEJO *et al.*, 2010), and 100% and 95,6% (FIGUEROA-SANTIAGO *et al.*, 2011). Commercial kits offer advantages over in-house assays: they save time and provide quality control reagents for better reproducibility within and between laboratories, which hampers the comparison between in-house and commercial tests. Furthermore, only very few commercial kits, such as the DRG *F. hepatica* IgG ELISA test evaluated here, are presently available for the diagnosis of human fascioliasis. Comparing DRG test results only with commercial assays results in sensitivity and specificity below the 100% sensitivity, respectively, specificity obtained by SeroFluke strips (MARTINEZ-SERNANDEZ *et al.*, 2011).

The results obtained show the test to be highly sensitive and specific. The sensitivity of this assay did not detect differences in positive samples from different epidemiological situations. Nevertheless, PPVs calculated for diverse epidemiological situations are very different. Thus, PPVs in hypoendemic areas were below 2,2%, in mesoendemic situations PPVs oscillated between 2,2% and 71,2%, while in hyperendemic situations PPVs were above 71,17%, making this test recommendable in such situations. Contrarily, NPVs calculated for diverse epidemiological situations were similar. NPVs for hypoendemic areas reached values above 99,9%, in mesoendemic situations NPVs oscillated between 99,5% and 99,9%, while in hyperendemic situations NPVs were below 99,5%.

4.2. FIELD EVALUATION OF A COPROANTIGEN DETECTION TEST FOR FASCIOLIASIS DIAGNOSIS AND SURVEILLANCE IN HUMAN HYPERENDEMIC AREAS OF ANDEAN COUNTRIES

The present chapter evaluates the coproantigen technique MM3-COPRO ELISA under field conditions for human fascioliasis diagnosis, using classical coprological techniques for egg detection (rapid sedimentation and Kato-Katz) and the Copro-ELISA test as gold standards in human hyperendemic areas of Andean countries. Thus, two endemic areas were chosen: Huacullani (Bolivia) representing the Altiplanic pattern with high prevalences and intensities, and the rural areas of Cajamarca (Peru), representing the valley pattern with high prevalences but with low intensities.

4.2.1. MATERIALS AND METHODS

To evaluate the MM3-coproantigen detection test we used faecal samples collected from two endemic areas of Andean countries. Stool samples obtained are detailed in section 2.2.2. in Materials & Methods.

Identification of true positive and true negative cases was carried out by using two criteria: i) finding of *F. hepatica* eggs in faeces applying the Kato-Katz technique (see 2.3.2.2.); ii) egg finding plus MM3-COPRO ELISA test results. The protocol used for MM3-COPRO ELISA is detailed in section 2.3.4.1.3. in chapter Materials & methods.

Statistical analyses were done using SPSS Statistics version 19 software. For the evaluation of categorical variables, the chi-square test or Fisher's exact test was used. Bivariant correlations (Pearson's correlation) were calculated to assess the relationship between optical density (OD) and epg of *F. hepatica*. A P value less than 0.05 were considered significant. Theoretical positive predictive values (PPV) and negative predictive values (NPV) were calculated from sensitivity and specificity values obtained using only classical coprological tests for the identification of *F. hepatica* eggs in feces as ''gold standard''. The formulas were used for their calculation are detailed in section 2.3.5.6 in Materials & Methods.

4.2.1.1. INSTITUTIONAL ETHICAL REVIEW PROCEDURE

Faecal samples from schoolchildren used in the diagnostic analyses were obtained after informed consent had been given from the local authorities of the communities, the children's parents and the heads and teachers of each school, following the principles expressed in the Declaration of Helsinki. Each community surveyed was informed in previous meetings about the public health relevance and impact of the intestinal and hepatic parasitic infections, and on the need for the development of control schemes at national, regional, district and village levels.

In Huacullani, activities were performed in collaboration with the Servicio Departamental de Salud La Paz (SEDES L.P.) and the Unidad de Epidemiología of the Bolivian Ministry of Health and Sports (MSyD). Consent was previously obtained from the Comisión de Etica de la Investigación (CEI) of the Comité Nacional de Bioética of Bolivia.

In Cajamarca, the initiative was performed in collaboration with the Dirección Regional de Salud of Cajamarca, and the Dirección General de de Zoonosis, Ministerio de Salud (MINSA), Lima. Consent was previously obtained from

the Dirección General de Zoonosis, MINSA, Lima and Centro de Investigación y Control de *Enfermedades Transmisibles of* Universidad Nacional de Cajamarca, Cajamarca.

4.2.2. RESULTS

Diagnostic parameters of the MM3-COPRO ELISA were estimated by choosing coprology and copro-ELISA as the "gold standard" assays to detect *F. hepatica* infection in humans. Positive cases of the MM3-COPRO ELISA and egg detection techniques of *F. hepatica* infection and performance characteristics of the MM3-COPRO ELISA according to study site are summarized in table 4.4.

Huacullani positive cases were globally 24,71% using the MM3-COPRO ELISA and 21,51% applying an egg detection technique (Kato-Katz). No significant differences were encountered between either % (P=0.093).

Cajamarca positive cases were globally 11,05% using the MM3-COPRO ELISA and 5,60% employing egg detection techniques (rapid sedimentation and Kato-Katz). Significant differences were detected between both % (P=0,007).

Differences between the two local patterns were detectable, i.e. significant differences were found when comparing MM3-COPRO ELISA positive cases % from Huacullani and Cajamarca (P=0,0025), and also when comparing egg detection positive cases % from Huacullani and Cajamarca (P=0,001).

In Huacullani, sensitivity and specificity were 94,68%, respectively, 98,48%. In Cajamarca, these results were 94,73%, 93,58%, respectively.

In Huacullani, of 437 samples assayed, 89 showed the presence of eggs through the Kato-Katz technique (20,37%). The MM3-COPRO ELISA was positive in 108 samples (24,71 %), which included samples with *Fasciola* eggs (89) and without *Fasciola* eggs (19), i.e. 82,40% of the children who were positive for the MM3-COPRO ELISA were also positive through the Kato-Katz procedure. It should be emphasized that there were five children shedding eggs with emissions of 48, 72, 96, 120 and 1248 epg, whose MM3-COPRO ELISA results were negative (1,14%). The stool sample showing 1248 epg was repeatedly re-analysed, and a negative result was always obtained with the MM3-COPRO ELISA test.

In Cajamarca, of 362 samples assayed, 18 showed the presence of eggs through the rapid sedimentation and Kato-Katz techniques (4,97%), whereas the MM3-COPRO ELISA was positive in 40 samples, which included the samples with

Fasciola eggs (18) and without *Fasciola* eggs (22), i.e. 45,0% of the children who were positive for the MM3-COPRO ELISA were also positive through coprological egg detection procedures. Interestingly, one child shed eggs (by the rapid sedimentation technique) but was negative for the MM3-COPRO ELISA. The remaining 321 MM3-COPRO ELISA negative samples, however, included 237 negative samples, and 84 positive samples for parasitic infections other than *Fasciola*. They involved one or more parasitic protozoans (*Blastocystis hominis*, *Chilomastix mensnilii*, *Giardia intestinalis*, *Entamoeba histolytica*, *E. coli*, *Endolimax nana*, *Iodamoeba buetschlii*) and helminth species (*Strongyloides stercoralis*, *Ascaris lumbricoides*, *Trichuris trichiura*, *Enterobius vermicularis* and *Hymenolepis nana*).

In Huacullani, the geometric mean egg content in *F. hepatica* positive samples was 164,09 epg, and the arithmetic mean was 334,98 (with SD of ± 992,56), with a range of 24 to 8088 epg (Table 4.4). In these samples from Huacullani, epg data were distributed into two groups: a high burden group (≥400 epg) of samples whose respective infected children were in need to be hospitalized for prevention follow up of potential post-treatment colics, and a low burden group (<400 epg) of samples whose respective infected children should not be hospitalized.

In Cajamarca, the geometric mean egg content in *F. hepatica* positive samples was 89,80 epg, and the arithmetic mean was 116,47 (with SD of ± 84,80), with a range of 16 to 376 epg (table 4.4), i.e. samples may be considered as belonging to a low burden group as their epg counts are <400.

Endemic area	Sensitivity %	Specificity %	KATO-KATZ Na AM ± SD Range GME	positive cases % MM3-COPRO	positive cases % Egg detection	MM3-COPRO + Egg detection +	MM3-COPRO + Egg detection -	MM3-COPRO - Egg detection +	MM3-COPRO - Egg detection -
Huacullani (N=437)	94,68	98,48	94 334,98±992,56 24-8088 142,17	24,71	21,51	89	19	5	324
Cajamarca (N=362)	94,73	93,58	17 116,47±84,80 16-376 89,80	11,05	5,60	18	22	1	321

Table 4.4- Positive cases (%) of MM3-COPRO ELISA and egg detection techniques of *F. hepatica* infection and performance characteristics of MM3-COPRO ELISA by study site. PPV = Positive predictive value; NPV = Negative predictive value; Na = total number of positive children with *Fasciola* infection by Kato–Katz; N = Number of children analysed; AM = arithmetic mean; GM = geometric mean

The OD values obtained for individual *F. hepatica* positive and negative faecal samples from Huacullani and Cajamarca are shown in Figure 4.5 and Figure 4.6, respectively. Positive samples with *F. hepatica* eggs showed OD values above the cut-off value except in five cases in Huacullani (determined by the Kato-Katz technique), and one case in Cajamarca (determined by the rapid sedimentation and Kato-Katz technique).

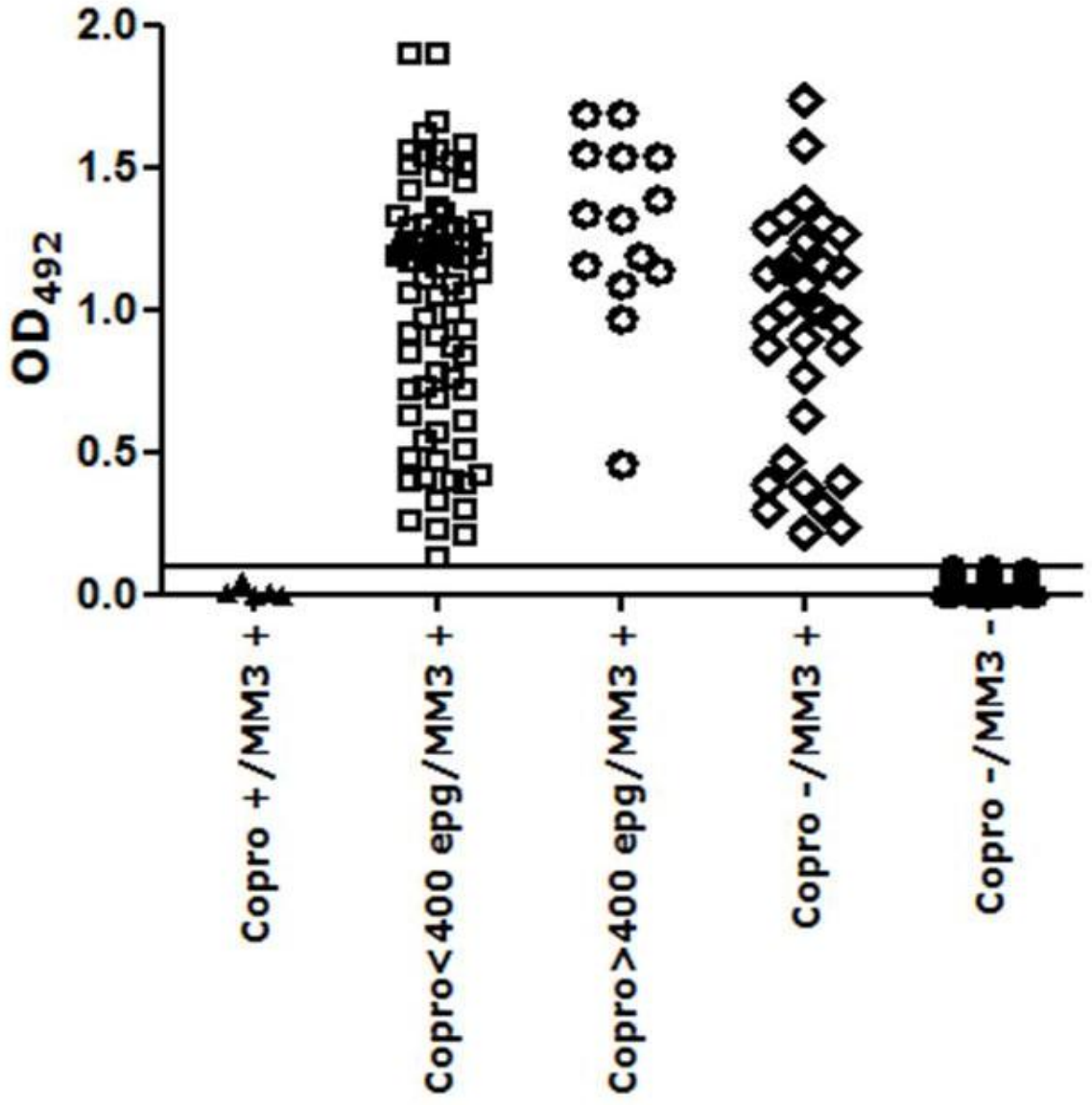

Figure 4.5- MM3-COPRO ELISA in faeces from children (n = 436) from Huacullani (Northern Bolivian Altiplano). Data points represent the mean absorbance at 492 nm obtained from three replicates of each sample tested. The line represents the cut-off value 0,097 units of OD at 492 nm.

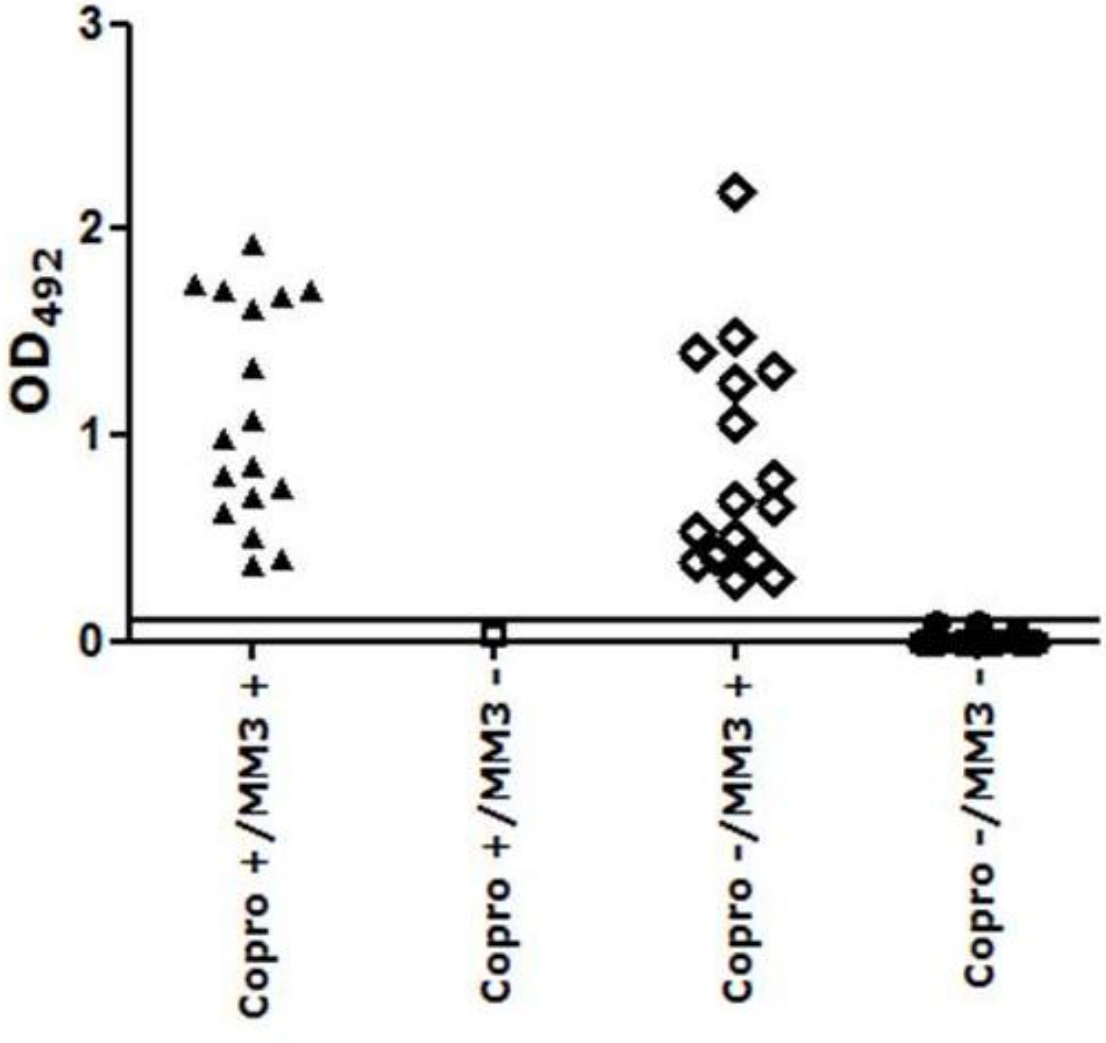

Figure 4.6- MM3-COPRO ELISA in faeces from children (n = 362) from Cajamarca valley (Peru). Data points represent the mean absorbance at 492 nm obtained from three replicates of each sample tested. The line represents the cut-off value 0,097 units of OD at 492 nm.

In Huacullani, in children who were positive in egg emission, the bivariant correlation between OD and epg data from low and high burden groups was carried out separately. A significant positive correlation was detected only between OD and low burden (r^2 = 0,20) (Figure 4.7), but no significant positive correlation was detected when considering OD and high burden (r^2 = 0,01) (Figure 4.8).

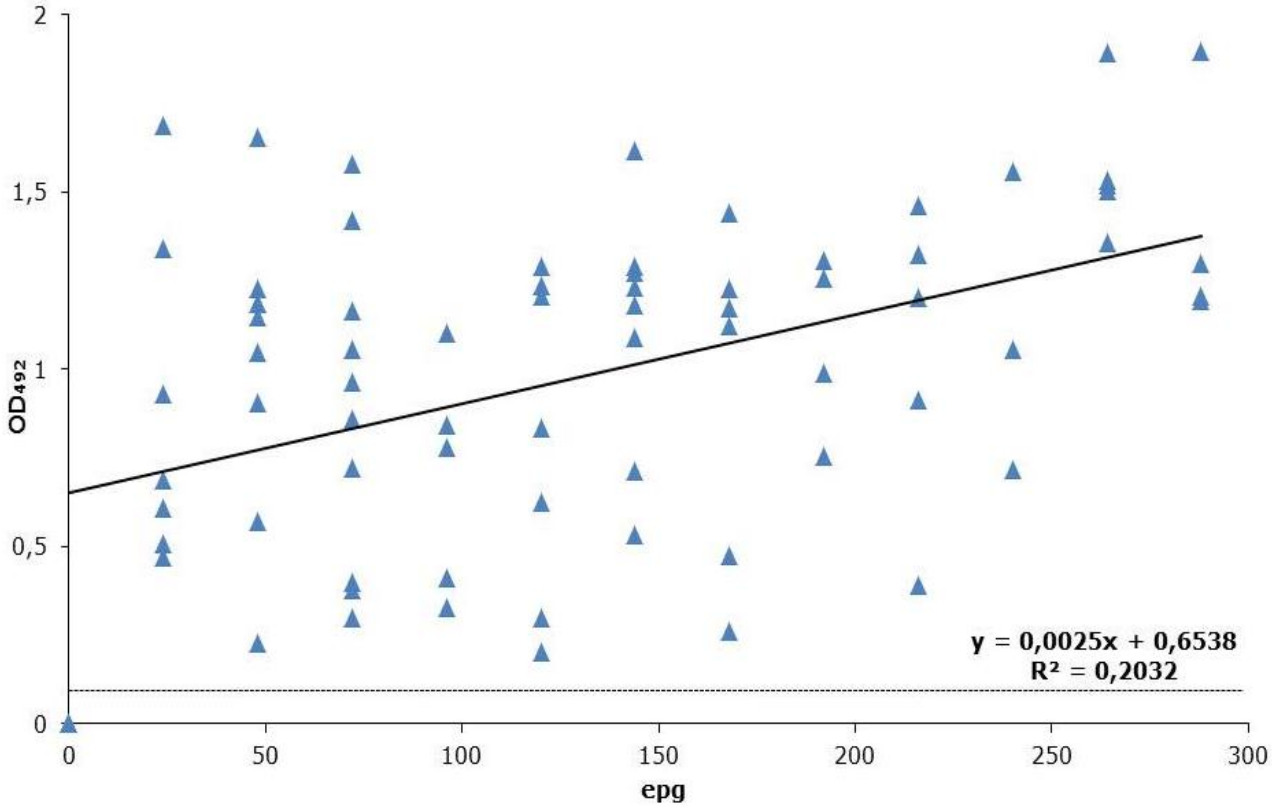

Figure 4.7- MM3-COPRO ELISA and intensity of *F. hepatica* infection in the low burden group of Huacullani. Data points represent the mean absorbance at 492 nm from egg positive children from Huacullani. epg represents the egg count per gram of feces. The dotted line represents the cut-off value 0,097 units of OD at 492 nm.

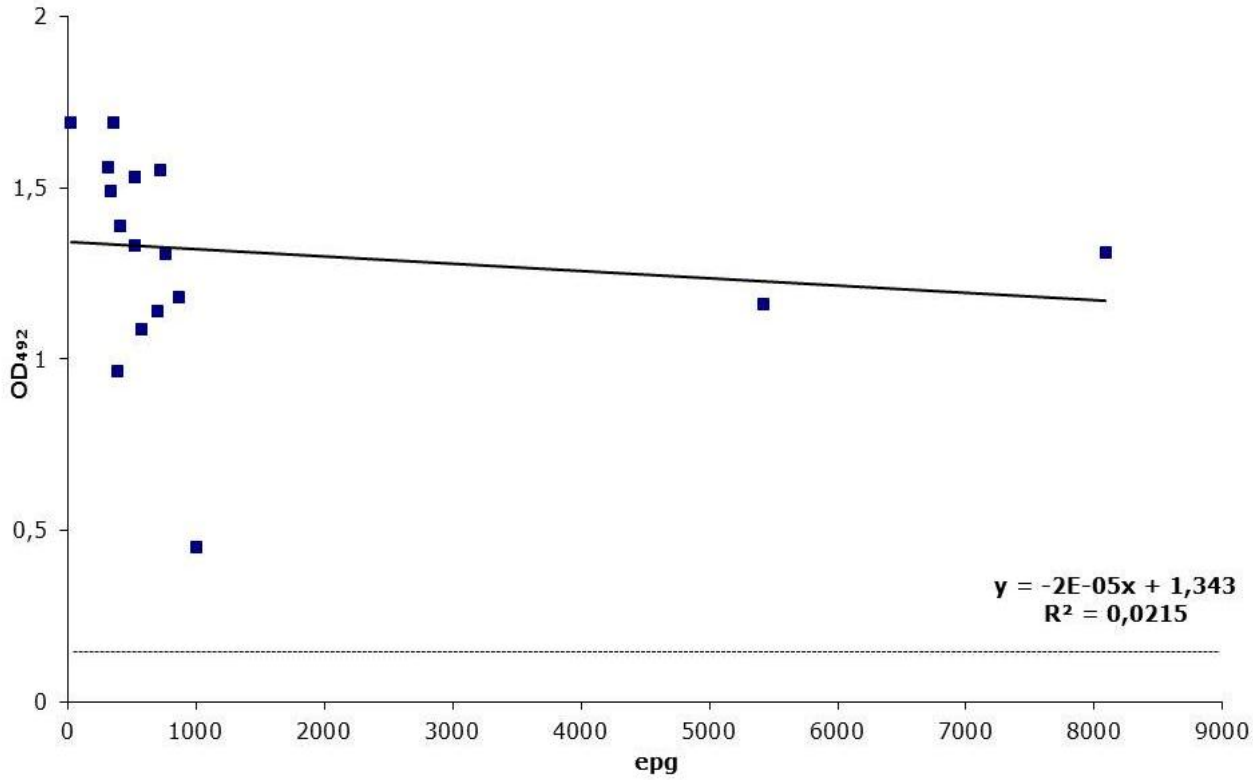

Figure 4.8- MM3-COPRO ELISA and intensity of *F. hepatica* infection in the high burden group of Huacullani. Data points represent the mean absorbance at 492 nm from egg positive children from Huacullani. epg represents the egg count per gram of feces. The dotted line represents the cut-off value 0,097 units of OD at 492 nm

In Cajamarca, in children who were positive in egg emission, the bivariant correlation between OD and epg data (low burden) was carried out. No significant positive correlation between OD and low burden (r^2 = 0,05) was detected (Figure 4.9).

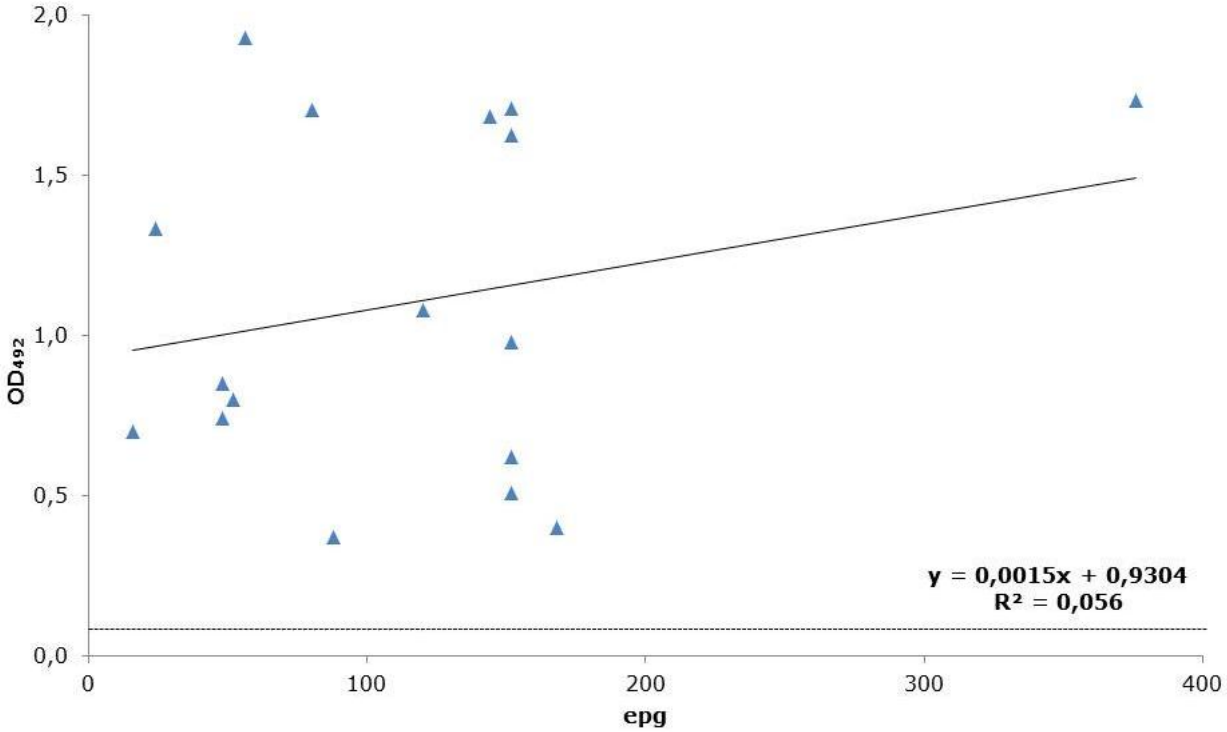

Figure 4.9- MM3-COPRO ELISA and intensity of *F. hepatica* infection in the low burden group of Cajamarca. Data points represent the mean absorbance at 492 nm from egg positive children from Cajamarca. epg represents the egg count per gram of feces. The dotted line represents the cut-off value 0,097 units of OD at 492 nm.

Theoretical PPVs and NPVs vs fascioliasis prevalence are represented in figure 4.10, showing the expected PPVs and NPVs depending on whether the test was used in low, medium or high prevalence scenarios in both highly endemic localities.

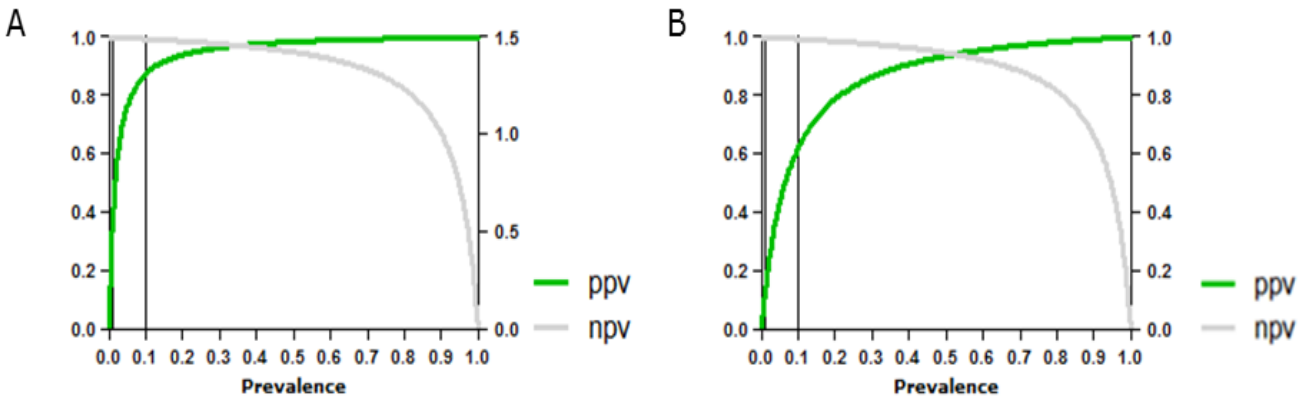

Figure 4.10-. Theoretical PPVs and NPV values vs fascioliasis prevalence. Curves show the expected PPVs (yellow continuous line) and NPV (red continuous line) values in low (below 1%), medium (between 1% and 10%) or high (above 10%) prevalence scenarios (expressed in vertical lines) in Huacullani (A) and Cajamarca (B).

4.2.3. DISCUSSION

Sensitivity is defined as the proportion of subjects with the disease who have a positive test for the disease. A sensitive test will rarely miss people with the disease. Specificity is the proportion of people without the disease who have a negative test result. A specific test will rarely misclassify people as having the disease when they do not (FLETCHER & FLETCHER, 2005). Knowing true positive and true negative cases is essential when calculating sensitivity and specificity, respectively. The identification of true positive and true negative cases was carried out by using classical coprological tests for the identification of *F. hepatica* eggs in faeces. Nevertheless, in the case of human fascioliasis, the application of the rapid sedimentation or Kato-Katz techniques may result in false negative cases. The aetiological diagnosis based on egg detection in stools is complicated because parasite eggs are not found along the prepatent period (LEVINE *et al.,* 1980; CHEN & MOTT, 1990; MAS-COMA *et al.,* 1999b), when juvenile worms migrate through the intestinal wall to the peritoneal cavity (at one week), penetrate the liver parenchyma (at five to seven weeks), and pass into the biliary tract where they ultimately reach maturity (at two months and more). Previous studies have even estimated that it takes at least a three-to-four-month period for *F. hepatica* flukes to attain sexual maturity in humans (WEI, 1984; CHEN & MOTT, 1990). Once the worms have matured, diagnosis still remains difficult because commonly employed microscopic techniques for quantitative diagnosis of *Fasciola* eggs are very specific but rather insensitive. On the other hand, in some cases it is also difficult during the biliary stage due to intermittent excretion of parasite eggs. Faecal egg counts are known to follow inter- and intraindividual variations in fascioliasis (EL-

MORSHEDY *et al.,* 2002; VALERO *et al.,* 2011). In our case, we used the Kato-Katz technique as a "gold standard" because it is considered the best available for quantitative analysis, although taking into account that it is admittedly rather imperfect.

When liver-flukes are located in the bile ducts, ES products are released, being eliminated via faeces. The detection of these products by means of a sandwich-ELISA reflects the installation of flukes in the bile ducts and the presence of the biliary stage of the disease (ESPINO & FINLAY, 1994). No differences were detected between prevalence results obtained using egg detection techniques and the MM3-COPRO ELISA in Huacullani, where egg intensities are higher according to the typical feature of the Altiplanic pattern. On the contrary, such differences were detected in Cajamarca, probably as a consequence of the low egg intensities characteristic of the valley pattern, i.e. in Cajamarca low burdens are the common feature and therefore the higher probability of infected subjects to intermittently shed very few eggs and remain unnoticed.

Five and one case of egg emission were detected as being negative using the MM3-COPRO ELISA in Huacullani and Cajamarca, respectively, including one case of very high egg count (1248 epg) in the Bolivian locality. Such cases pose a question mark. Two possible explanations include (i) an intermittent release of the ES products from the liver to the intestine through the choledoc, and (ii) the existence of food remains in the intestine masking or interfering with the detection of the fluke ES antigens. However, the sensibility of antigens to high temperatures during the transport or/and an inadequate handling of the samples at a given moment throughout the whole procedure cannot be ruled out.

In both Huacullani representative of the fascioliasis Altiplanic pattern and Cajamarca representing the fascioliasis valley pattern, cases of coproantigens present in the faeces of humans without the appearance of *F. hepatica* eggs in stools were detected. Previous time-course studies in animals on the detection of *F. hepatica* coproantigens by ELISA indicated that coproantigens were detectable prior to patency (ESPINO *et al.,* 1997). Furthermore, a marked increase in the levels of these coproantigens around the beginning of the faecal egg output was observed (VALERO *et al.,* 2009b). Considering the positivity/negativity of MM3-COPRO ELISA and the presence/absence of eggs in faeces, two situations were established in the current study: the Altiplanic pattern with a correlation between positivity of the MM3-COPRO ELISA and the presence of eggs, and the valley pattern with a larger

number of positive cases applying the MM3-COPRO ELISA but without presence of eggs in faeces.

The Altiplanic pattern, characterized by higher prevalences and intensities, showed no statistical differences between the percent of children who were positive in coproantigens and eggs in faeces. Thus, it may be concluded that the majority of children with liver-flukes in the biliary ducts shed eggs. Nevertheless, in the valley pattern, characterized by high prevalences but low intensities, differences were detected between the percentage of children who were positive in coproantigens and the reduced number of these children that shed eggs in faeces. This suggests that children did not shed eggs or only a much reduced, undetectable number of them even though they presented parasites in their biliary canals. In Europe, for instance, the diagnosis of human fascioliasis is frequently established using serological tests, because the detection of *F. hepatica* eggs in stools is not always possible. Thus, in an epidemiological survey from 1970 to 1999 to record cases of human fascioliasis detected in the Limousin region (central France), egg detection in stools was positive in only 27,6% of a total of 711 subjects with fascioliasis (RONDELAUD *et al.,* 2006).

Negativisation of results in the MM3-COPRO ELISA, which takes approximately one to three week in animals, is usually considered when determining the efficacy of anthelminthic treatment of biliary fascioliasis (MEZO *et al.,* 2004). Contrarily, serological methods have limitations when determining the efficacy of anthelminthic treatment because the presence of antibodies indicates previous exposure to the parasite rather than the existence of a current infection. Additionally, once the anthelminthic treatment has been administered, several months have to go by for the serological antibody-detecting tests to become negative. Hence, the detection of specific antigens in faeces enables the confirmation of a current infection, whereas antibody detection tests need to be complemented by another technique to confirm the results obtained in treated subjects. However, future studies should be carried out to determine the time required for negativisation of MM3-COPRO ELISA results after effective treatment in humans.

The MM3-COPRO ELISA is also a reliable method for detecting *F. gigantica* coproantigens in faecal samples from experimentally infected sheep (VALERO *et al.,* 2009b). Although most reported cases of human fascioliasis are caused by *F. hepatica*, infections by *F. gigantica* have also been reported (VALERO *et al.,* 2009a). The fact that the MM3-COPRO ELISA can detect infections by both species

may be of great value to ensure diagnosis of human and animal fascioliasis in countries where *F. gigantica* predominates, or where both species of *Fasciola* are present (VALERO *et al.,* 2009a; UBEIRA *et al.,* 2009).

Determining a patient's parasitic burden is crucial given the necessity to monitor the drug treatment in order to prevent a hepatic colic as the consequence of the massive expulsion of liver-flukes (WHO, 2007), similarly as in other helminth diseases (MONTRESOR *et al.,* 1998). The Kato-Katz is usually employed as a coprological quantitative technique. Nevertheless, this technique has a low sensitivity, and the elaboration of several slides from the same individual stool sample is indispensable. Additionally, the microscopic egg count is rather slow in cases of heavy egg burdens. For all of these reasons, the application of the Kato-Katz technique in community surveys becomes problematic because (i) it is pronouncedly time consuming when the number of samples is high, and (ii) requires an additional technique to increase the sensitivity in areas where subjects shed a very low number of eggs in an intermittent way.

Results obtained in the samples from Huacullani showed that the concentration of coproantigens in faeces is correlated with epg in the low burden group (<400 epg). This result agrees with a previous study using the MM3-COPRO ELISA in cattle, which showed that the concentration of coproantigens in faeces is also correlated with the number of flukes found in the livers of animals collected after slaughter (MEZO *et al.,* 2004), as well as with the results of positive correlation found with another coproantigen test in fascioliasis infected patients in Cuba (ESPINO & FINLAY, 1994). Nevertheless, our findings in the high burden group (≥400 epg) showed that the concentration of coproantigens in faeces is not correlated with epg. This result agrees with the absence of any correlation between egg shedding in human samples from Hospital patients, measured by the Kato-Katz technique, and coproantigen concentration, measured by the MM3-COPRO ELISA (UBEIRA *et al.,* 2009). One possible explanation for this discrepancy may be that the positive cases analysed in Cuba (ESPINO & FINLAY, 1994) probably corresponded to recent infections of less than a year of evolution (early chronic stage), whereas our samples were from patients with chronic infections, in which egg excretion is probably more erratic. It must be kept in mind that fasciolid flukes may survive up to 13,5 years inside humans (MAS-COMA *et al.,* 1999b), and the pattern of egg shedding is not linear but fluctuates between maximum and minimum values (VALERO *et al.,* 2002, 2011). Comparatively, the kinetics of coproantigen release versus the kinetics of egg shedding showed a similar pattern but with a two-week time-lag in epg (VALERO *et al.,* 2009b). Moreover, the

influence of the crowding effect on *Fasciola* egg shedding should also be taken into account (VALERO *et al.,* 2006b).

In Cajamarca, chronic fascioliasis in valley samples, coproantigen levels did not show a good correlation with epg. Therefore, the use of only a coproproantigen technique appears to be insufficient to evaluate the fluke burden.

In these hyperendemic areas, the number of subjects who participate in surveys of this kind is very large, which implies the problem of transporting and preserving the faecal samples, as the coproantigen degrades at ambient temperature within a few days and the faecal material cannot be treated with any classical fixative. One possible alternative is freezing the samples at -20º C, but this poses the additional problems of (i) the difficulty and expensiveness of transporting frozen samples to the laboratory where the determination should take place, and (ii) the inappropriateness of frozen samples for the diagnosis of several coinfecting parasites. Another solution is the use of Coproguard®, which has been demonstrated to be convenient for sample preservation in this kind of surveys including the application of a coproantigen detection test (UBEIRA *et al.,* 2009).

Current efforts for the control of human fascioliasis need diagnostic techniques which allow for high sensitivity and specificity, mass screening, detection in the acute phase, early detection of treatment failure or re-infection in post-treated subjects, and usefulness for surveillance programmes. Our results indicate that a coproantigen-detection test such as the MM3-COPRO ELISA fulfils all these aspects. It provides a good tool to detect biliary fascioliasis in humans under field conditions in Andean hyperendemic countries, including a higher sensitivity than egg detection techniques, especially in areas where burdens tend to be low, such as in areas of the valley transmission pattern. Hence, the MM3-COPRO ELISA appears not only to be useful for individual diagnosis in hospitals, but also in human surveys in fascioliasis endemic areas characterized by low to high parasitic burdens.

CONCLUSIONS

CONCLUSIONES

5. CONCLUSIONS

5.1. SUMMARY

Fascioliasis is a zoonotic parasitic disease caused by *Fasciola hepatica* and *F. gigantica*. Of both species, *F. hepatica* is the only one described in the Americas, where human fascioliasis endemic areas are mainly located in high altitude areas of Andean countries. Emergence of human fascioliasis prompted a worldwide control initiative including a pilot study in several countries. Given the necessity to characterize the fasciolid populations involved, the overall aim of the present research is, first, the phenotypic characterization of faciolid adults and eggs implicated in human endemic areas, where this initiative has been implemented, through a computer image analysis system (CIAS) applied on the basis of standardized measurements. Second, new immunological techniques and their potential use as immunological diagnostic tests in those areas are evaluated. The specific results have been grouped into five parts.

A) Characterization of eggs of both *F. hepatica* and *F. gigantica* for their differential diagnosis. The influence of both the geographical location and the host species (human and livestock) were analysed in eggs shed in faeces from fascioliasis human endemic areas, where only *F. hepatica* is present (the Northern Bolivian Altiplano and the Cajamarca valley in Peru), and from areas where *F. hepatica* and *F. gigantica* coexist (the Kutaisi region of Georgia, the Nile Delta in Egypt, and the Quy Nhon province in Vietnam). The study revealed that eggs shed by humans show morphological traits different from eggs shed by animals. In humans, *F. hepatica* eggs are bigger and *F. gigantica* eggs are smaller than reported to date from livestock, and their measurements overlap when compared. The material analysed in this study shows that the size of eggs shed by humans from Georgia and Egypt corresponds to the *F. hepatica* morph, while the size of eggs shed by humans from Vietnam corresponds to the *F. gigantica* morph. Measurements of *F. hepatica* and *F. gigantica* eggs originating from humans and animals from sympatric areas overlap, and, therefore, they do not allow differential diagnosis when within this overlapping range.

B) Study of the correlation between egg-shedding and uterus development in *F. hepatica* human and animal isolates. Uterus area (UA) development of adult *F. hepatica* obtained at different days post infection (dpi) in a Wistar rat model with isolates obtained from cattle, sheep, pigs and humans from

the endemic human fascioliasis zone of the Northern Bolivian Altiplano was analysed and compared with the number of eggs shed per gram of faeces as obtained through the Kato–Katz technique. The multiple regression model showed that UA is dependent on dpi and isolate. The evolution of UA vs dpi followed a damped model. This work shows a positive correlation between liver fluke UA and egg production. The complete absence of eggs in the uteri of some parasite individuals at 300 dpi was observed, which corresponds to the cessation of egg shedding in the advanced chronic stage.

C) Study of *F. hepatica* phenotypic characterization in Andean human endemic areas. The phenotypic features of fasciolid adults infecting sheep present in the Cajamarca Valley and Mantaro Valley (valley transmission patterns) and the Northern Bolivian Altiplano (altiplanic transmission pattern) were analysed. The aforementioned highland populations were compared to standard lowland natural and experimental populations of European origin. The results showed that two phenotypic patterns could be distinguished in *F. hepatica* adult size: the valley pattern (Cajamarca and Mantaro, Peru) and the altiplanic pattern (Northern Altiplano, Bolivia). Furthermore, the Andean valley population and European standard populations presented phenotypic homogeneity. The Altiplano population showed a large size range with a pronouncedly lower minimum size, indicating that uterus gravidity is reached at a smaller size than in valley populations.

D) Study of the DRG *Fasciola hepatica* IgG ELISA test as a serological diagnostic tool of human fascioliasis in different epidemiological situations To validate this kit, the sera of 54 fascioliasis cases, originating from three endemic areas, were used: (i) a hyperendemic *F. hepatica* area where humans usually shed a large number of parasite eggs in faeces (11 sera); (ii) an epidemic *F. hepatica* area where humans usually shed small amounts of parasite eggs (24 sera) and (iii) an overlap area of both *Fasciola* species where human shedding of parasite eggs in faeces is usually scarce or non-existent (19 sera). One hundred and sixty-eight patients with other parasitic infections and 89 healthy controls were also analysed. Sensitivity and specificity of the DRG assay were 95,3% (95% confidence intervals, 82,9–99,2%) and 95,7% (95% confidence intervals, 92,3–97,5%), respectively. No correlation between egg output and the optical density (OD) values of the test was observed.

E) Field evaluation of the MM3 coproantigen detection test for fascioliasis diagnosis and surveillance in human hyperendemic areas of Andean countries. As part of the above-mentioned control initiative, two hyperendemic areas were chosen: Huacullani, Northern Altiplano, Bolivia, representing the Altiplanic transmission pattern with high prevalences and intensities; the Cajamarca valley, Peru, representing the valley pattern with high prevalences but low intensities. Coprological sample collection and study procedures were analysed to improve individual diagnosis, subsequent treatments and surveillance activities. Therefore, a coproantigen-detection technique (MM3-COPRO ELISA) was evaluated, using classical techniques for egg detection for comparison. A total of 436 and 362 stool samples from schoolchildren of Huacullani and Cajamarca, respectively, were used. Positivity in samples from Huacullani was 24,77% using the MM3-COPRO technique, and 21,56% applying Kato-Katz. Positivity in samples from Cajamarca was 11,05% using MM3-COPRO, and 5,24% using rapid sedimentation and Kato-Katz. In Huacullani, using Kato-Katz as gold standard, sensitivity and specificity were 94,68% and 98,48%, respectively, and applying Kato-Katz and COPRO-ELISA test together, values were 95,68% and 100%. In Cajamarca, using rapid sedimentation and Kato-Katz together, results were 94,73% and 93,58%, and using rapid sedimentation, Kato-Katz and copro-ELISA together, values were 97,56% and 100%, respectively. There was no correlation between coproantigen detection by OD and infection intensity by eggs per gram of feces (epg) in Cajamarca low burden cases (<400 epg), nor in Huacullani high burden cases (≥ 400 epg), although there was in Huacullani low burden cases (<400 epg). Six cases of egg emission appeared negative by MM3-COPRO, including one with a high egg count (1248 epg). It can be concluded that the coproantigen-detection test allows for high sensitivity and specificity, fast mass screening, detection in the chronic phase, early detection of treatment failure or reinfection in post-treated subjects, and is convenient in surveillance programmes. However, this technique falls short when evaluating the fluke burden on its own.

5.2. CONCLUSIONS

The conclusions obtained from our work are numerous and varied according to their different contents. To facilitate their presentation, they are listed according to groups depending on their objectives.

A. With regard to the results obtained in the study on fluke egg characteristics for the diagnosis of human and animal fascioliasis by *F. hepatica* and *F. gigantica*:

- Fasciolid egg size in human stool samples ought to be corrected in books and monographs related to medical parasitology and/or tropical medicine as well as in guides for clinicians and diagnosticians. When dealing with human fascioliasis globally, *F. hepatica* egg length/width data should be 100,6–162,2/65,9–104,6 µm in human stools and 73,8–156,8/58,1–98,1 µm in animal faeces when referring to areas where *F. gigantica* is absent (as in the Americas and Europe). When referring to areas where both fasciolid species are present (as in many parts of Africa and Asia), *F. hepatica* egg length/width data should be 106,5–171,5/63,9–95,4 µm in human stools and 120,6–163,9/69,2–93,8 µm in animal faeces, and *F. gigantica* egg length/width data should be 150,9–182,2/85,1–106,2 µm in human stools and 130,3–182,8/74,0–123,6 µm in animal faeces.

- For areas where both fasciolid species co-exist, the size of fasciolid eggs shed by humans may be intermediate between the reported data for *F. hepatica* and that for *F. gigantica* in humans and such situations might be interpreted as infections by intermediate or hybrid forms.

- With regards to *F. gigantica*, egg length/width data should also be increased to 129,6–204,5/61,6–112,5 µm in animal faeces when referring to areas where *F. hepatica* is absent (as in great parts of Africa)

- Future studies in other areas are likely to modify the above-mentioned maximum and minimum size measurements for fasciolid eggs shed in humans stools, but the present exhaustive study suggests that only very slight differences may be found, if any.

- In endemic areas of Asia and Africa where both fasciolid species overlap, a human subject may be simultaneously infected by fluke adults

belonging to both *F. hepatica* and *F. gigantica*. Consequently, an appropriate coprological diagnosis in such an area may not rely on the measurement of only one or very few eggs. Whenever possible, measuring a sufficient amount of eggs to appropriately assess egg size variability could facilitate the verification of whether only one or the other, or perhaps both species simultaneously infect the patient. Moreover, it has to be considered that, if adult flukes of both species co-infect the patient, intermediate hybrid eggs may additionally appear in stools in case some flukes have crossbred.

B. With regard to the results obtained in the study on the correlation between egg-shedding and uterus development in *F. hepatica* human and animal isolates:

   - Our work shows a relationship between liver fluke uterus area and egg production.

   - The variability detected in egg production in different *F. hepatica* isolates in the experimental Wistar model, implies the necessity to characterize the isolates employed in fascioliasis studies in which egg production is used as a biological tag.

C. With regard to the results obtained in the study on *F. hepatica* phenotypic characterization in Andean human endemic areas, analysing valley versus altiplanic patterns in liver flukes from sheep from Cajamarca and Mantaro, Peru:

   - Flukes from Andean endemic areas may be catalogued according to two phenotypic patterns, the valley pattern including Andean human endemic areas of high altitude (Cajamarca: 2500–3100 m; Mantaro: 3400–3500 m) where *F. hepatica* egg production by the fluke oogenotop and minimum body size when reaching gravidity are similar to those in lowland populations of Europe (Spain, Poland). Furthermore, the Altiplanic pattern including Andean human endemic areas of very high altitude (Northern Altiplano: 3800–4100 m) where *F. hepatica* egg production by the fluke oogenotop begins earlier and minimum body size when reaching gravidity is smaller than in lowland populations of Europe (Spain, Poland) as well as in Andean valleys (Cajamarca, Mantaro).

D. With regard to the results obtained in the study on the DRG *Fasciola hepatica* IgG ELISA test as a serological diagnostic tool of human fascioliasis in different epidemiological situations:
   - The DRG test could be used both as an individual serodiagnostic test for human fascioliasis when backed up by a clinical history, together with a second diagnostic technique in certain situations of possible crossreactions (patients co-infected with other helminths) and in future large-scale epidemiological studies of human fascioliasis worldwide (recommended for mass screening, especially when considering its convenient handling).

E. With regard to the results obtained in the study on the field evaluation of a coproantigen detection test for fascioliasis diagnosis and surveillance in human hyperendemic areas of Andean countries:
   - Considering the positivity/negativity of MM3-COPRO ELISA and the presence/absence of eggs in faeces, two situations were established in the current study: the Altiplanic pattern with a correlation between positivity of the MM3-COPRO ELISA and the presence of eggs, and the valley pattern with a larger number of positive cases applying the MM3-COPRO ELISA but without presence of eggs in faeces.
   - The Altiplanic pattern, characterized by higher prevalences and intensities, showed no statistical differences between the percentage of children who were positive in coproantigens and eggs in faeces. Thus, it may be concluded that the majority of children with liver-flukes in the biliary ducts shed eggs. Nevertheless, in the valley pattern, characterized by high prevalences but low intensities, differences were detected between the percentage of children who were positive to coproantigens and the reduced number of these children that shed eggs in faeces. This suggests that children did not shed eggs or only a much reduced, undetectable number even despite presenting parasites in their biliary canals.
   - The present validation of the MM3-COPRO ELISA is expected to facilitate the improvement of human fascioliasis diagnosis in endemic areas. The practical application of this sensitive and convenient method for large scale surveillance in the control programmes in the Northern Bolivian Altiplano and Cajamarca could improve screening of human fascioliasis in these endemic areas by detecting infected humans in the biliary stage of the disease, as a large number of samples can easily be processed. Keeping in mind that the most affected subjects are usually children, the

attainment of faecal samples is easier and faster than taking blood samples, which is considered invasive. The former does not pose difficulties for community elders, school head teachers and parents who usually give their consent. Moreover, to many of these indigenous communities, blood extraction is culturally not acceptable.

- Our experience in Huacullani and Cajamarca indicates that MM3-COPRO ELISA offers the easiest and fastest way to adequately face large mass screenings, by initially applying the coproantigen technique to all of the coprological samples obtained in the community survey and thereafter applying the Kato-Katz technique only or first to the coproantigen positive samples. Those subjects in whose coproantigen positive samples no eggs were found are better recommended to be treated anyway. This allows for a quick selected treatment action, lending the positive effects of (i) fast respond to the communities surveyed that verify that infected subjects are treated in only a few days after the survey was made, and (ii) reduces the probability of drug resistance appearance. The remaining coproantigen negative samples may finally be analyzed for the eventual detection and subsequent selected treatment of very few subjects shedding eggs, although this last step will unavoidably be time-consuming.
- The coproantigen-detection test allows for high sensitivity and specificity, fast large mass screening capacity, detection in the chronic phase, early detection of treatment failure or reinfection in post-treated subjects, and usefulness in surveillance programmes. However, this technique falls short when evaluating the fluke burden on its own.
- Future studies are needed in Cajamarca (and other endemic areas in valleys of the Andean countries) to verify whether in these cases the non-detection of eggs implies that the parasite (i) has not reached the biliary ducts or is located in the bile-ducts but oviposition has not yet started (suggesting a more or less recent infection) or (ii) oviposition is taking already take place but with only very low egg numbers or with intermittent shedding (indicating that subjects present only one or a very few flukes in the chronic stage).

5.3. CONCLUSIONES

Las conclusiones que cabe extraer de nuestro trabajo son numerosas y variadas. Para facilitar su exposición, procedemos a enumerarlas por grupos según sus diferentes objetivos.

A. En lo que se refiere a las características morfológicas de los huevos de los fasciólidos como herramientas en el diagnóstico de la fascioliasis humana y animal causada por *F. hepatica* y F*. gigantica:*

- El tamaño de los huevos de fasciólidos en muestras humanas debe ser corregido en libros y monografías relacionados con la Parasitología Médica y/o con la Medicina Tropical, así como en guías para clínicos y analistas. Cuando se trate globalmente de Fascioliasis humana, los valores de longitud/anchura de los huevos de *F. hepatica* en zonas donde *F. gigantica* no está presente (como es el caso de las Américas y Europa), deben ser de 100,6-162,2/65,9-104,6 µm en muestras humanas y de 73,8-156,8/58,1-98,1 µm en heces de animales. Cuando nos referimos a áreas en las cuales ambas especies de fasciólidos están presentes (como es el caso de muchas zonas de África y Asia), los valores de longitud/anchura de los huevos de *F. hepatica* deben ser de 106,5-171,5/63,9-95,4 µm en muestras humanas y de 120-163,9/69,2-93,8 µm en heces de animal, mientras que los valores de longitud/anchura de los huevos de *F. gigantica* deben ser de 150,9-182,2/85,1-106,2 µm en muestras humanas y de 130,3-182,8/74,0-123,6 µm en heces animales.

- En áreas donde ambas especies de fasciólidos coexisten, el tamaño de los huevos de fasciólido emitidos por humanos puede ser de un valor intermedio entre *F. hepatica* y *F. gigantica*, interpretándose esta situación como una infección por formas hibridas o intermedias.

- Los valores de longitud/anchura de los huevos de *F. gigantica* procedentes de heces animales de áreas donde *F. hepatica* está ausente (como ocurre en la mayor parte de África) son 129,6-204,5/61,6-112,5 µm.

- Futuros estudios en otras áreas quizás puedan modificar el tamaño mínimo y máximo de las medidas realizadas en los huevos de fasciólidos encontrados en muestras humanas, si bien el exhaustivo estudio

realizado sugiere que sólo encontraremos ligeras diferencias respecto a los datos anteriormente indicados.

- En el caso de áreas endémicas, tanto de Asia como de África donde ambas especies de fasciólidos coexisten, un paciente puede estar afectado simultáneamente por adultos de *F. hepatica* y *F. gigantica*. En consecuencia, un diagnóstico apropiado en este tipo de áreas no debe consistir en la medida de unos pocos huevos, sino que, en la medida de lo posible, se debe realizar las medidas de una gran cantidad de huevos para asegurar la variabilidad del tamaño del huevo y poder facilitar la verificación de la infección del paciente por una u otra especie, o de quizás ambas especies simultáneamente. Más aun, hay que considerar también que, si el paciente está infectado por adultos fasciólidos de ambas especies, cabe la posibilidad de que algunos de los adultos de ambas especies se hayan cruzado, apareciendo huevos híbridos o intermedios en la muestra.

B. En lo que respecta a los estudios sobre la correlación entre la emisión de huevos por heces y el desarrollo uterino de *F. hepatica* en aislados humanos y animales:

- Nuestro estudio experimental demuestra una relación directa entre el tamaño del útero de *F. hepatica* y el número de huevos por gramo de heces emitidos.

- Las diferencias detectadas en la producción de huevos entre diferentes aislados de *F. hepatica* en el modelo experimental Wistar, implica la necesidad de caracterizar los aislados empleados en los estudios de la fascioliasis en donde la producción de huevos se utiliza como un marcador biológico.

C. En lo que se refiere a la caracterización fenotípica de *F. hepatica* en áreas Andinas humanas endémicas y el análisis del patrón valle versus patrón altiplánico de fasciólidos procedentes de ovinos:

- Los adultos de *F. hepatica* de zonas endémicas Andinas procedentes de ovinos se pueden catalogar de acuerdo a dos patrones fenotípicos: el patrón valle, incluyendo áreas Andinas endémicas humanas de alta altitud (Cajamarca: 2500-3100 m; Mantaro: 3400-3500 m) en donde la producción de huevos de *F. hepatica* y el tamaño corporal mínimo para

alcanzar la madurez es similar a las poblaciones en baja altitud europeas (España, Polonia). Además, el patrón Altiplánico, incluyendo áreas Andinas endémicas humanas de gran altitud (Altiplano Norte: 3800- 4100 m) donde la producción de huevos de *F. hepatica* comienza antes y el tamaño corporal mínimo para alcanzar la madurez es menor que el que presenta tanto las poblaciones europeas de baja altitud (España, Polonia) como las poblaciones de los valles Andinos (Cajamarca, Mantaro).

D. En lo que se refiere a la evaluación del test DRG ELISA IgG *Fasciola hepatica* para el diagnóstico de la Fascioliasis humana en diferentes situaciones epidemiológicas cabe destacar:

- El test DRG puede ser utilizado, en situaciones de posibles reacciones cruzadas (pacientes co-infectados con otros helmintos), tanto como una prueba de serodiagnóstico individual respaldado con el historial clínico del paciente y con una segunda técnica de diagnóstico, como, en el futuro, en estudios epidemiológicos a gran escala en todo el mundo (sobre todo en estudios epidemiológicos masivos).

E. En lo que se refiere a la evaluación de campo de un test de detección de coproantígeno para el diagnóstico y la vigilancia de la Fascioliasis en áreas hiperendémicas de países andinos:

- En el presente estudio se establecen dos situaciones considerando la positividad/negatividad del ELISA MM3-COPRO y la presencia/ausencia de huevos en heces. La primera es el patrón Altiplánico, donde existe una correlación positiva entre los casos positivos del ELISA MM3-COPRO y la presencia de huevos. La segunda es el patrón valle, con un gran número de casos positivos utilizando el ELISA MM3-COPRO, pero sin presencia de huevos en las heces.
- En el patrón Altiplánico, caracterizado por altas prevalencias e intensidades, no han encontraron diferencias estadísticas entre el porcentaje de niños positivos por coproantigeno y el porcentaje de niños positivos por la presencia de huevos del parásito en heces. Estos resultados permiten concluir que en el patrón Altiplánico la gran mayoría de niños presentan adultos parásitos en los conductos biliares que emiten huevos. Por el contrario, en el caso del patrón valle, caracterizado por altas prevalencias pero bajas intensidades, se han detectaron diferencias estadísticas entre el porcentaje de niños positivos

por coproantigenos y el reducido porcentaje de niños que emitían huevos del parásito en heces. Esto sugiere en el patrón valle, los niños no emiten huevos o emiten muy pocos huevos a pesar de presentar parásitos adultos en el canal biliar.

- Con la validación del ELISA MM3-COPRO se espera facilitar el diagnóstico de la fascioliasis humana en áreas endémicas. La aplicación de este sensible y conveniente método en la vigilancia a gran escala de los programas del Altiplano norte de Bolivia y Cajamarca permitirá mejorar el screening de la fascioliasis humana en esas áreas endémicas y facilitará la detección de humanos parasitados en la fase biliar de la enfermedad al permitir procesar un gran número de muestras fácilmente. Cabe recordar que la mayoría de la población afectada por la enfermedad suele ser niños, por lo que la obtención de muestras fecales es más rápida y fácil que la toma de muestras de sangre, la cual es considerada invasiva. Los ancianos de la comunidad, directores y profesores de escuela y los profesores suelen dar su consentimiento para la obtención de muestras fecales. Además, en muchas comunidades indígenas, la extracción de sangre es culturalmente inaceptable.
- Nuestra experiencia en Huacullani y Cajamarca indica que el ELISA MM3-COPRO es una herramienta fácil y rápida para detectar la enfermedad en fase crónica a gran escala, utilizando inicialmente la técnica de detección de coproantígeno en todas la muestras coprológicas obtenidas en la comunidad estudiada, para posteriormente aplicar la técnica de Kato-Katz únicamente o en primer lugar en las muestras positivas por la anterior técnica. Se recomienda tratar a aquellos sujetos en los cuales la técnica de detección de coproantígeno es positiva aunque no se encuentre huevos en heces. De esta manera se asegura una rápida respuesta de selección de tratamiento obteniendo la ventaja de (i) una rápida respuesta en las comunidades estudiadas, donde los sujetos infectados son tratados pocos días después del estudio, y (ii) reduce la probabilidad de la aparición de resistencia al medicamento. El resto de muestras negativas se analizaran para la detección y posterior tratamiento de los sujetos que emiten pocos huevos, aunque este último paso consumirá mucho tiempo.
- El test de detección de coproantígeno presenta una alta especificidad y sensibilidad, capacidad para realizar la detección de la enfermedad en fase crónica a gran escala, una rápida detección del fallo terapéutico o

de posibles re-infecciones, siendo además también útil en los programas de vigilancia. No obstante, esta técnica falla en la evaluación de la carga parasitaria.

- Es necesario realizar más estudios en Cajamarca (y otras áreas endémicas en los valles de países Andinos) para verificar si la ausencia de detección de huevos en heces implica que el parásito (i) no ha alcanzado los conductos biliares o están en los conductos biliares pero no ha comenzado la oviposición (lo que sugiere una infección más o menos recientes) o (ii) se está produciendo la oviposición pero o bien la cantidad de huevos que se emiten con las heces es muy baja o bien la emisión es intermitente (lo que indica que el sujeto presenta muy pocos adultos en fase crónica).

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ANEXO

ANNEX

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Fluke egg characteristics for the diagnosis of human and animal fascioliasis by *Fasciola hepatica* and *F. gigantica*

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ABSTRACT

In trematodiases, shape and size of the fluke eggs shed with faeces are crucial diagnostic features because of their typically reduced intraspecific variability. In fascioliasis, the usual diagnosis during the biliary stage of infection is based on the classification of eggs found in stools, duodenal contents or bile. The aim of the present study is to validate the identification of *Fasciola* species based on the shape and size of eggs shed by humans, characterizing their morphometric traits using a computer image analysis system (CIAS). The influence of both the geographical location and of the host (human and livestock) has been analysed. Coprological studies were carried out in fascioliasis human endemic areas, where only *F. hepatica* is present (the northern Bolivian Altiplano and the Cajamarca valley in Peru), and where *F. hepatica* and *F. gigantica* coexist (the Kutaisi region of Georgia, the Nile Delta in Egypt, and the Quy Nhon province in Vietnam). Classically, it is considered that at the abopercular end of the shell of *Fasciola* eggs there is often a roughened or irregular area. Nevertheless, results show that the frequency of the presence of this feature in *F. hepatica* is population-dependent, and therefore is not a pathognomonic criterion in diagnosis. The study reveals that eggs shed by humans show morphological traits different from eggs shed by animals. In humans, *F. hepatica* eggs are bigger and *F. gigantica* eggs are smaller than reported to date from livestock, and their measurements overlap when compared. The material analysed in this study shows that the size of eggs shed by humans from Georgia and Egypt corresponds to the *F. hepatica* morph, while the size of eggs shed by humans from Vietnam corresponds to the *F. gigantica* morph. Measurements of *F. hepatica* and *F. gigantica* eggs originating from humans and animals from sympatric areas overlap, and, therefore, they do not allow differential diagnosis when within this overlapping range. In this sense, the new results should aid clinicians since the application of the classic egg size range in human samples may lead to erroneous conclusions. Fasciolid egg size in human stool samples ought to be corrected in books and monographs related to medical parasitology and/or tropical medicine as well as in guides for clinicians and parasitic disease diagnosis analysts.

1. Introduction

Fascioliasis is an important parasitic disease caused by two liver fluke species: *Fasciola hepatica* and *F. gigantica* (Trematoda). *Fasciola hepatica* has a cosmopolitan distribution, mainly in temperate zones, while *F. gigantica* is found in tropical regions of Africa and Asia. Thus, the two fasciolid species overlap in large regions (Mas-Coma and Bargues, 1997). Fascioliasis in livestock has always been recognized as a veterinary problem on a worldwide scale, but in humans the disease was only considered of secondary importance until the end of the 1980s owing to the relative small number of human reports. Only about 2000 human cases were reported in the 25 years previous to 1990 (Chen and Mott, 1990). This scenario dramatically changed after the initiative launched by the World Health Organization at the beginning of the 1990s. At present, estimations for all continents reach 17 million people to be infected, and this may even be an underestimation if the total lack of data concerning numerous Asian and African countries is considered (Mas-Coma, 2005).

Today, several geographical areas have been described as endemic for the disease in humans, including hypoendemic, mesoendemic and hyperendemic situations, with prevalences and intensities ranging from low to very high (Mas-Coma, 2005). In these human endemic areas, children and females are most severely affected by both prevalences and intensities (Mas-Coma et al., 2005). In developed countries, patients are diagnosed in hospitals or other health centres usually during the acute phase or at the beginning of the chronic phase. On the contrary, infected subjects detected in surveys in human endemic areas of developing countries are mainly in the advanced stage of chronicity,

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when not already re-infected due to the high contamination risk, as the liver fluke is able to survive up to 13.5 years in human beings (Mas-Coma et al., 2005). The public health impact of this scenario is reaching an importance never expected before, owing to (i) the great pathogenicity of long-term liver fluke infection proven experimentally (Valero et al., 2003, 2006a, 2008) and (ii) the immune-modulation of fasciolids in the acute phase (Brady et al., 1999) and their immune suppression effect in chronic and advanced chronic phases (Girones et al., 2007), which is in the background of co-infections with other parasitic and infectious diseases (Mas-Coma et al., 2005).

WHO decided in October 2006 to launch a worldwide initiative against this disease (WHO, 2007) based on the success of obtaining a donation of Egaten®, the only highly efficient drug presently available for human treatments (WHO, 2008). After an initial pilot scheme in Vietnam, Egypt, Peru and Bolivia, this initiative is currently being extended to many other countries. Therefore, the capacity to correctly diagnose human fascioliasis becomes crucial. Unfortunately, there are some unavoidable difficulties in human fascioliasis diagnosis in human endemic areas of developing countries: (i) indirect tests, either serological or using coproantigens, are useless for intensity estimation; (ii) immunological tests are generally expensive and require a minimum of laboratory infrastructure; and (iii) indirect tests have so far never proved to be able to differentiate between infection by *F. hepatica* and *F. gigantica*, an important aspect considering the higher pathogenicity of the latter due to its larger fluke size but also because of their different epidemiology, transmission and control characteristics. Molecular tests have shown to be useful for differential diagnosis (Marcilla et al., 2002), but are often unaffordable, too.

Therefore, direct coprological analysis for the detection and identification of fasciolid eggs continues to be the most appropriate diagnostic strategy for both detection of infection and estimation of intensity, not only for those human endemic rural areas but also for the implementation of the WHO treatment drive. This conclusion is reached even in spite of the recognized lower sensitivity of egg detection in faecal samples and its uselessness for the diagnosis of patients in the acute phase (Hillyer, 1999), as well as the lack of an accurate relationship between egg counts per gram of faeces and the fluke burden (Valero et al., 2006b).

Moreover, in areas of Africa and Asia where the two fasciolid species overlap, fluke egg size was believed to be the only method allowing the differential diagnosis between *F. hepatica* and *F. gigantica*. However, identifying fluke adults obtained during an endoscopy either by microscopic morphometry (Periago et al., 2006) or by molecular tools (Marcilla et al., 2002) may also be performed nowadays, although this is not only difficult but may also be problematic in humans. Moreover, the infrastructure for such techniques is evidently not available in rural endemic areas. The use of serological tests or the lack of a calibrated microscope for measurements with an ocular micrometer explains why subjects diagnosed from areas where both species co-exist are currently referred to as infected by *Fasciola* sp. (Mas-Coma et al., 2005). In such a situation it is surprising that an analysis of the size of eggs shed by humans has never been carried out, so that in fact an extrapolation of the size of the fluke eggs shed by livestock species has always been used for human diagnosis.

Each trematode species has its own particular egg shape and the length and width of the eggs are generally within a specific range (WHO, 1991). In fascioliasis, the usual diagnosis during the biliary stage of infection is based on the classification of eggs found in stools, duodenal contents or bile. However, large variations were first observed in the size of *F. hepatica* eggs in livestock from different geographical locations (Tinar, 1984). Furthermore, it has been experimentally shown that the final host species (sheep, cattle, pig and donkey) decisively influences the size of the *F. hepatica* eggs even within the same endemic area (northern Bolivian Altiplano) (Valero et al., 2001a).

The problem has even been further increased by the confirmation of the existence of morphologically intermediate forms between the two fasciolid species and genetic hybrids of both in overlapping areas, endemic for animals (Itagaki et al., 2005; Semyenova et al., 2006) and humans in Africa (Periago et al., 2008) and Asia (Ashrafi et al., 2006; Le et al., 2008). The existence of these intermediate forms has been molecularly verified to be not only common but even the rule in these overlapping areas (Bargues et al., in prep.) and poses a question mark on whether egg characteristics are suitable as a tool for the differential diagnosis of fascioliasis caused by either species. A concrete example of this problem has been recently emphasized in the diagnosis of fascioliasis in humans (Inoue et al., 2007).

The aim of the present study is to validate the identification of *Fasciola* species based on the shape and size of eggs shed by humans, characterizing their morphometric traits using a computer image analysis system (CIAS). The influence of both the geographical origin (samples from South America, Europe, Africa and Asia) and the host species (human and livestock) has been analysed. Added to this global analysis, the measurements of eggs shed by humans from several geographical areas are also compared with the eggs from livestock of the same geographical origins to illustrate the situation within a local endemic area. The areas selected for this study are important human fascioliasis endemics zones, where our team had the opportunity to carry out field work within the WHO initiative. In the New World, the northern Bolivian Altiplano (Mas-Coma et al., 1999) and the Cajamarca valley in Peru (Espinoza et al., 2007) where chosen because of their high prevalences and intensities in humans caused by *F. hepatica* only. In Europe, the Kutaisi region in Georgia was chosen as *F. hepatica* is widely distributed there, but *F. gigantica* was also sporadically detected several decades ago (Gigitashvili, 1969) and human fascioliasis is currently emerging (Zenaishvili et al., 2004; Valero et al., in prep.). In Africa, the study was performed in the Nile Delta region of Egypt, where both *F. hepatica* and *F. gigantica* are encountered, and endemic areas present high prevalences and intensities in humans (Esteban et al., 2003; Curtale et al., 2007). In Asia, the analysis was carried out in Vietnam, where this disease has emerged in humans throughout the whole country in recent years (De et al., 2003; Le et al., 2008; Valero et al., in prep.).

2. Materials and methods

Non-embryonated eggs from humans and livestock analysed in this study originated from 13 isolates of Fasciolidae (see Table 1), including one from Bolivia, one from Peru, two from Georgia, three from Egypt and two from Vietnam. Four previously described animal-isolates from Bolivia (Valero et al., 2001a) were included for comparative purposes. As fixation is known to influence size and shape of helminth eggs, for the present study egg materials used were always without fixation, suspended in water, preserved in darkness at 4 °C until required and measured in the shortest time possible, within the next fortnight after obtaining them.

2.1. Egg materials from humans

A total of 1049 eggs from human faeces were analysed. The material from Bolivia, Peru, and Egypt originated from school surveys, the material from Georgia originated from total population surveys, and material from Vietnam originated from adult patients of the Quy Nhon Hospital. The following materials were analysed: 154 eggs from 5 children (6–12 years old) from Huacullani (in the northern Altiplano, Bolivia), 167 eggs from 4 children (7–14 years

Table 1
Comparative morphometrical data on *Fasciola* eggs from infected humans of the Northern Altiplano (Bolivia), Cajamarca Valley (Peru), Kutaisi region (Georgia), Nile Delta (Egypt) and Quy Nhon (Vietnam) and from animals from the same areas. All values are shown as range with mean ± standard deviation in parenthesis. *n* = sample size. Significant differences obtained when comparing each egg measurement from human samples in pairs with those of the animal hosts from the same endemic area by post hoc test (Bonferroni) in Bolivia (*) and Egypt (¥) and by *t*-test in Georgia (£) and Vietnam (§) ($P < 0.005$). Data from "pure" specimens of *F. gigantica* from Burkina Faso are included for comparison.

Isolate	EL (μm)	EW (μm)	EP (μm)	ER (μm s)	EA (μm^2)	EL/EW
Bolivia-human (*n* = 154)	119.8–159.5 (139.7 ± 8.2)	67.6–102.1 (76.9 ± 5.2)	321.2–429.5 (366.5 ± 19.5)	1.1–1.4 (1.3 ± 0.0)	6623.3–12166.4 (8356.4 ± 847.6)	1.4–2.2 (1.8 ± 0.1)
F. hepatica-Bolivia-cattle (*n* = 168)	105.3–155.9 (132.0 ± 10.5)*	61.7–82.5 (71.14 ± 4.3)*	270.6–422.9 (340.0 ± 33.4)*	–	5286.5–9676.8 (7170.2 ± 802.5)*	1.6–2.3 (1.8 ± 0.2)
F. hepatica-Bolivia-sheep (*n* = 104)	114.8–151.1 (130.8 ± 7.1)*	65.5–81.4 (72.6 ± 3.9)*	294.2–368.2 (327.6 ± 15.0)*	–	5998.2–8608.5 (7238.0 ± 532.8)*	1.5–2.1 (1.8 ± 0.1)
F. hepatica-Bolivia-pig (*n* = 186)	73.8–148.6 (123.8 ± 11.3)*	58.1–82.6 (71.8 ± 4.4)*	47.8–360.0 (313.7 ± 20.9)*	–	3988.7–8626.9 (6837.0 ± 820.3)*	1.1–2.0 (1.7 ± 0.2)*
F. hepatica-Bolivia-donkey (*n* = 161)	96.4–140.8 (125.4 ± 8.3)*	63.3–84.7 (75.0 ± 3.7)*	272.1–350.4 (318.8 ± 16.3)*	–	5562.6–8686.2 (7177.4 ± 646.1)*	1.3–2.0 (1.7 ± 0.1)*
Peru-human (*n* = 167)	100.6–161.0 (138.4 ± 9.9)	65.9–104.6 (80.2 ± 6.6)	296.4–441.2 (369.0 ± 23.3)	1.2–1.4 (1.3 ± 0.1)	5779.3–12433.8 (8554.1 ± 1068.3)	1.3–2.1 (1.7 ± 0.2)
Georgia-human (*n* = 53)	104.5–162.2 (140.1 ± 11.8)	72.1–96.6 (82.1 ± 4.7)	310.1–394.1 (366.4 ± 17.5)	1.1–1.3 (1.2 ± 0.0)	6594.6–10876.5 (8833.9 ± 1012.1)	1.3–2.0 (1.7 ± 0.1)
F. hepatica-Georgia-cattle (*n* = 117)	116.3–156.8 (140.2 ± 10.1)	72.2–98.1 (83.4 ± 6.9)	337.6–415.4 (373.0 ± 16.2)£	1.1–1.4 (1.2 ± 0.1)	7191.2–11315.0 (8883.4 ± 721.8)	1.3–2.0 (1.7 ± 0.2)
Egypt-human (*n* = 608)	106.5–171.5 (139.4 ± 11.0)	63.9–95.4 (76.7 ± 5.4)	276.5–404.0 (346.0 ± 20.5)	1.1–1.3 (1.1 ± 0.0)	5540.9–11482.6 (8308.7 ± 857.2)	1.4–2.5 (1.8 ± 0.2)
F. hepatica-Egypt-cattle (*n* = 35)	120.6–163.9 (146.7 ± 14.2)¥	69.2–93.8 (80.3±6.4)¥	317.9–419.8 (380.9 ± 33.6)¥	1.2–1.4 (1.3 ± 0.1)¥	6547.5–10957.8 (9093.7 ± 1386.9)¥	1.6–2.1 (1.8 ± 0.1)
F. gigantica-Egypt-cattle (*n* = 73)	130.3–175.0 (149.4 ± 8.3)¥	74.0–123.6 (94.6±6.8)¥	358.6–465.7 (408.9 ± 19.8)¥	1.1–1.4 (1.2 ± 0.1)¥	7893.6–15603.4 (10996.0 ± 1067.9)¥	1.3–2.0 (1.6 ± 0.1)¥
Vietnam-human (*n* = 67)	150.9–182.2 (165.3 ± 6.2)	85.1–106.2 (95.9 ± 4.4)	394.8–453.3 (416.9 ± 11.8)	1.1–1.2 (1.1 ± 0.0)	11065.4–14537.8 (12152.0 ± 671.5)	1.5–2.0 (1.7 ± 0.1)
F. gigantica-Vietnam-cattle (*n* = 101)	156.2–182.8 (169.9 ± 5.3)	90.6–114.9 (104.1 ± 5.7)	405.2–487.3 (454.9 ± 15.6)§	1.1–1.3 (1.2 ± 0.0)§	11217.4–15518.2 (13487.4 ± 942.8)§	1.4–1.9 (1.6 ± 0.1)§
F. gigantica-Burkina Faso-cattle (*n* = 142)	129.6–204.5 (156.8 ± 1.1)	61.6–112.5 (89.4 ± 0.7)	335.5–471.8 (390.1 ± 2.2)	1.0–1.3 (1.1 ± 0.0)	7846.3–15890.7 (11144.1 ± 124.3)	1.3–2.6 (1.8 ± 0.0)

old) from Santa Rosa de Chaquil (in the valley of Cajamarca, Peru), 53 eggs from 3 individuals (10–40 years old) from Akhali Terjola and Itkhvisi (in the Kutaisi region, Georgia), 608 eggs from 13 children (8–11 years old) from Abis, Ola Garbea, Hosh Essa, and El Waquad (in the Nile Delta, Egypt), and 67 eggs from 2 adults (17, 27 years old) from Quy Nhon (Vietnam). The surveys were made at random on a given day among all consenting individuals. A clean 30 ml plastic, wide-mouthed, numbered container with a snap-on lid was given to each individual, who was then asked to try to fill the container with his/her own faeces and to return it immediately. Stool specimens were collected from all consenting individuals and personal data was noted on delivery of the container. Faecal specimens were transported to the laboratory within the following 5 h. From each stool sample, a Kato–Katz slide (helm-Test®, AK test, AK Industria e Comèrcio Ltda, Belo Horizonte, Brazil) was taken. Fluke eggs were recovered from the positive samples.

2.2. Egg materials from livestock

Data in this paper corresponds to eggs from liver flukes from domestic animals living in the same or neighbouring areas to those where human samples were collected. For comparison with eggs of human samples from the northern Bolivian Altiplano and the Peruvian Cajamarca valley, data from *F. hepatica* eggs from the most common reservoir host species (104 eggs from sheep, 168 from cattle, 186 from pigs and 161 from donkeys) in the human hyperendemic area of the Bolivian Altiplano (Valero et al., 2001a) were used. From Georgia, a total of 117 *F. hepatica* eggs obtained from 3 cattle in the Kutaisi region were included in the study. In the Nile Delta region of Egypt, 35 *F. hepatica* eggs and 73 *F. gigantica* eggs from 3 and 4 cattle, respectively, were analysed. A total of 101 *F. gigantica* eggs from 3 cattle from Vietnam's area of Quy Nhon were added. For comparison with *F. gigantica*, data known to be from "pure" specimens of that species were also used: 142 eggs from cattle in the Bobo Dioulasso area, Burkina Faso, where *F. hepatica* is absent and *Radix natalensis* is the only lymnaeid vector species present (Periago et al., 2006).

2.3. Criteria for the specific classification of eggs from humans and animals

In Bolivia and Peru, the only liver fluke species present infecting both humans and livestock in the Bolivian Altiplano (Mas-Coma et al., 1999) and the Cajamarca valley (Espinoza et al., 2007) is *F. hepatica*, because *F. gigantica* never colonized the New World.

In Georgia, Egypt and Vietnam, however, special precautions were taken because of the distribution overlap of the two fasciolid species in each one of the respective endemic areas where egg samples studied came from. Adult fasciolid specimens tend to crossbread despite being hermaphroditic (Hurtrez-Bousses et al., 2004). So, the problem in these overlap areas is that adults of the two species hybridise and hybrid adult flukes found are known to show morphologically intermediate forms (Ashrafi et al., 2006; Periago et al., 2008; Le et al., 2008). Eggs in these endemic areas may phenotypically differ from eggs typical of one or the other pure, parental species. Hence, the only way to assure the specific classification of eggs was by obtaining the eggs directly from the fluke adult and afterwards processing this adult for specific classification. Unfortunately, this was only feasible for animal samples but not for human samples. Therefore, adult liver flukes were obtained from the livers of cattle from slaughterhouses in localities also furnishing the human samples here studied. The flukes were classified as *F. hepatica* or *F. gigantica* following the criteria of Periago et al. (2006, 2008). To avoid immature eggs from the uterus which could phenotypically differ from the mature eggs found in bile and faeces as those used for diagnosis, eggs were obtained from mature adult flukes by applying a light pressure on the acetabulum to force mature eggs to come out through the genital pore. When needed to obtain more eggs, a small hole in front of the acetabulum can be made with two needles to facilitate the ejection of eggs. It shall be taken into account that, in *Fasciola*, the uterus is not only an organ for egg maturation but also for egg storage (Valero et al., 2001b). This means that eggs in the final part of the uterus are (i) completely shaped, (ii) mature, and (iii) viable. The absence of differences in size, maturity and viability between eggs from the final uterine part and eggs from bile and faeces was verified in different animal individuals. To obtain eggs in that way is easy because mature fasciolids are very big flukes and most of its uterus is postacetabular but it ends preacetabularily. Eggs obtained by this simple technique are the same size and shape than the ones found in bile and faeces (i.e., no immature eggs included among those obtained by that technique). Results showed that only *F. hepatica* was concerned in Georgia, forms of both fasciolid species were involved in Egypt, and only *F. gigantica* forms were present in the animals analysed in Vietnam.

2.4. Measurement techniques

The measurements were taken by means of CIAS, including a computer connected to a 3CCD colour video camera (Sony DXC-930P) linked to a microscope, using image analysis software (ImagePro® Plus 4.5, Media Cybernetics Inc., Silver Spring, USA). According to the standardized methodology (Valero et al., 2005), egg characteristics studied were: egg length (EL), egg width (EW), egg perimeter (EP), egg area (EA), EL/EW ratio, and egg roundness (ER).

The egg roundness measurement ($ER = EP^2/4\pi EA$) was used to quantify the egg shape. It is a measurement of how circular an object is (the expected perimeter of a circular object divided by the actual perimeter). A circular object will have a roundness of 1.0, while more irregular objects will have larger values (Periago et al., 2008).

2.5. Statistical analyses

Comparison of the average egg measurements between human and animal samples was carried out using the one-way ANOVA, a post hoc test (Bonferroni) and *t*-test (software SPSS v.15). Morphometric data was explored using two multivariate analyses in five measurements (EL, EW, EP, EA, EL/EW):

(A) The principal component analysis (PCA), a technique for summarizing most of the variation in a multivariate dataset in few dimensions (Rohlf and Marcus, 1993; Klingenberg, 1996; Dujardin and Le Pont, 2004). The first principal component (PC1) is the lineal combination that accounts for the maximum variance. Geometrically, it corresponds to the direction of the longest axis through the scatter of data points. Subsequent principal components take up maximal variance, subject to being orthogonal to all preceding component axes (Klingenberg, 1996). Size-free canonical discriminant analysis was used on the covariance matrix of transformed logarithms. This technique consists of regressing each character separately on the within group first principal component (PC1), which is a multivariate estimate of size (Bookstein, 1989; Dos Reis et al., 1990). The analyses were carried out using the BAC v.2 software (Dujardin, 2002). The statistical methodology used for egg measurements is very similar to that performed by Periago et al. (2008) on adults of *F. hepatica* and *F. gigantica* species from the same geographical area.

(B) All the populations were also studied by means of discriminant analysis using the geographic area of origin and host species as the grouping variables. A stepwise discriminant function pro-

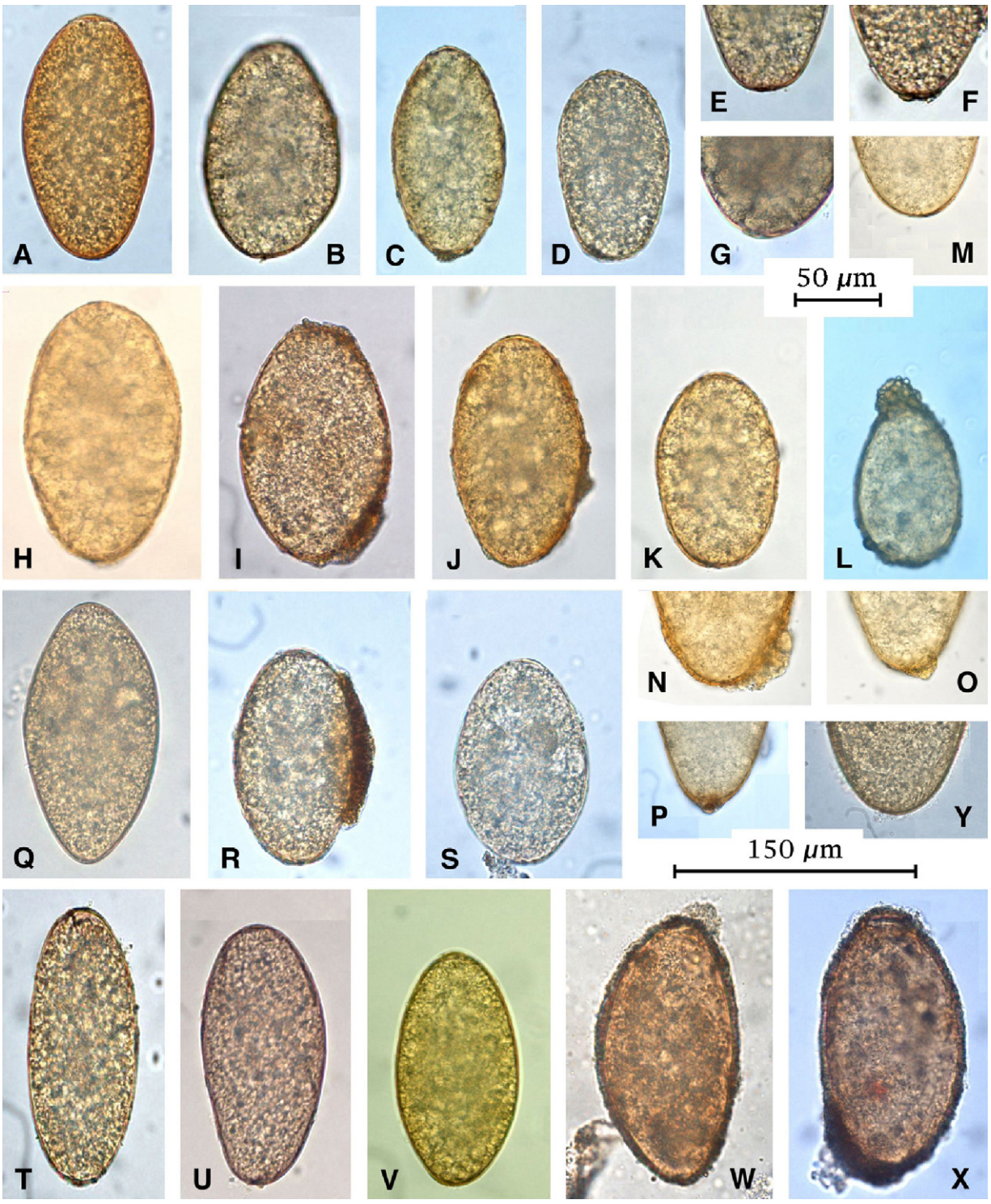

Fig. 1. Shape and size variability of fasciolid eggs shed in human stools: (A–G) *Fasciola hepatica* of the Northern Altiplano, Bolivia; (H–P) *F. hepatica* of the Cajamarca Valley, Peru; (Q–S) *F. hepatica* of the Kutaisi region, Georgia; (T–V) fasciolid eggs (mainly *F. hepatica* phenotypes) of the Nile Delta region, Egypt; (W–Y) *F. gigantica* of the Quy Nhon area, Vietnam. Note the presence of lateral irregularities on the egg shell surface in I, J and R, presence of such irregularities on the abopercular end of the shell in C, E–G, N–P and X, and their absence on the same abopercular shell end in M and Y. Scale bars: A–D, H–L, Q–X = 150 μm; E–G, M–P, Y = 50 μm.

cedure was applied using Wilks' λ (SPSS v.15). Values were considered statistically significant when $P < 0.05$.

3. Results

In the egg samples from *Fasciola* species shed by humans, the abopercular end of the shell is often roughened or irregular (more or less pronounced) (Fig. 1). Nevertheless, this irregularity is sometimes laterally located. The morphological analysis of the eggs shows that this irregularity on the shell surface is very common in Bolivia (99 cases with a protuberance out of 154 eggs: 64.3%) as well as in Peru (121 cases out of 167: 72.5%). This irregularity is less frequent in the human samples from Egypt (30.1%). However, it is very infrequent in the human samples from Georgia (it was

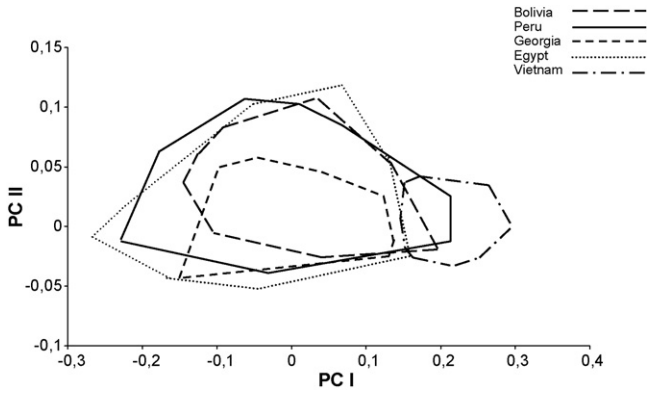

Fig. 2. Factor map corresponding to *Fasciola* eggs shed by humans from Bolivia, Peru, Georgia, Egypt and Vietnam. Samples are projected onto the first (PC1, 74%) and second (PC2, 19%) principal components. Each group is represented by its perimeter.

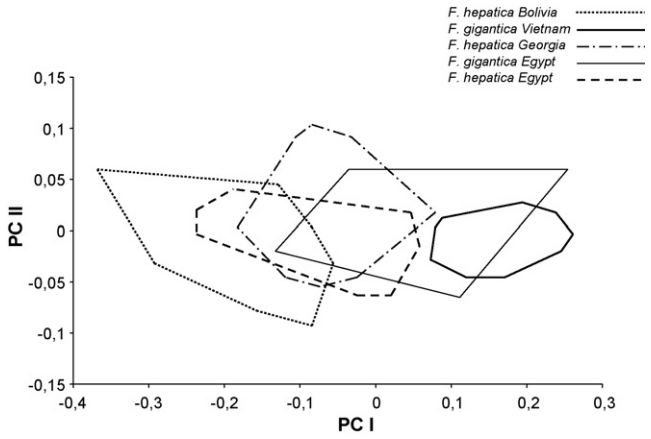

Fig. 3. Factor map corresponding to *Fasciola* eggs from naturally infected cattle from Bolivia, Georgia, Egypt and Vietnam. Samples are projected onto the first (PC1, 94%) and second (PC2, 5%) principal components. Each group is represented by its perimeter.

detected in only one egg out of 53: 1.9%) and Vietnam (only in eight eggs out of 67: 11.9%).

3.1. Study of the influence of the geographical location

The size of *F. hepatica* eggs obtained from human and animal samples are shown in Table 1. The study of the influence of the geographical location on the size of fasciolid eggs shed by humans was carried out by PC1 analysis and canonical discriminant analysis. For comparison, a similar study with the same purpose on the size of fasciolid eggs obtained in cattle infections was also carried out:

(A) *Eggs from humans*: The fasciolid egg variables from human samples from Bolivia, Peru, Georgia, Egypt and Vietnam all significantly correlated with PC1, which contributed 78% to the overall variation. The resulting factor maps (Fig. 2) clearly illustrate global size differences in the populations analysed. Two zones can be distinguished: one zone is made up of Bolivia, Peru, Georgia and Egypt, while the other zone consists only of Vietnam and overlaps with the Bolivian and Peruvian samples. The results show that Georgian and Egyptian data is included in *F. hepatica* global size. Canonical discriminant functions had statistically significant values of Wilks' λ ($P < 0.001$). The functions y_1 and y_2, including 99.4% of accumulate variance, are the following:

$$y_1 = 0.908\mathrm{EL} + 0.696\mathrm{EW} - 5.366\mathrm{EP} + 3.668\mathrm{EA} + 1.693\frac{\mathrm{EL}}{\mathrm{EW}}$$

$$y_2 = 0.825\mathrm{EL} + 1.225\mathrm{EW} - 0.401\mathrm{EP} - 2.139\mathrm{EA} + 0.684\frac{\mathrm{EL}}{\mathrm{EW}}$$

The discriminant linear functions (axes 1, y_1 and 2, y_2) clearly differentiate eggs of *F. hepatica* from Bolivia and Peru from eggs of *F. gigantica* from Vietnam. The results of the classification according to the predicted group obtained with these discriminant functions are given in Table 2. Specimens from Georgia overlap with some specimens from Bolivia, Peru, Egypt and Vietnam. Nevertheless, specimens from Egypt form a different group from either *F. hepatica* or *F. gigantica* standard specimens and only overlap with some specimens from Vietnam. The average data obtained in human samples shows very similar values in all egg measurements from Bolivia, Peru, Georgia and Egypt.

Summing up, the length and width range of *F. hepatica* eggs from humans in altitude Andean areas, where only *F. hepatica* is present, is 100.6–161.0/65.9–104.5 μm, values very close to values obtained from human samples from Georgia (104.5–162.0/72.1–96.6 μm) and from Egypt (106.5–171.5/63.9–95.4 μm), where *F. hepatica* and *F. gigantica* co-exist. The human samples from Vietnam have the highest values, 150.9–182.2/85.1–106.2 μm, overlapping with those obtained from all other human samples analysed.

(B) *Eggs from cattle*: The fasciolid egg variables from cattle samples from Bolivia, Georgia, Egypt and Vietnam all significantly correlated with PC1, which contributed 94% to the overall variation. The resulting factor maps (Fig. 3) clearly illustrate global size differences in the populations analysed. This factor map shows that, although the egg size of *F. hepatica* and *F. gigantica* from cattle does not overlap when allopatric populations from geographically extremely distant areas are compared, e.g.

Table 2
Predicted group classification results of eggs of human origin from: (1) *Fasciola hepatica*, Bolivia; (2) *Fasciola hepatica*, Peru; (3) *Fasciola hepatica*, Georgia; (4) *Fasciola sp.*, Egypt; (5) *Fasciola gigantica*, Vietnam.

	1	2	3	4	5	Total number
Original						
1	103	36	11	3	1	154
2	61	92	12	2	0	167
3	7	25	7	15	2	56
4	0	0	3	596	9	608
5	0	0	0	0	67	67
%						
1	66.9	23.4	7.1	1.9	0.6	100.0
2	36.5	55.1	7.2	1.2	0.0	100.0
3	12.5	44.6	12.5	26.8	3.6	100.0
4	0.0	0.0	0.5	98.0	1.5	100.0
5	0.0	0.0	0.0	0.0	100.0	100.0

Table 3
Predicted group classification results of eggs of cattle origin from: (1) *Fasciola hepatica*, Bolivia; (2) *Fasciola hepatica*, Georgia; (3) *Fasciola hepatica-like*, Egypt; (4) *Fasciola gigantica-like*, Egypt; (5) *Fasciola gigantica*, Vietnam.

	1	2	3	4	5	Total number
Original						
1	57	9	1	1	0	68
2	0	0	0	9	92	101
3	15	56	40	6	0	117
4	1	2	3	60	7	73
5	8	4	20	3	0	35
%						
1	83.8	13.2	1.5	1.5	0.0	100.0
2	0.0	0.0	0.0	8.9	91.1	100.0
3	12.8	47.9	34.2	5.1	0.0	100.0
4	1.4	2.7	4.1	82.2	9.6	100.0
5	22.9	11.4	57.1	8.6	0.0	100.0

Table 4
Predicted group classification results of eggs from different definitive animal host species and humans sampled in Bolivia: (1) *Fasciola hepatica*, cattle; (2) *Fasciola hepatica*, sheep; (3) *Fasciola hepatica*, donkey; (4) *Fasciola hepatica*, pig; (5) *Fasciola hepatica*, human.

	1	2	3	4	5	Total number
Original						
1	19	22	7	12	8	68
2	16	45	26	13	4	104
3	0	13	36	12	0	61
4	3	18	23	41	1	86
5	20	2	5	1	126	154
%						
1	27.9	32.4	10.3	17.6	11.8	100.0
2	15.4	43.3	25.0	12.5	3.8	100.0
3	0.0	21.3	59.0	19.7	0.0	100.0
4	3.5	20.9	26.7	47.7	1.2	100.0
5	13.0	1.3	3.2	0.6	81.8	100.0

Bolivia and Vietnam, the egg size of *F. hepatica* and *F. gigantica* overlaps when sympatric populations are compared, e.g. from Egypt. Canonical discriminant functions had statistically significant values of Wilks' λ ($P < 0.001$). The functions y_1 and y_2, including 97.7% of accumulate variance, are the following:

$$y_1 = -1.028\text{EL} + 2.228\text{EW} - 0.103\text{EP} - 1.654\text{EA} + 2.299\frac{\text{EL}}{\text{EW}}$$

$$y_2 = -3.469\text{EL} - 0.053\text{EW} - 0.300\text{EP} + 2.911\text{EA} + 3.089\frac{\text{EL}}{\text{EW}}$$

The discriminant linear functions (axes 1, y_1 and 2, y_2) clearly differentiate eggs of *F. hepatica* from Bolivia and eggs of *F. gigantica* from Vietnam. The results of the classification according to the predicted group obtained with these discriminant functions are given in Table 3. Specimens from Georgia overlap with some specimens from Bolivia and Egypt. Nevertheless, *F. gigantica* specimens from Egypt form a different group but overlap with some specimens of *F. hepatica* from Bolivia, Georgia and Egypt and *F. gigantica* from Vietnam.

3.2. Study of the influence of the host species

The study of the influence of the host species on the egg size was carried out by PC1 analysis in two geographical areas, namely Bolivia and Vietnam. *Fasciola hepatica* egg variables from the northern Bolivian Altiplano from humans and domestic animals (sheep, cattle, pig and donkey) all significantly correlated with PC1, thus contributing 86% to the overall variation. The resulting factor maps (Fig. 4) clearly illustrate global size differences in the human population analysed versus domestic animal host species, including a bigger size in eggs from humans. Canonical discriminant functions had statistically significant values of Wilks' λ ($P < 0.001$). The functions y_1 and y_2, including 95.0% of accumulate variance, are the following:

$$y_1 = 0.074\text{EL} + 1.930\text{EW} - 1.394\text{EP} - 1.224\text{EA} + 1.518\frac{\text{EL}}{\text{EW}}$$

$$y_2 = -3.915\text{EL} + 3.853\text{EW} + 0.867\text{EP} - 1.563\text{EA} + 5.366\frac{\text{EL}}{\text{EW}}$$

The discriminant linear functions (axes 1, y_1 and 2, y_2) clearly differentiate the egg population of *F. hepatica* from humans and the egg population of *F. hepatica* from other host species. The results of the classification according to the predicted group obtained with these discriminant functions are given in Table 4. Specimens from humans form a different group but overlap with some specimens either from sheep, cattle, pig or donkey. The analysis of the average of all absolute egg measurements obtained from the northern Bolivian Altiplano shows that *F. hepatica* egg average values from human samples are greater than the average values of eggs from the remaining host species. The significant differences obtained when comparing pair-wise each egg measurement from human and animal samples using the Bonferroni test are shown in Table 1. The results confirm that the *F. hepatica* egg size in human samples possesses its own morphological identity.

Fasciola gigantica egg measurements from Vietnamese humans and cattle all significantly correlated with PC1, contributing 74% to the overall variation. The resulting factor maps (Fig. 5) clearly illustrate global size differences in the human population analysed, including a global size of *F. gigantica* eggs from humans smaller than that of eggs from cattle. Canonical discriminant functions had statistically significant values of Wilks' λ ($P < 0.001$). The results of the classification according to the predicted group obtained with these discriminant functions are given in Table 5. The significant

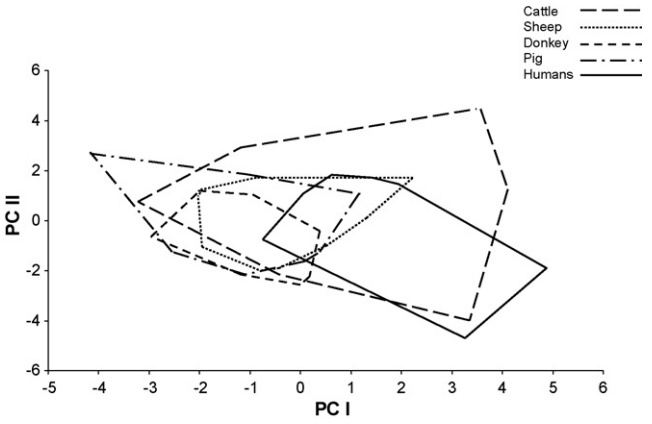

Fig. 4. Factor map corresponding to *Fasciola* eggs from humans and livestock (cattle, sheep, pig and donkey) from the same endemic area in the northern Bolivian Altiplano. Samples are projected onto the first (PC1, 86%) and second (PC2, 11%) principal components. Each group is represented by its perimeter.

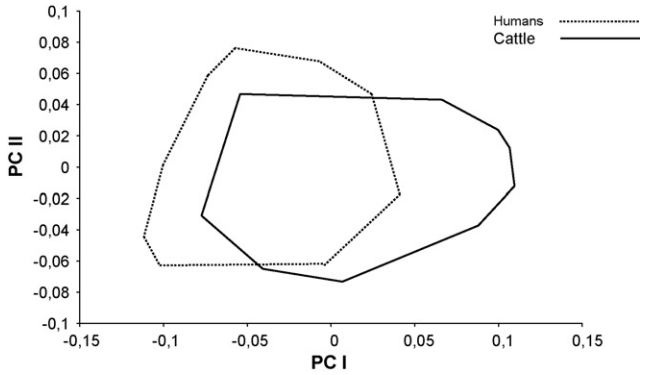

Fig. 5. Factor map corresponding to *Fasciola* eggs from humans and cattle from the same endemic area in Vietnam. Samples are projected onto the first (PC1, 70%) and second (PC2, 13%) principal components. Each group is represented by its perimeter.

Table 5
Predicted group classification results of eggs from Vietnam: (1) *Fasciola gigantica*, human; (2) *Fasciola gigantica*, cattle.

	1	2	Total number
Original			
1	67	0	67
2	6	95	101
%			
1	100.0	0.0	100.0
2	5.9	94.1	100.0

differences obtained when comparing pair-wise each egg measurement from human and animal samples using the *t*-test are shown in Table 1. The results confirm that the *F. gigantica* egg size in the human samples possesses its own morphological identity, and that *F. gigantica* eggs from human samples have smaller average values than the average values of eggs from cattle.

4. Discussion

Until the end of the 1990s, human fascioliasis was merely considered a rarely encountered disease sporadically acquired in animal endemic areas. Thus, the majority of published studies have dealt only with individual cases or family group reports. In such situations, eggs are usually present in a very small number at irregular intervals. Sometimes, repeated stool examinations might even be necessary to detect eggs (Arjona et al., 1995). However, prevalences and intensities are pronouncedly higher, especially in children, in human hyperendemic areas (Mas-Coma et al., 2005). Unfortunately, studies performed in those areas include only large scale human epidemiological surveys, but so far there is no report including an exhaustive egg morphometric analysis. Eggs of *Fasciola* species are operculated, large, light yellowish-brown, broadly ellipsoidal and non-embryonated when excreted in faeces, and their length and width are generally within a specific range, which is one of the most frequently used criteria for the diagnosis of human fascioliasis.

Classically, it is considered that at the abopercular end of the shell of *Fasciola* eggs there is often a roughened or irregular area that is not seen in *Fasciolopsis* eggs (Ash and Orihel, 1997). Nevertheless, our observations show that the frequency of the presence of this feature in *F. hepatica* is population-dependent, and therefore is not a pathognomonic criterion in diagnosis. Whether the peculiar abopercular and/or lateral irregularities in the egg shell surface observed in this study are related to the geographical origin or due to egg production abnormalities caused by human treatment (as, for instance, the usual albendazole treatments in the two Andean countries) could neither be analysed nor established.

Fascioliasis is traditionally considered a worldwide veterinary problem, and consequently the egg size range found in the literature applied to human diagnosis was in fact obtained through the analysis of samples from domestic animal hosts: *F. hepatica* 130–148/60–90 μm (Boray, 1982), 130–145/70–90 μm (WHO, 1991) or 130–150/63–90 μm (Mas-Coma and Bargues, 1997), and *F. gigantica* 150–196/90–100 μm (Boray, 1982; Mas-Coma and Bargues, 1997). Therefore, the borderlines allowing differentiation between the two species were traditionally considered to be 150 μm in length and 90 μm in width, lower values representing *F. hepatica* and higher values *F. gigantica*.

Our study reveals that humans, in comparison to other host species, have a decisive influence on the size of *F. hepatica* and *F. gigantica* eggs, showing a greater variation than the above-mentioned classic size range, i.e. in humans *F. hepatica* eggs are bigger and *F. gigantica* eggs are smaller than the classic sizes reported. Moreover, these intraspecific size variabilities overlap when both species are compared. These *Fasciola* egg-size variabilities also intersect with the size of eggs belonging to other trematode species able to infect humans and presenting a similar morphology, e.g. species of the genera *Fasciolopsis*, *Gastrodiscoides* (Mas-Coma et al., 2005) and *Echinostoma* (WHO, 1991), and may lead to confusion in egg classification when carried out by inexperienced personnel, if other diagnostic criteria are not considered (see, for example, Belisario et al., 2007).

The overlap distribution of *F. hepatica* and *F. gigantica* also led to a long ranging controversy on the taxonomic identity of the *Fasciola* species found in Far East countries, in which some resemble *F. hepatica*, whereas others resemble *F. gigantica*, with morphologically intermediate forms also being present and involving phenomena such as abnormal gametogenesis, diploidy, triploidy and mixoploidy, parthenogenesis, and hybridisation events between different genotypes (see review in Mas-Coma and Bargues, 1997). At present, the easiest way to genetically confirm that a specimen collected in nature is an hybrid is by detecting introgression. Introgression refers to the permanent incorporation of genes from one set of differentiated populations into another, i.e. the incorporation of alien genes into a new, reproductively integrated population system (Dowling and Secor, 1997; Mas-Coma and Bargues, 2009). In *Fasciola*, the existence of hybrid forms was confirmed when it was shown that Japanese fasciolids from animals presented ribosomal DNA sequences almost identical to those of one fasciolid species and mitochondrial DNA sequences almost identical to those of the other fasciolid species (Itagaki and Tsutsumi, 1998; Itagaki et al., 1998). Moreover, in Asian areas sympatric for both liver fluke species, a large overlap of egg measurements was detected (Watanabe, 1962; Sahba et al., 1972; Kimura et al., 1984; Srimuzipo et al., 2000). In Vietnam, the adult flukes found in animals and human patients fall into two morphological categories, one typical of *F. gigantica* and the other closely resembling *F. hepatica* (Le et al., 2008). Le et al. (2008) reported data indicating that hybrid/introgressed populations of *Fasciola* occur in Vietnam and that these are implicated in human infection. The egg material analysed in our study shows that the size of eggs shed by humans from Vietnam only corresponds to the *F. gigantica* morph.

Fasciola gigantica is the main fasciolid species in Africa. Morphometric values of fasciolid eggs from European and African bovines were analysed by Periago et al. (2006) using CIAS. The results showed that the range of egg measurements intersects between *F. hepatica* and *F. gigantica* allopatric populations (EL: 107.3–152.7 μm in *F. hepatica* from Spain, 100.2–155.6 μm in *F. hepatica* from the French island of Corsica, and 129.6–204.5 μm in *F. gigantica* from Burkina Faso; EW: 52.4–89.1 μm in *F. hepatica* from Spain, 55.0–87.7 μm in *F. hepatica* from Corsica, and 61.6–112.5 μm in *F. gigantica* from Burkina Faso). In Egypt, both *F. hepatica* and *F. gigantica* are present, but usually the terminology *Fasciola* sp. is used for human fascioliasis in the Nile Delta (Esteban et al., 2003; Curtale et al., 2007). Phenotypic *F. hepatica*/*F. gigantica* intermediate forms of liver fluke adults from this human endemic area have been described in cattle, and the *F. hepatica* morph appears to be more frequent than the *F. gigantica* morph in this animal species (Periago et al., 2008). This fact agrees with our results since the size of eggs shed by humans from Egypt corresponds to the *F. hepatica* morph.

In conclusion, measurements of *F. hepatica* and *F. gigantica* eggs obtainable with a microscope such as EL, EW and EL/EW, originating from both humans and animals, partly overlap in areas where both fasciolid species co-exist, such as in Egypt, and therefore caution must be taken when using egg size as the only criterion for differential diagnosis (Table 1). A similar conclusion is reached when comparing other measurements obtainable by CIAS such as EP, ER and EA (Table 1). In this sense, our findings should aid clinicians in these areas since the application of the classic egg size range in human samples may lead to erroneous conclusions.

The measurements of the fluke eggs found in the stools shed by a Japanese patient (Inoue et al., 2007) constitute a good example to illustrate the problem here in question. The living fluke removed by balloon extraction after endoscopic sphincteromy showed morphometric characteristics fitting *F. hepatica*. The ribosomal DNA ITS-2 and ITS-1 sequences (fragments of 327 and 351 bp of the complete 364 and 432 bp spacer sequences, respectively) obtained from a fluke egg pull isolated from the patient's faeces were also in agreement with this fasciolid species. The mitochondrial COI sequence (unfortunately only a short 493-bp fragment and not the complete 1533-bp gene sequence) obtained was also close to *F. hepatica*.

Although all these features indicated that the parasite was *F. hepatica*, the egg size of 153–175/75–95 μm (average 164.7/83.7 μm) (Inoue et al., 2007) did not fit the egg size characteristics traditionally considered for *F. hepatica* (130–150/63–90 μm), but it did for *F. gigantica* (150–196/90–100 μm) (Mas-Coma and Bargues, 1997). When compared with the new egg size data reported for human samples in the present study, the size of the Japanese eggs fits well with the egg size noted for *F. hepatica* in areas where both fasciolid species overlap, except for the maximum range for the egg length (175 μm), which very slightly surpasses that detected in humans from Egypt (171.5 μm). The egg width average of the Japanese fluke (83.7 μm) is closer to that of *F. hepatica* in Peruvian (80.2 μm) or Georgian subjects (82.1 μm), and the minimum range for the Japanese egg width (75 μm) is pronouncedly smaller than the minimum egg width (85.1 μm) noted for *F. gigantica* in Vietnamese subjects. This comparison suggests that the Japanese fluke was probably a specimen from a long-term originated, introgressed lineage in which the introgression was not detectable in the short COI sequence fragment obtained. It shall be taken into account that introgression may involve the substitution of only a part of the mitochondrial genome, not necessarily of all mtDNA genes, and that an accumulation of many genetically different introgressed hybrid forms resulting from back-crossing may be expected in an area of overlap (Mas-Coma and Bargues, 2009).

Fasciolid egg size in human stool samples ought to be corrected in books and monographs related to medical parasitology and/or tropical medicine as well as in guides for clinicians and diagnosis analysts. When dealing with human fascioliasis globally, *F. hepatica* egg length/width data should be 100.6–162.2/65.9–104.6 μm in human stools and 73.8–156.8/58.1–98.1 μm in animal faeces when referring to areas where *F. gigantica* is absent (as in the Americas and Europe). When referring to areas where both fasciolid species are present (as in many parts of Africa and Asia), *F. hepatica* egg length/width data should be 106.5–171.5/63.9–95.4 μm in human stools and 120.6–163.9/69.2–93.8 μm in animal faeces, and *F. gigantica* egg length/width data should be 150.9–182.2/85.1–106.2 μm in human stools and 130.3–182.8/74.0–123.6 μm in animal faeces. For areas where both fasciolid species co-exist, the size of fasciolid eggs shed by humans may be intermediate between the above-mentioned data for *F. hepatica* and that for *F. gigantica* in humans in overlapping areas and such situations might be interpreted as infections by intermediate or hybrid forms. With regards to *F. gigantica*, egg length/width data should also be increased to 129.6–204.5/61.6–112.5 μm in animal faeces when referring to areas where *F. hepatica* is absent (as in great parts of Africa). Future studies in other areas may perhaps modify the above-mentioned maximum and minimum size measurements for fasciolid eggs shed in human stools, but the present exhaustive study suggests that only very slight differences may be found, if any.

Finally, it has to be added that in endemic areas of Asia and Africa where both fasciolid species overlap, a human subject may be simultaneously infected by fluke adults belonging to both *F. hepatica* and *F. gigantica*. Consequently, an appropriate coprological diagnosis in such an area shall not rely on the measurement of only one or very few eggs. Whenever possible, measuring a sufficient amount of eggs to appropriately assess egg size variability could facilitate the verification of whether only one or the other, or perhaps both species simultaneously have infecting the patient. Moreover, it has to be considered that, if fluke adults of both species co-infect the patient, intermediate hybrid eggs may additionally appear in stools in case some flukes have crossbred.

Conflict of interest

All authors declare no conflicts of interest.

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Short communication

MM3-ELISA evaluation of coproantigen release and serum antibody production in sheep experimentally infected with *Fasciola hepatica* and *F. gigantica*

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ABSTRACT

During an experimental infection of sheep with *Fasciola hepatica* or *F. gigantica*, MM3-SERO and MM3-COPRO ELISA tests were applied to compare the kinetics of antibody production and coproantigen release between the 2nd and 32nd week post-infection (wpi). The Kato-Katz technique was used to measure the kinetics of egg shedding by both *Fasciola* species (eggs per gram of feces, epg). The kinetics of IgG antibodies for all sheep infected with *F. hepatica* and *F. gigantica* followed a similar pattern. Optical density (OD) increased rapidly between the 4th until the 12th wpi, when the highest values were reached and then decreased slowly until the 32nd wpi. Coproantigen levels increased above the cut-off value between 6 and 9 wpi in the *F. hepatica* group, and between 9 and 11 wpi in the *F. gigantica* group. The comparison between coproantigen levels and epg indicated that *F. hepatica*-infected sheep had detectable amounts of coproantigens 4–7 weeks before patency (egg shedding), while *F. gigantica*-infected sheep had detectable amounts of coproantigens 3–6 weeks before patency. When comparing the kinetics of coproantigen release vs the kinetics of epg, a similar pattern emerged, but with a two-week time-lag in epg, for both *F. hepatica* and *F. gigantica* infections. The amount of coproantigen release by each adult was not burden dependent for *F. hepatica* infection (burden of 33–66 adults), while it was for *F. gigantica* infection (burden of 17–69 adults). The results demonstrate the usefulness of the MM3-SERO and MM3-COPRO ELISAs as tools for the diagnosis of early as well as long-term fascioliasis infections, and suggest that they can potentially be applied to human fascioliasis even in countries where *F. hepatica* and *F. gigantica* co-exist. These tests can be employed not only in the diagnosis, but also in studies on epidemiology as well as pathogenesis and treatment in animals and humans since they allow post-treatment infection monitoring.

1. Introduction

Fasciola hepatica and *F. gigantica* are two trematode species having an important impact on public health due to the infections they cause in humans and livestock. *F. hepatica* has a cosmopolitan distribution, mainly in

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temperate zones, while *F. gigantica* is found in tropical regions of Africa and Asia (Torgerson and Claxton, 1999; Mas-Coma et al., 2005). Although the majority of cases is attributed to *F. hepatica*, human infections with *F. gigantica* are also present in many countries (Ashrafi et al., 2006a,b; Le et al., 2007).

The diagnosis of fascioliasis is complicated due to the liver fluke's biological cycle within the definitive host. After ingestion of metacercariae, juvenile worms migrate through the intestinal wall to the peritoneal cavity, penetrate the liver parenchyma through which they migrate (time depends on the host species), and pass into the biliary tract, where they ultimately reach maturity and start oviposition. Consequently, eggs are present in stool several weeks after the ingestion of metacercariae.

Immunodiagnosis has traditionally been used in animal and human fascioliasis, and numerous methods and techniques have been developed for the detection of *F. hepatica* infection (see global review in Hillyer, 1999). The majority of methods based on coproantigen detection are applied to *F. hepatica* infection, but only few are applied to *F. gigantica* infection (Endah Estuningsih et al., 2004). The MM3-SERO and MM3-COPRO ELISA tests developed by Mezo et al. (2004, 2007) to detect serum antibodies and fecal antigens have shown to be highly specific, sensitive and simple for the diagnosis of acute and chronic *F. hepatica* infections in cattle and sheep. However, the usefulness of these methods for diagnosis of *F. gigantica* infections has never been investigated. In the present study, these two tests are evaluated by analyzing the kinetics of antibody production and coproantigens released during an experimental infection of sheep with *F. gigantica* and *F. hepatica* for comparative purposes.

2. Materials and methods

2.1. Experimental procedures

F. hepatica and *F. gigantica* metacercariae were experimentally obtained in the Department of Parasitology of the University of Valencia as previously described (Mas-Coma et al., 2001). *Fasciola gigantica* and its snail host *Lymnaea callaudi* originated from Egypt, while *F. hepatica* and the lymnaeid *Galba truncatula* originated from Spain.

The experimental group consisted of 12 four-to-five-week-old sheep (Guirra autochthonous breed) divided into three groups of four animals each. Two groups were infected *per os* with 200 *F. hepatica* or *F. gigantica* metacercariae, respectively, and the third group was used as control. Food and water were provided *ad libitum*. Permission for animal research was obtained from the Ethics Animal Research Committee of the University of Valencia. Animal ethics guidelines were strictly adhered to in the care of the animals. Post-mortem examinations were carried out on all animals. The bile ducts and liver parenchyma were examined and the flukes inside were collected.

2.2. Fecal samples

Fecal pellets were collected fresh from each animal on the day of infection and once a week, starting at 5 weeks post-infection (wpi). Fecal egg detection was carried out by analyzing 3–8 Kato-Katz slides (Helm-Test®, AK test, AK Industria e Comércio Ltda, Belo Horizonte, Brazil) per weekly sample (Valero et al., 2002). The average egg output per day was calculated as eggs per g of feces (epg). For coproantigen determination, aliquots of each fecal sample were stored frozen at −20 °C until use.

2.3. Serum samples

Whole blood was collected from the external jugular every 2 weeks from the day of infection (week 0) until 32 wpi. The blood samples were centrifuged at 760 *g* at 4 °C for 10 min, and the obtained serum samples were stored at −80 °C until analysis.

2.4. ELISA procedures

Coproantigen detection in fecal samples was carried out using the MM3-COPRO ELISA test following the procedure described by Mezo et al. (2004). The presence of anti-*Fasciola* IgG antibodies in serum from infected sheep was determined using the MM3-SERO capture ELISA following the procedure described by Mezo et al. (2007). Commercial versions of MM3-COPRO (BIO K 201) and MM3-SERO (BIO K 211) are available from Bio-X Diagnostics (La Jemelle, Belgium).

2.5. Statistical analysis

Each assay was repeated twice, and the results were expressed as the mean OD_{492} of each determination. Fluke *F. hepatica* vs *F. gigantica* burden was compared by the non-parametric Mann–Whitney test. IgG levels in *F. hepatica* and *F. gigantica*-infected sheep vs control were compared using the *T* test. Bivariant correlations (Pearson's correlation) were calculated for coproantigen levels vs epg values corresponding to a two-week time-lag in both *F. hepatica* and *F. gigantica* sheep groups. The relationship between antigen concentrations (ng/ml) divided by the parasitic burden vs parasitic burden in both *F. hepatica* and *F. gigantica* infection was analyzed by linear and curvilinear regressions. Egg counts were conducted after converting individual values to logarithms ($\log e$) with the $(n + 1)$ transformation [ln(epg)] (Stephenson, 1987). Statistical analyses were carried out with SPSS 15 and Origin 6.0 (Microcal Software, Inc., Northampton, MA, USA). Results were considered statistically significant when $p < 0.05$.

3. Results

Experimental monitoring confirmed that all sheep infected with metacercariae developed a liver-fluke infection. In the *F. hepatica* infection group, the fluke recovery percentage with respect to the number of metacercariae inoculated ranged from 16.5% to 33%. The egg shedding prepatent period (PP) ranged from 10 to 14 wpi (12.3 ± 1.7). One *F. gigantica*-infected sheep was put down in the 13th wpi due to development of clinical fascioliasis, presenting a fluke recovery of 25%, and a PP of 11 wpi. In the rest of the *F. gigantica*-infected sheep the fluke

recovery percentage ranged from 8.5% to 34.5%, and the PP ranged from 14 to 16 wpi (15.0 ± 1.0). The *F. hepatica* burden recovered at necropsy oscillated between 33 and 66 adult flukes, with a mean ± SD of 51.5 ± 13.7. The *F. gigantica* burden oscillated between 17 and 69 adult flukes (44.0 ± 21.6), without any significant differences between the two groups (Mann–Whitney test, $p < 0.05$).

The kinetics of IgG antibodies recognizing the antigen captured by mAb MM3 was studied every two weeks between the 2nd and 32nd wpi. The kinetics of antibodies for all sheep infected with *F. hepatica* and *F. gigantica* followed the same pattern. OD increased rapidly between the 4th and the 12th wpi, when the highest values were reached, and then decreased slowly until the 32nd wpi (Fig. 1). OD values for control sheep always remained below the cut-off value. Using the *T* test, significant differences between infected and control sheep were assessed ($p < 0.05$).

The kinetics of coproantigens recognized by mAb MM3 was studied weekly between the 2nd and 32nd wpi. In the *F. hepatica* group, coproantigen levels increased quickly above the cut-off, reaching higher OD values, although showing strong week-to-week fluctuations in the course of the experiment (Fig. 1). The comparison between coproantigen levels and fecal egg counts (epg) indicated that *F. hepatica*-infected sheep had detectable amounts of coproantigens between the 6th and the 9th wpi (at 6 wpi coproantigens were detected in 25% of sheep, at 7 wpi in 50%, at 8 wpi in 75%, and at 9 wpi in 100%), whereas eggs began to appear in feces from weeks 10 to 14 (at 10 wpi eggs were detected in 25% of sheep, at 11 wpi in 25%, at 12 wpi in 50%, at 13 wpi in 75% and 14 wpi in 100%). Comparing both methods, the MM3 test allowed detection of infection four to seven weeks before PP. Comparatively, the kinetics of coproantigen release vs the kinetics of egg shedding showed a similar pattern but with a two-week

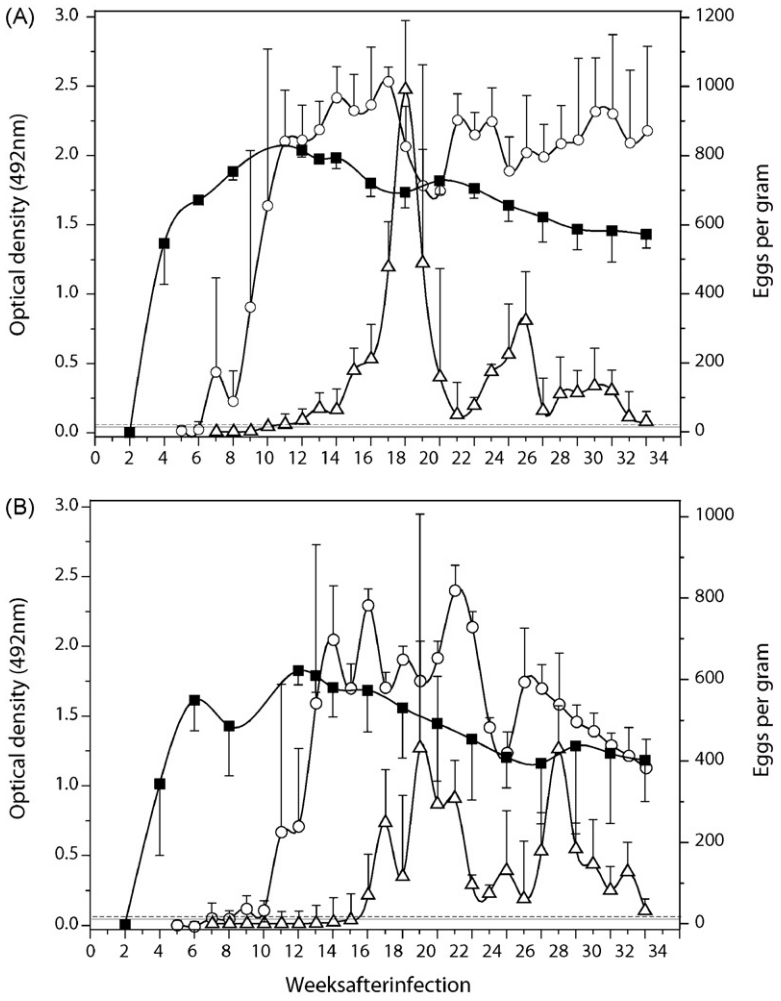

Fig. 1. Mean OD values (±SD) obtained in MM3-SERO (closed squares), MM3-COPRO (open circles) and eggs per gram of feces (open triangles) from experimentally infected sheep at different times after infection (weeks). (A) *Fasciola hepatica* infection. (B) *Fasciola gigantica* infection. OD values obtained with MM3-SERO and MM3-COPRO for sera and fecal samples from uninfected controls maintained below the cut-off values during the entire experiment. Cut-off value for MM3-COPRO (OD = 0.082) was calculated as 4 standard deviations (SD) from the mean absorbance values for the fecal samples obtained from the same animals used in this study during the first 4 wpi. Cut-off value for MM3-SERO (DO = 0.074) was previously established (Mezo et al., 2007). Cut-off values for MM3-COPRO and MM3-SERO ELISAs are represented as dashed and continuous horizontal lines, respectively.

time-lag in epg (Fig. 1). To verify this fact, a significant positive correlation ($r = 0.92$, $p < 0.05$) was observed when contrasting coproantigen levels and the epg values of two weeks later.

Concerning the *F. gigantica*-infected group, it is noteworthy that the coproantigen detection took place precociously at 7 wpi for the sheep that was put down in the 13th wpi. In the remaining groups, coproantigen release increased quickly until maximum values were reached. Similar to *F. hepatica*, OD values showed strong week-to-week fluctuations along the experiment (Fig. 1). The comparison between coproantigen levels and fecal egg counts (epg) indicated that *F. gigantica*-infected sheep presented detectable amounts of coproantigens from the 7th to the 11th wpi (at 7 wpi coproantigens were detected in 25% of sheep, at 8 wpi in 25%, at 9 wpi in 50%, at 10 wpi in 75%, and at 11 wpi in 100%), whereas eggs began to appear in feces from weeks 11 to 16 (at 11 wpi egg detection was possible in 25% of sheep, at 12 wpi in 25%, at 13 wpi in 25%, at 14 wpi in 50%, at 15 wpi in 75%, and at 16 wpi in 100%). Comparing both methods, the MM3 ELISA test allows detection of *F. gigantica* infection 3–6 weeks before PP. The comparison of the kinetics of fecal excretion of the antigens vs the kinetics of egg shedding showed a similar pattern but with a two-week time-lag in epg, similar to that seen in the *F. hepatica*-infected group (Fig. 1). A significant positive correlation ($r = 0.89$, $p < 0.005$) was also observed when contrasting coproantigen levels and the epg values of 2 weeks later.

For *F. hepatica* no significant correlation was observed between the amount of the coproantigen released per fluke and parasite burden in any of the correlation models tested. In contrast, in *F. gigantica* infection, the exponential model $y(x) = y_0 + a\exp(-x/b)$, where $y_0 = 2.029 \pm 0.625$, $a = 66.122 \pm 3.229$, $b = 18.426 \pm 1.297$ ($R^2 = 0.999$; $p < 0.001$) provided a suggestive description of the inverse relationship between the coproantigen released per fluke and parasitic burden.

4. Discussion

The main goal of the present study is to evaluate the application of MM3-SERO ELISA and MM3-COPRO ELISA for the diagnosis of *F. gigantica* infection. Our study shows that MM3-SERO ELISA detects the presence of IgG antibodies in sheep infected with 200 metacercariae of either *F. gigantica* or *F. hepatica* at 4 wpi. Our results also show that the kinetics of IgG levels follow the same pattern regardless of the *Fasciola* species, although their patency periods vary. Anti-*Fasciola* IgG antibodies are detected in all infected animals as early as 4 wpi, and reach their maximum values around the 12th wpi. These results are in agreement with other studies on fluke-specific IgG (Santiago and Hillyer, 1988; Chauvin et al., 1995; Moreau et al., 1998; Zhang et al., 2004; Raadsma et al., 2007). Comparatively, it is also observed that the anti-Fasciola IgG response is slightly lower in the animals infected with *F. gigantica,* which can be explained by small antigenic differences between the species-specific antigens recognized by mAb MM3, or by differences in the kinetics of each antigen released by each species.

Similar to *F. hepatica* (Mezo et al., 2007), MM3-COPRO ELISA proves to be useful for monitoring coproantigen release in feces of sheep infected with *F. gigantica*. In our study, the first detection of *F. gigantica* coproantigens took place 3–6 weeks before the appearance of eggs in feces, which was similar to the 4–7 weeks observed for *F. hepatica*. Moreover, coproantigen release was maintained along the entire duration of the experiment (32 wpi). These results underline the usefulness of the MM3-COPRO ELISA test for the diagnosis of both early and long-term fascioliasis in endemic *F. gigantica* areas. Similar to our results, several studies have already shown that the detection of *F. hepatica*/*F. gigantica* coproantigens through ELISA is prior to patency, and with a marked increase in the levels of these antigens around the beginning of the fecal egg output (Espino et al., 1998; Moustafa et al., 1998; Endah Estuningsih et al., 2004).

In *F. hepatica* infection, the maturation time of juvenile flukes in the bile duct and the initiation of egg laying may depend on the infection dose (see review in Valero et al., 2006). In this sense, it was reported that in sheep infected with 200 metacercariae, the egg shedding patency period was 63 days, whereas in those with heavy infections (i.e. infected with 2000 metacercariae) eggs appeared 13–15 weeks after ingestion (Boray, 1969). The crowding effect becomes manifest through a delayed fluke migration from the liver parenchyma into the common bile duct. Using a dose of 200 metacercariae, a density-dependence phenomenon for the coproantigen release by *F. gigantica* can be observed. However, this is not the case for *F. hepatica,* which might be related to the greater individual biomass of the former.

The results of the present study show the suitability of MM3-SERO and MM3-COPRO for the diagnosis of *F. hepatica* and *F. gigantica* infections in sheep, which are also of interest in human infections. Once confirmed for human use, MM3-SERO and MM3-COPRO tests may potentially be applied in human fascioliasis endemic areas, even in countries where *F. hepatica* and *F. gigantica* co-exist. It is expected that these tests may be useful not only for individual diagnosis in hospitals, but also for human surveys in epidemiological studies as well as for analyses on pathogenesis, treatment efficiency and surveillance as they allow the continuous monitoring of the infection status.

Conflicts of interest

The authors declare no conflicts of interest.

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MM3-ELISA Detection of *Fasciola hepatica* Coproantigens in Preserved Human Stool Samples

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Abstract. In this study, we evaluate the MM3-COPRO method for detection of *Fasciola* coproantigens in human fecal samples, and the usefulness of a new preservative/diluent, CoproGuard, developed for preservation of *Fasciola* coproantigens. The MM3-COPRO assay was evaluated with 213 samples from healthy patients, 30 *Fasciola* positive fecal samples (according to the Kato-Katz method), and 83 samples from patients with other parasitic infections. All *Fasciola* positive specimens were detected with the MM3-COPRO assay (100% sensitivity) and there was no cross-reactivity with other common parasites present in the clinical specimens analyzed (100% specificity). The use of CoproGuard enhanced coproantigen extraction without affecting the detection limit of the assay, and the antigenicity of *Fasciola* coproantigens in fecal samples stored at 37°C was retained throughout the entire observation period (120 days). We concluded that the MM3-COPRO ELISA combined with the use of CoproGuard may be a very useful tool for the diagnosis of human fascioliasis.

INTRODUCTION

The genus *Fasciola* includes the species *F. hepatica* and *F. gigantica*, both of which can cause infections in humans and animals.[1,2] In general, human fascioliasis is less frequent than animal fascioliasis, but the former is re-emerging, with reported cases in more than 51 countries throughout the world.[3–5] Indeed, fascioliasis is recognized as a major public health problem by the World Health Organization (WHO), with an estimated 17 million people infected worldwide,[4,6,7] and 180 million at risk from infection.[8]

Although damage depends on the number of infecting flukes,[9,10] this trematode can produce considerable pathology, which is first provoked by young flukes migrating through the liver parenchyma (acute phase), and then by the adults established in the biliary ducts (latent/chronic phase).[11] Pathologic changes have also been observed in the gallbladder, which may sometimes harbor adult flukes.[12,13]

Human fascioliasis may be suspected after examination of epidemiologic and clinical data. During this phase the illness may present with prolonged fever, epigastric and right upper quadrant pain, hepatomegaly, urticaria, leukocytosis, mild eosinophilia to hypereosinophilia, and anemia.[3,12,14,15] In addition, the computer tomography (CT) scan may reveal single or multiple hypodense liver lesions that change over time on sequential scans.[15–17] The latent/chronic phase of human fascioliasis is frequently asymptomatic.[18] However, when present, several clinical, laboratory, and radiologic findings related to biliary obstruction and cholangitis can be observed. These include upper abdominal pain, intermittent jaundice, intrahepatic cystic abscesses with prolonged fever, eosinophilic cholecystitis, and extrahepatic cholestasis with elevation of liver enzymes.[15]

Several coprologic techniques for showing the presence of eggs in stools have long been used by various authors to confirm the diagnosis of human fascioliasis. However, these methods are often unreliable because parasite eggs are not found in feces until 3–4 months after infection,[14] and their low sensitivity to low-burden infections—which limits their clinical application—is recognized.[3,18] The enzyme-linked immunosorbent assay (ELISA) methods developed for determination of *Fasciola* coproantigens in stool samples from animals and humans provide an alternative to coprologic examination.[19,20] One of these methods is MM3 capture ELISA (MM3-COPRO), which we previously showed to be useful for detection of *Fasciola hepatica* and *Fasciola gigantica* coproantigens in experimental and natural *Fasciola* infections of sheep and cattle.[21,22] This test proved to be highly sensitive (confirmed by necropsy) and specific (no cross reaction was observed with antigens from other helminths), and enabled detection of *Fasciola* infections 1–5 weeks before patency.[21] Furthermore, other researchers recently tested a commercial version of the test, and its usefulness for detection of *F. hepatica* infections in cattle was confirmed under field conditions.[23] However, until now MM3-COPRO has not been evaluated for coproantigen measurement in fecal samples from infected humans.

MM3-recognized *Fasciola* coproantigens in human stools are probably more concentrated than in cattle, but determination of these antigens may be complicated by the action of proteases in the samples, or by the presence of substances that may interfere in the capture ELISA, such as lipids, antibodies, salts, organic residues, and other compounds that may be present in different proportions in human and animal fecal samples. In the present study, we evaluated the usefulness of the MM3-COPRO method for detection of *Fasciola* coproantigens in human stools,[21] and also tested the validity of a new fecal preservative (CoproGuard) specifically developed to prevent degradation of the MM3-recognized *Fasciola* coproantigens in stool samples over time.

MATERIAL AND METHODS

Fecal samples. Parasitologically negative fecal samples (213 samples), positive fecal samples containing parasites other than *Fasciola* (83 samples; see Table 1), and positive fecal samples containing different amounts of *Fasciola* eggs (30 samples) were obtained from the *Complejo Hospitalario Universitario de Santiago de Compostela* (Service of Microbiology) and from the *Departamento de Parasitología, Facultad de Farmacia,*

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TABLE 1
Parasite content in the fecal samples ($N = 113$) used to evaluate the sensitivity and specificity of MM3-COPRO ELISA*

	F. hepatica	Protozoa	*S. stercoralis*	*H. nana*	*A. lumbricoides*	*E. vermicularis*	*T. trichiura*	TOTAL
Fasciola hepatica	30							30
Protozoa		35						35
Strongyloides stercoralis		1	2					3
Hymenolepis nana		12		7				19
Ascaris lumbricoides		3		2†	11			16
Enterobius vermicularis				1†		4		5
Trichuris trichiura		1			2		2	5
TOTAL	30	52	2	10	13	4	2	113

*The presence of oocysts of *Cryptosporidium parvum* and/or cysts and trophozoytes of *Giardia intestinalis* or *Entamoeba histolytica* was referred to here as "protozoa." (†): indicates the presence of *Entamoeba coli* cysts in the samples.

Universidad de Valencia, Spain, respectively. The presence of parasite forms in fecal samples was tested by microscopy by 1) direct examination of fresh stools, 2) the phenol-auramine stain,[24] 3) the Kato-Katz method (see later), and 4) the sodium acetate-acetic acid-formalin (SAF)-ethyl acetate sedimentation technique as recommended by the manufacturer of the Para-Pack Plus SAF specimen collection kit (Meridian Biosciene Europe, Nice, France). To avoid possible cross-reactions with other parasites, mixed infections of *Fasciola* and other parasites were not included in the study. The *F. hepatica* positive and negative samples used in this study were collected during a 5-year period (2004–2008) and stored frozen at –20°C without addition of preservatives. All the *F. hepatica* positive samples corresponded to chronic cases of fascioliasis of an unknown period of evolution. Eleven samples were obtained from adult patients (> 18 years of age; range: 19–77 years) and 19 samples were obtained from young patients (< 18 years of age; range: 7–17 years). The overall mean age was 23.5 years of age (SD = 19.16).

Kato-Katz determinations. Eggs were detected in fresh stools after analysis of 3–8 Kato-Katz slides (Helm-Test, AK test, AK Industria e Comércio Ltda, Belo Horizonte, Brasil) per sample, depending on the concentration of *Fasciola* eggs. The average egg output was calculated as eggs per g of feces (epg).

Processing of fecal samples for ELISA determinations. To research the conditions of coproantigen extraction, a pool of 5 parasitologically negative fecal samples was mixed with *F. hepatica* excretory-secretory antigens (ESAs), obtained from adult cultured *Fasciola*, as previously described.[21] The sample-antigen mixtures were then homogenized with the aid of a spatula, and diluted 1:10 v/v in distilled water or in CoproGuard solution to provide fecal suspensions of final concentrations of 10, 25, or 50 ng/mL diluent. The samples were then resuspended on a vortex mixer, and centrifuged for 10 min at 1,500 *g*. Finally, the supernatants were collected and stored at –20°C until analysis by MM3-COPRO ELISA. The CoproGuard preservative contains biocidal substances, proteins, and surfactants, and is available from the Laboratorio de Parasitología, Universidad de Santiago de Compostela, Spain.

To test the stability of *Fasciola* coproantigens in fecal samples, aliquots of 5 individual positive human stools were diluted 1:10 v/v with 1) distilled water; 2) 0.2% sodium azide (VWR International, Barcelona, Spain) in distilled water; 3) 0.03% thimerosal (VWR International) in distilled water, or 4) CoproGuard. All of the samples were resuspended on a vortex mixer, centrifuged at 1,500 *g* for 10 min and the supernatants divided in aliquots, which were stored at 4°C, room temperature (RT), frozen at –20°C, or at 37°C (to accelerate degradation of the fecal samples diluted in CoproGuard). Finally, the stored samples were assayed in duplicate at regular intervals by use of the MM3-COPRO ELISA. The results were expressed as percentage of optical density (OD) obtained for each test sample with respect to the OD obtained for control samples stored frozen at –20°C.

To evaluate the conditions of incubation for the MM3-COPRO ELISA, a pool of 5 negative fecal samples were mixed with *F. hepatica* ESAs and diluted 1:10 in CoproGuard to a final concentration of 25 ng/mL. The coproantigen was then extracted as described previously, and subjected to MM3-COPRO ELISA under several incubation conditions. The following times and temperatures were considered: 1 hr at RT, 2 hr at RT, and one night at 4°C. The effect of orbital shaking (100 rpm) was also tested.

MM3-COPRO ELISA detection of *Fasciola hepatica* coproantigens in fecal samples. The MM3-ELISA test was performed as previously described with some modifications.[21] Polystyrene microtiter 1 × 8 F strip plates (Greiner Bio-One GmbH, Frickenhausen, Germany) were coated overnight with 100 µL/well of a solution containing 10 µg/mL of rabbit anti-*Fasciola* polyclonal IgG antibody in phosphate buffered saline (PBS) (wells from odd-numbered rows), or with 100 µL/well of a solution containing 10 µg/mL of IgG polyclonal antibodies from non-immunized rabbits (wells from even-numbered rows). Uncoated sites were blocked with 1.5% of sodium caseinate in (PBS) for 1 hr at RT, and each fecal supernatant (100 µL) was then added in quadruplicate (2 odd-numbered wells plus 2 even-numbered wells), and incubated overnight at 4°C. After washing 6 times with PBS containing 0.2% Tween-20 (PBS-T), 100 µL of a solution containing 0.3 µg of biotinylated MM3 antibodies in PBS-T plus 1% bovine serum albumin (PBS-T-BSA) was added to each well and incubated for 1 hr at 37°C. After washing as above, bound MM3 antibody was detected by incubation, first with peroxidase-conjugated neutravidin (Pierce, Rockford, Illinois; dilution 1:2000 in PBS-T-BSA) for 1 hr at 37°C, and then with the substrate (buffered H_2O_2 and o–phenylenediamine [OPD], Sigma-Aldrich, Madrid, Spain). After incubation for 20 min at RT, the reaction was stopped by addition of 3 N H_2SO_4. Finally, the optical density (OD) was measured at 492 nm. The OD value for each sample was calculated as OD1–OD2, where OD1 is the mean for the 2 even-numbered wells (coated with anti-*Fasciola* polyclonal antibodies), and OD2 is the mean for the 2 odd-numbered wells (coated with irrelevant polyclonal antibodies).

Limit of detection of the assay. The limit of detection of the MM3-COPRO ELISA was calculated by testing serial dilutions of known amounts of *F. hepatica* ESAs in

CoproGuard. Protein concentrations were determined with the MICRO BCA Protein Assay Kit (Pierce), with the BSA internal standard provided in the kit. The OD values obtained were used to produce a calibration curve with a Sigmoidal Logistic function (Microcal Origin 6.0 software, Microcal Software, Inc., MA), with which the MM3-recognized coproantigen concentrations in stool samples were calculated. The concentration value obtained at the intersection point of the standard curve with the cut-off value of the MM3-COPRO ELISA was considered as the limit of detection of the assay. The concentrations of ESAs assayed ranged from 0.27 to 400 ng/mL. The cut-off value was calculated as the mean OD plus 4 standard deviations from the OD values obtained with the 213 coprologically negative fecal samples.

The antigen concentrations in positive fecal samples were calculated by interpolating the mean OD values obtained in the MM3-COPRO ELISA in the standard logistic curve described previously (see also Figure 4).

Statistics. Multiple comparisons between groups were made with the Tukey-Kramer Multiple Comparison Test available in the Microcal Origin 6.0 software package. The relationship between *Fasciola* egg output (Kato-Katz values) and OD values, measured by the MM3-COPRO test, was calculated by a linear correlation test with the same software package.

Ethical aspects. The present study was approved by the Ethics Committee of the University of Santiago de Compostela.

RESULTS

MM3-COPRO ELISA parameters. The results presented in Figure 1 showed that the yield of coproantigens extracted from stool samples with CoproGuard was higher than classic extraction with distilled water, for all concentrations tested ($P < 0.01$). As expected, the results also showed that the use of shaking increased the OD values obtained when fecal samples were incubated at RT ($P < 0.01$), but that this procedure was not relevant when samples were incubated overnight at 4°C (Figure 2). In addition, we did not observe any significant differences in OD values among samples incubated for 1 or 2 hours at RT.

Stability of the *F. hepatica* coproantigens in preserved and non-preserved human fecal samples, and limit of detection of the MM3-COPRO ELISA. The stability of *Fasciola* coproantigens in human fecal samples was investigated in 5 Kato-Katz *F. hepatica* positive samples (range 7.1 to 25.1 ng/mL, determined at 1:10 v/v dilution in CoproGuard) maintained under different preservation conditions, including refrigeration and the use of biocides. The stability of the antigen was observed during a period of 5 weeks, except for samples preserved in CoproGuard, which were observed for 17 weeks (Figure 3). Comparison of the different preservation conditions revealed that even when maintained at 37°C, only the antigenicity of coproantigens in the samples diluted with CoproGuard did not vary throughout the observation period (Figure 3). In contrast, biocides such as sodium azide and thimerosal did not preserve the antigenicity, as the start signal decreased to approximately 30% by the end of the observation period. When the samples were maintained at 4°C, the *F. hepatica* coproantigens retained about 70% of their initial antigenicity after 5 weeks. Significant differences ($P < 0.01$) between the OD values obtained for samples stored at 4°C and those obtained for samples stored with either sodium azide or thimerosal were observed. The OD signal produced by samples stored in CoproGuard at 37°C tended to be higher than that produced by the corresponding frozen controls (Figure 3); this suggests that extraction of the *F. hepatica* antigen from the fecal particles is facilitated at the higher temperature.

With respect to the total protein content in the *F. hepatica* ESAs, a detection limit of 1.1 ng/mL was obtained for *F. hepatica* coproantigens diluted in CoproGuard, as deduced from

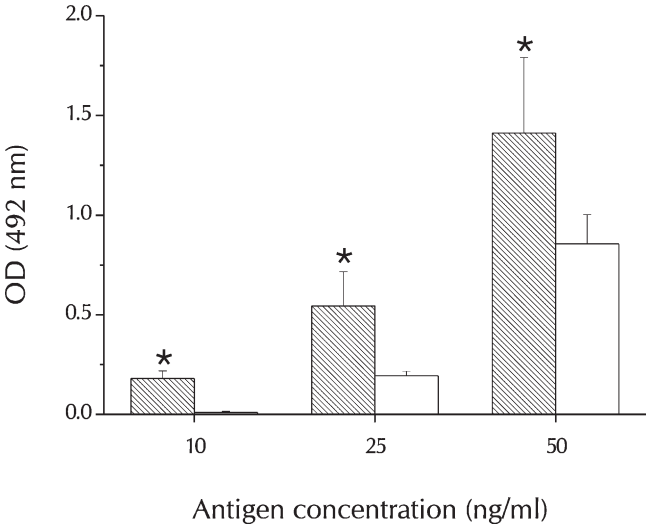

FIGURE 1. Effect of the use of CoproGuard or distilled water for extraction of *Fasciola hepatica* coproantigens from fecal samples mixed with *F. hepatica* excretory-secretory antigens (ESAs) at different concentrations. After antigen mixing, the samples were diluted 1:10 in distilled water or in CoproGuard, centrifuged, and assayed by the MM3-COPRO ELISA, with an incubation time of 2 hr at room temperature (RT) and orbital shaking (100 rpm). Vertical bars represent mean optical density (OD) values ± SD obtained for water extraction (white bars) and CoproGuard extraction (crossed bars). (*): $P < 0.01$.

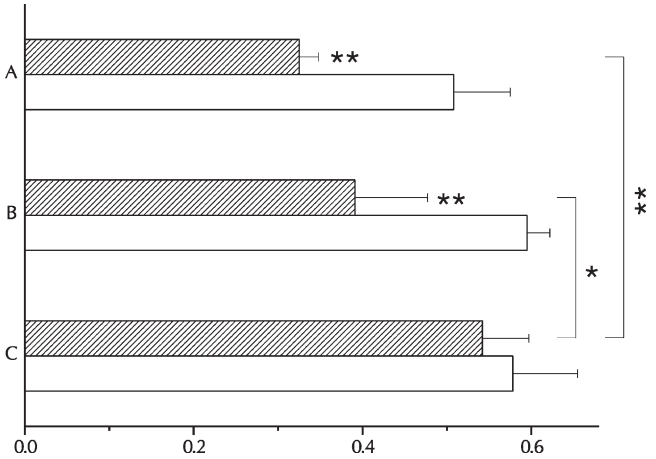

FIGURE 2. Effect of the incubation time and shaking on the MM3-COPRO ELISA detection of *Fasciola hepatica* coproantigens. Pooled fecal samples containing 25 ng/mL of excretory-secretory antigens (ESAs) were diluted 1:10 in CoproGuard and tested by MM3-COPRO ELISA under the following incubation conditions: (**A**) 1 hr at room temperature (RT); (**B**) 2 hr at RT; (**C**) overnight at 4°C. Horizontal bars represent mean optical density (OD) values ± SD obtained for the different enzyme-linked immunosorbent assay (ELISA) conditions assayed. White bars indicate orbital shaking (100 rpm); crossed bars indicate that orbital shaking was not used. (*): $P < 0.05$; (**): $P < 0.01$.

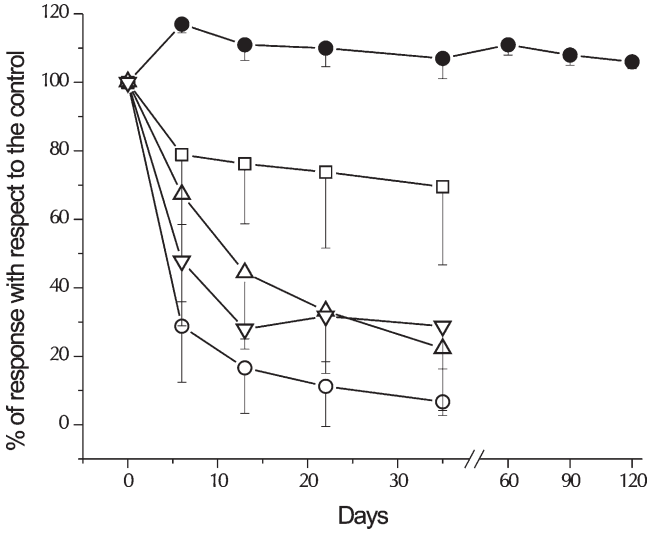

FIGURE 3. Analysis of the stability of *Fasciola hepatica* coproantigens in fecal samples under different storage conditions. Kato-Katz positive fecal suspensions containing 7.1 to 25.1 ng/mL of *F. hepatica* coproantigens were preserved in CoproGuard at 37°C (filled circles), 0.2% sodium azide in distilled water at room temperature (RT) (open triangles), 0.03% thimerosal in distilled water at RT (inverted triangles), or in distilled water without biocides, at RT (open circles) and at 4°C (open squares). The values shown were obtained from the mean optical densities (ODs) ± SDs of 5 individual fecal samples, and expressed as percentages with respect to control. Enzyme-linked immunosorbent assay (ELISA) determinations were carried out after up to 35 days, except for samples stored in CoproGuard (120 days).

the intersection of the curve shown in Figure 4 with the cut-off value of 0.097 OD (horizontal line), previously calculated for the 213 samples from uninfected subjects (see Figure 5). This detection limit was not influenced by the use of the CoproGuard as diluent/preservative, as the same response was obtained by diluting *F. hepatica* ESAs in PBS (data not shown).

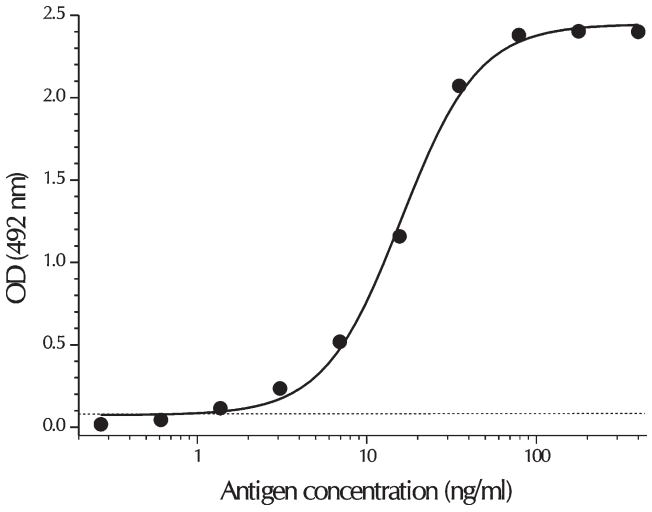

FIGURE 4. Calibration curve for the MM3-COPRO ELISA, obtained by plotting optical density (OD) at 492 nm against known concentrations (0.27–400 ng/mL) of whole *Fasciola hepatica* excretory-secretory antigens (ESAs) diluted in CoproGuard. Experimental OD values were adjusted to a sigmoidal (logistic) curve ($r^2 = 0.997$) with the Microcal Origin 6.0 software package. The dashed line shows the cut-off value calculated for human fecal samples (OD = 0.097; see Figure 5).

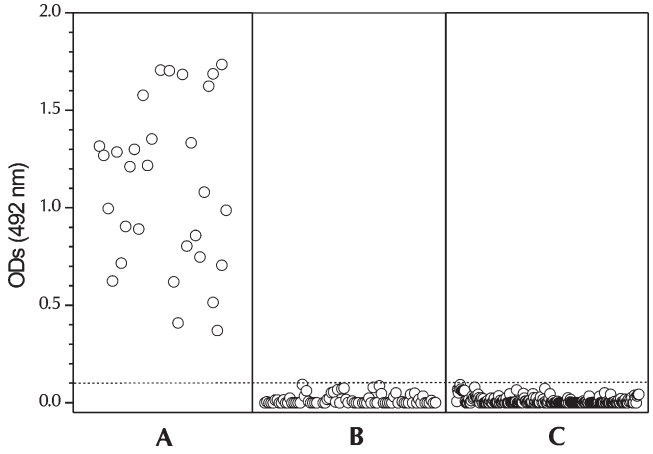

FIGURE 5. Individual MM3-COPRO ODs obtained for individual *Fasciola hepatica* positive and negative fecal human samples. (**A**) *Fasciola* positive samples ($N = 30$); (**B**) *Fasciola* negative samples from patients with other parasitic infections ($N = 83$); (**C**) fecal samples from uninfected subjects ($N = 213$). The dashed line shows the cut-off value (OD = 0.097) calculated for group C (mean OD plus 4 SD mean).

Sensitivity and specificity of the MM3-COPRO test. The sensitivity and specificity of the MM3-COPRO ELISA were evaluated by comparing the OD values obtained with *Fasciola* positive and *Fasciola* negative samples, as shown in Table 1. For this, 30 coprologically positive *Fasciola* samples and 83 samples from patients with parasitic infections other than *Fasciola* were analyzed. These included 35 samples containing one or more pathogenic protozoans (*Giardia intestinalis*, *Entamoeba histolytica*, and *Cryptosporidium parvum*) and 48 samples from patients reported to be infected by one or more helminth species (*Strongyloides stercoralis*, *Ascaris lumbricoides*, *Trichuris trichiura*, *Enterobius vermicularis*, and *Hymenolepis nana*). Among these, 15 samples corresponded to mixed infections of helminths and protozoans. Considering helminths alone, 22 samples corresponded to patients with double infections.

The OD values obtained for individual *F. hepatica* positive and negative fecal samples are shown in Figure 5. As can be seen, only positive samples with *F. hepatica* eggs, as determined by the Kato-Katz technique, showed OD values above the cut-off value. The mean value for positive samples was 14.83 ± 6.08 ng/mL with a range from 4.5 to 25.5 ng/mL. Considering the obtained cut-off value of 1.1 ng/mL, the MM3-COPRO ELISA was able to discriminate as positive 100% of the positive fecal samples evaluated by the Kato-Katz technique. Indeed, among the *Fasciola* positive samples, 27/30 samples (90%) presented OD values higher than 0.5 OD, and the lowest OD value obtained (0.37 OD) was 3.8 times higher than the cut-off value. No differences were observed between the OD values corresponding to fecal samples from patients with parasitic infections other than *Fasciola* and fecal samples classified as negative by microscopy ($P = 0.198$). The mean egg content in the *F. hepatica* positive samples was 130 ± 20 epg, with a range of 16 to 496 epg. There was no significant correlation between OD values of the *Fasciola* positive samples and the egg output determined by the Kato-Katz technique (Figure 6; $P = 0.12$).

DISCUSSION

Antibody-based antigen detection of *Fasciola* antigens may be advantageous over microscopic examination in terms of

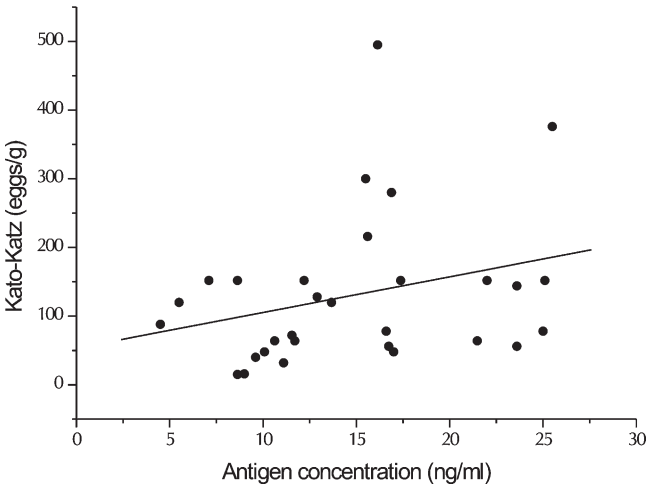

FIGURE 6. Plot of the number of eggs per gram in positive *Fasciola* samples, determined by the Kato-Katz method, against the antigen concentration measured by the MM3-COPRO ELISA. The linear regression analysis did not reveal any significant correlation ($P = 0.12$).

sensitivity and facility of use, particularly for epidemiologic studies in endemic areas. Moreover, because the release of *Fasciola* coproantigens takes place before egg shedding,[20,25] immunologic methods are also preferable to egg examination for detection of acute infections. However, because microscopic examination is currently the only method that is 100% specific for diagnostic of human fascioliasis, we used the Kato-Katz method as a "gold standard" for comparisons in the present study.

In the last two decades, several ELISA methods based on the use of polyclonal and monoclonal antibodies have been reported to be useful for detection of ESAs from *F. hepatica* and *F. gigantica* in sheep and cattle feces.[19,21,26,27] Among these, the ELISA method based on the use of the ES78 monoclonal antibody is the only method that has been successfully adapted for detection of human cases of fascioliasis,[20] although the method is not still available commercially. In this study, we optimized our previous MM3-COPRO ELISA method for detection of *Fasciola* coproantigens in human stools.[21] With CoproGuard as diluent/preservative, the test showed a detection limit of 1.1 ng/mL, which is more than one order lower than that reported for the ES78 sandwich ELISA (15 ng/mL).[20] This low detection limit enabled the MM3-COPRO ELISA to detect 100% of positive samples in 1:10 dilutions rather than the 1:4 dilutions used in the ES78 method. Because fecal antigen suspensions are dense at low dilutions, the use of more diluted antigen preparations is advantageous in terms of preventing obstruction of the aspiration systems and facilitating washing, particularly when automatic ELISA devices are used. The 1.1 ng/mL detection limit obtained for the MM3-COPRO ELISA in the present study was only slightly higher than that previously reported for bovine samples (0.6 ng/mL).[21] We attribute these small differences to the changes in the secondary ELISA reagents used with the MM3-COPRO ELISA in the present study.

As regards the specificity of the MM3-COPRO ELISA, there were no problems related to cross-reactivity when common protozoans or helminths were present (see Figure 5). This is not surprising because the specificity of the test mainly depends on the recognition of a single epitope by the MM3 monoclonal antibody on the *Fasciola* coproantigens, and the test already proved to be highly specific in previous studies with fecal samples of bovine and ovine origin.[21,28] Nevertheless, it will be necessary to carry out new studies in countries where other types of human trematodosis occur (e.g., opisthorchiasis, chonorchiasis, echinostomiasis) to confirm the usefulness of the MM3-COPRO assay in countries where these diseases are endemic.

As deduced from the results shown in Figures 1 and 3, the use of the CoproGuard diluent/preservative improved antigen extraction, thus rendering the antigen more accessible to the anti-*Fasciola* capture polyclonal antibodies immobilized in the ELISA plate. This effect was probably partly mediated by the presence of tensoactive agents in the composition of the preservative. Moreover, CoproGuard was able to preserve the antigenicity of the fecal samples stored either at RT or at 37°C, which may be very useful for facilitating sample collection and storage in endemic areas in tropical and subtropical countries where the high temperatures and long distances from hospitals may make adequate storage of samples difficult. To our knowledge, this is the first preservative capable of maintaining the antigenicity of *Fasciola* coproantigens for ELISA determinations.

The antigenicity of *F. hepatica* coproantigens was not preserved by biocides such as sodium azide or thimerosal, but degradation was lowered when samples were refrigerated at 4°C. However, we observed that the antigens are relatively stable in some stools, as can be deduced from the large standard deviations observed for samples not preserved in CoproGuard (Figure 3). This suggests that degradation of MM3-recognized *Fasciola* coproantigens depends on the presence of particular protease species, or other factors, which differ for each patient. Interestingly, in previous studies carried out with CoproGuard in our laboratory, we have observed that this preservative is also suitable for preserving *F. hepatica* antigens in fecal samples from cattle and sheep (results not shown).

We have recently showed that the MM3-COPRO ELISA is also a reliable method of detecting *F. gigantica* coproantigens in fecal samples from experimentally infected sheep.[22] Although most reported cases of human fascioliasis are caused by *F. hepatica*, infections by *F. gigantica* have been reported in countries such as Vietnam, Iran, and Egypt.[29–31] The fact that the MM3-COPRO ELISA can detect infections by both species may be of great value to ensure diagnosis of human and animal fasciolosis in countries where *F. gigantica* predominates, or where both species of *Fasciola* are present.

One of the main difficulties with the current methods available for diagnosis of human fascioliasis is in detecting chronic infections or infections where only one or a few adult flukes are present in liver ducts (low-burden infections). In these cases egg shedding may be very low or absent,[2] which makes diagnosis by direct coprologic analysis difficult or impossible. This may be further complicated because egg excretion in *Fasciola* infections is frequently intermittent.[11,25] Interestingly, the high sensitivity and favorable signal-to-noise ratio shown by the MM3-COPRO ELISA is very promising for detection of these cases, although field studies must be carried out in areas where fascioliasis is endemic to confirm the usefulness of the test.

Finally, it should be emphasized that quantitative analysis of *Fasciola* eggs in feces (Kato-Katz) is frequently used for indirect estimation of fluke burden before treatment of human infections, to prevent obstruction of biliary ducts in patients

with heavy infections.[32,33] Fecal egg counting may also be used to monitor the efficiency of flukicide treatment.[34] In a previous study with MM3-COPRO ELISA in cattle, it was observed that the concentration of coproantigens in feces is also correlated with the number of flukes found in the livers of animals collected after slaughter,[21] and that this method is better than egg counting for evaluating the efficacy of flukicide treatment. In the present study, we did not observe any correlation between egg shedding, measured by the Kato-Katz technique, and coproantigen concentration, measured by MM3-COPRO (see Figure 5). This result contrasted with the positive correlation reported by Espino and Finlany[20] for the ES78 test. One possible explanation for this discrepancy may be that the positive cases reported by Espino and Finlay probably corresponded to recent infections of less than a year of evolution, whereas our samples were from patients with chronic infections, in which egg excretion is probably more erratic. Moreover, the influence of the crowding effect on *Fasciola* egg shedding should also be taken into account.[35]

In summary, we provide experimental evidence that 1) the MM3-COPRO ELISA is highly specific and at least as sensitive as the Kato-Katz method for diagnosis of *Fasciola* infections in humans, and 2) the detected coproantigens can be stored at RT in CoproGuard. However, further field studies are necessary to evaluate whether the MM3-COPRO test is as sensitive as in sheep and cattle for detection of low-burden *Fasciola* infections in humans.

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Correlation between egg-shedding and uterus development in *Fasciola hepatica* human and animal isolates: applied implications

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ABSTRACT

The emission of *Fasciola hepatica* eggs in faeces is usually subject to oscillations along time in animals as well as humans. Thus, looking for alternative biological markers reflecting eggs shed per gram of faeces (epg) with lower oscillations may be useful. This study analyzes the possible relationship between liver-fluke uterus area and epg. Uterus area (UA) development of adult F. hepatica obtained at different days post infection (dpi) in a Wistar rat model with isolates obtained from cattle, sheep, pigs and humans from the endemic human fascioliasis zone of the Northern Bolivian Altiplano was analyzed and compared with the number of eggs shed per gram of faeces as obtained through the Kato–Katz technique. The morphometric study of the UA of liver flukes was carried out using image analysis software. The multiple regression model shows that UA is dependent on dpi and isolate. The evolution of UA vs dpi followed a damped model. This work shows a positive relationship between liver-fluke UA and egg production. The complete absence of eggs in the uteri of some parasite individuals at 300 dpi was observed, which corresponds to the cessation of egg shedding in the advanced chronic stage. The results obtained suggest the necessity to characterize the isolates employed with regard to geographical as well as host origin in fascioliasis studies in which egg production is used as a biological tag.

1. Introduction

Fascioliasis is an important human and animal disease caused by the trematode species *Fasciola hepatica* and *F. gigantica*. At present, fascioliasis is emerging or re-emerging in numerous regions. The highest human fascioliasis prevalence and intensities are encountered in the Northern Bolivian Altiplano (Mas-Coma et al., 1999), where *F. hepatica* is the only fasciolid species present (Valero et al., 1999), and *Galba truncatula* the only intermediate snail host species (Bargues and Mas-Coma, 2005). In this endemic region, sheep and cattle may be considered the main reservoir host species, with pigs and donkeys playing a secondary role (Mas-Coma et al., 1997). Valero and Mas-Coma (2000) have demonstrated that the viability and infectivity of metacercariae experimentally obtained from eggs shed by sheep, cattle, pigs and donkeys (i.e. their respective isolates) are similar, and that adult liver fluke body development in a common experimental animal model such as the Wistar rat (see Valero et al., 2000, 2003, 2006a,b, 2008) is not influenced by the animal species isolate source (Valero et al., 2001a). Uterus size vs age in *F. hepatica* experimental adult isolates from the Northern Bolivian Altiplano human hyperendemic area was studied in the Wistar model under standardized infective dose conditions with a very long follow-up of up to 300 days including the advanced chronic stage. This is the first study on the relationship between uterus size and epg measured by the Kato–Katz technique, a common diagnostic tool for human helminth infections. Liver fluke uterus area vs time

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has been modeled and the influence of different factors such as isolate and advanced chronic stage has been analyzed.

2. Materials and methods

2.1. Liver flukes from experimentally infected rats

Only *F. hepatica* and *G. truncatula* specimens from the Northern Bolivian Altiplano were used. Snails shedding the cercariae which gave rise to the metacercariae were from a laboratory-reared strain (in Heraeus–Vötsch HPS 1500 and HPS 500 climatic chambers; experimental conditions: temperature 20 °C; photoperiod 12/12 h light/darkness; RH 90%). These snails were in turn monomiracidially infected with *F. hepatica* eggs found in faeces of naturally infected sheep, cattle, pigs and humans (Mas-Coma et al., 2001). Thus 4 metacercariae isolates (cattle isolate, sheep isolate, pig isolate and human isolate) were obtained. These metacercariae were stored in fresh water at 4 °C in darkness until required. A total of 73 male Wistar (Iffa Credo, Barcelona, Spain) aged 4–5 weeks were used for infection with metacercariae inoculated orally by means of an orogastric syringe. Rats were housed in micro-isolator boxes (Iffa Credo, Barcelona, Spain) and maintained in a pathogen-free room, electrically heated with a 12/12 h light/darkness cycle (conditions in compliance with the European Agreement of Strasbourg, 18 March 1986). Food and water were provided *ad libitum*. Wistar rats were divided into 4 sets according to the isolate: cattle (12 rats), sheep (24), pig (19) and human (18), and were infected with doses of 20 metacercariae each. Infection was established by dissection and by detection of eggs in faeces. Dissection was carried out at different days post-infection (dpi) (between 40 and 300 dpi) to obtain worms at different stages of maturity, body size and gravid uteri. Infection was confined to the common bile duct from 40 dpi onwards. The detection of eggs in faeces was carried out by the Kato–Katz technique (Helm-Test®, AK test, AK Industria e Comercio Ltda, Belo Horizonte, Brazil). Infection intensity was characterized by the number of adults located in the liver. To avoid possible effects of liver fluke burden in egg production (Valero et al., 2006b) only material from low level infections (≤4 liver flukes/rat) was used. Likewise, to prevent possible host pathological effects in egg production (Valero et al., 2000, 2006a) only material from rats without lithiasis was used in this study.

2.2. Obtaining F. hepatica adults

The 73 rats were sacrificed at different times between 40 and 300 dpi (see Table 1). The following fluke numbers were obtained: (A) cattle isolate: 14 adults (50 dpi: 1 in one rat, 1 in another rat; 75 dpi: 1, 1; 100 dpi: 1, 1; 150 dpi: 3, 1; 200 dpi: 1, 1; 225 dpi: 1, 1; average: 1.2 ± 0.6, range: 1–3; (B) sheep isolate: 51 adults (40 dpi: 1, 1; 75 dpi: 3, 3, 2, 3; 100 dpi: 3, 2, 3, 1; 150 dpi: 2, 1, 1, 1; 225 dpi: 2, 2, 1; 300 dpi: 3, 3, 3, 4, 2, 2, 2; average: 2.1 ± 0.9; range 1–4; (C) pig isolate: 22 adults (40 dpi: 1, 1; 50 dpi: 1, 1; 75 dpi: 1, 1; 100 dpi: 1, 1; 150 dpi: 1, 1; 175 dpi: 1, 1, 1; 200 dpi: 2, 1; 225 dpi: 2, 1; 261 dpi: 2, 1; average: 1.2 ± 0.4; range 1–2; (D) human

Table 1
Comparison between uterus area (UA) development in mm² and egg shedding data along time in Wistar rats experimentally infected with *Fasciola hepatica*. Epg, average of eggs per gram of faeces/adult shed between 10 days prior to day when corresponding UA data was obtained (dpi, days post infection). All values shown as mean ± standard deviation; *N*, number of liver-flukes.

Isolate		40 dpi (*N*)	50 dpi (*N*)	75 dpi (*N*)	100 dpi (*N*)	150 dpi (*N*)	175 dpi (*N*)	200 dpi (*N*)	225 dpi (*N*)	261 dpi (*N*)	300 dpi (*N*)
Cattle	UA ± SD	–	2.0 ± 0.1 (2)	4.2 ± 1.1 (2)	9.5 ± 1.3 (2)	9.1 ± 3.5 (4)	–	11.5 ± 1.3 (2)	9.7 ± 1.5 (2)	–	–
	epg ± SD	–	42.1 ± 11.9	77.5 ± 14.8	268.8 ± 128.6	215.2 ± 146.74	–	223.2 ± 185.8	120.0 ± 196.6	–	–
Sheep	UA ± SD	1.8 ± 0.1 (2)	–	5.3 ± 1.8 (11)	6.8 ± 2.9 (9)	7.6 ± 1.0 (5)	6.6 ± 0.8 (7)	–	9.2 ± 2.7 (5)	–	6.2 ± 2.2 (19)
	epg ± SD	2.5 ± 3.1	–	251.7 ± 73.4	163.9 ± 47.1	260.6 ± 160.8	188.9 ± 66.6	–	230.1 ± 61.7	–	302.9 ± 122.4
Pig	UA ± SD	1.8 ± 0.2 (2)	3.9 ± 1.2 (2)	5.72 ± 1.2 (2)	7.1 ± 1.0 (2)	4.4 ± 0.1 (2)	8.8 ± 0.3 (3)	9.0 ± 0.9 (3)	7.4 ± 2.8 (3)	3.02 ± 0.9 (3)	–
	epg ± SD	32.0 ± 18.3	58.2 ± 33.2	114.9 ± 31.1	247.2 ± 58.5	96 ± 104.6	242.7 ± 33.2	227.2 ± 109.0	125.6 ± 63.1	143 ± 119.7	–
Human	UA ± SD	–	1.8 ± 1.2 (3)	9.0 ± 2.4 (3)	8.6 ± 2.7 (3)	12.7 ± 1.2 (3)	7.0 ± 3.6 (11)	–	10.0 ± 2.7 (3)	–	7.3 ± 0.7 (4)
	epg ± SD	–	21.4 ± 16.8	193.8 ± 225.6	272.5 ± 192.4	331.3 ± 272.4	301.7 ± 164.3	–	457.1 ± 174.4	–	527.9 ± 573.8

isolate: 29 adults (50 dpi: 1, 2; 75 dpi: 1, 2; 100 dpi: 1, 2; 150 dpi: 1, 2; 175 dpi: 2, 2, 2, 2, 3; 225 dpi: 1, 2; 300 dpi: 1, 1, 1; average: 1.6 ± 0.6; range 1–3). Liver fluke adult specimens were fixed with Bouin's solution between slide and cover-slip (placing the cover-slip on the parasite without external pressure), stained with Grenacher's borax carmine and mounted in Canada balsam (Panreac, Barcelona, Spain).

2.3. Follow-up study of eggs

Faecal pellets were collected from 73 rats fresh at 9:00 a.m. once a day from each animal and stored in closed Petri dishes to avoid drying before examination. Faecal egg detection was carried out by analyzing 1 Kato–Katz slide per daily sample and epg was calculated. Analyses were carried out daily from 30 dpi up to 300 dpi in the sheep and human isolates, up to 225 dpi in the cattle isolate, and up to 261 dpi in the pig isolate. In this experiment it was assumed that deparasitization had not taken place, and the number of adult flukes did not vary throughout the experiment. This premise is supported by the fact that the number of eggs detected in faeces did not decrease abruptly in any rat analyzed. Egg production per worm was calculated dividing eggs per gram of faeces by the number of worms detected in the common bile duct. The average egg output in all shedding per fluke and day and average of eggs per gram of faeces shed between 10 and 25 days prior to the day when corresponding UA data was obtained (=animal necropsy, 40, 50, 75, 100, 150,175, 200, 225, 261 and 300 dpi).

2.4. Measurement techniques and data analyses

Adult uterus measurements were carried out according to the method proposed for fasciolids by Valero et al. (2001b), using a Computer Image Analysis System (CIAS), including a computer work station-connected to a stereomicroscope equipped with a digital colour video camera (DX 20, Kappa) and employing image analysis software (Image-Pro1Plus 4.5, USA) (Valero et al., 2005). Fluke uterus area (UA) measurements are expressed in mm^2.

2.5. Analytical methods

Graphic plots of morphometric measurements against age (t) provide empirical ontogenetic trajectories for *F. hepatica* adults (Valero et al., 2006b). In the present study, an ontogenetic trajectory describes the change of UA as a function of its age in days. Ontogenetic trajectories are obtained as solutions of differential equations that express mathematical statements of growth rules of morphological variables (Alberch et al., 1979). These growth rules are explicit expressions of the derivative of the morphological variable referring to age (t). The solutions of these differential equations do not only contain parameters included in the growth rule but also initial conditions. A preliminary analysis of the results indicated that a "damped" model gives the best representation of the data. For UA, two models for ontogenetic trajectories, saturated (a) ($y = y_m [1 - z_0 \exp(-kt)]$) and logistic (b) ($y = y_m/[1 + z_0 \exp(-kt)]$) were verified, where y_m is the maximum value attained by UA, and z_0 and k are constants or parameters of the trajectories; z_0 is related to the initial value of uterus area (y), i.e. y_0 at $t = 0$, and exhibits a different expression in each case. The parameter k is a growth rate in either case. The Mc Curve fit statistics package was used to fit the models to data in place of the iterative trials as suggested by De Renzi (1988). This procedure minimizes the least square sum of residuals (sse), i.e. $(yi - \ddot{y}i)^2$, with the raw data yi's and their estimates ÿi's.

2.6. Statistical analyses

For a proper description, some transformations were necessary. Egg counts were considered after converting individual values to logarithms ($\log e$) with the $(n + 1)$ transformation ln(epg) (Stephenson, 1987). Bivariant correlations (Pearson's correlation) were calculated for UA and ln(epg). The analysis of the relation between UA obtained for a determined day p.i. and the transformed ln(epg) count (average of eggs per gram of faeces shed between 10 and 25 days prior to the day when corresponding UA data was obtained) was carried out using one-way ANOVA. Effect–size samples were controlled by power (Lenth, 2009). Adjusted non-lineal curves were tested using r^2 and sse. GRAPHPAD PRISM 5.0 was used to represent ontogenetic trajectories. To analyze the factors influencing the UA at different dpi, multiple regression analysis using UA as dependent variable was performed. The variables were dpi and type of isolate. Data processing was done with PASW Statistics 17 software. Results were considered statistically significant when $P < 0.05$.

3. Results

3.1. Follow-up study of eggs and uterus area (40–300 dpi)

The evolution of average epg/adult/day obtained for each isolate vs time is represented in Fig. 1. The results showed that faecal egg counts have strong day-to-day fluctuations in rats in all isolates. The ANOVA analysis of the correlation of UA vs ln(epg): (a) epg average shed between 10 day prior to the day when corresponding UA data was obtained; (b) epg average shed between 25 days prior to the day when corresponding UA data was obtained. The ANOVA correlation only proved to be statistically significant when UA was correlated with ln(epg) average of the last 10 days (cattle: $F = 10.233$, $P < 0.033$, power: 42%; sheep: 11.215, 0.020, 99%; pig: 11.215, 0.020, 52.2%; and human: 7.444, 0.041, 25.5%). The evolution of the UA according to dpi in rats inoculated with the 4 isolates and 10 days prior to day when corresponding UA data was obtained are shown in Table 1 and Fig. 2, respectively.

3.2. Uterus area growth

The multiple regression model shows that UA is dependent on dpi ($P < 0.000$) and isolate ($P < 0.000$). The general analysis of all parasite individuals aged 225–300 dpi shows an elevated dispersion of UA, which usually tends to decrease up to 225 dpi. Interestingly, the complete absence of eggs in the uteri of some parasite individu-

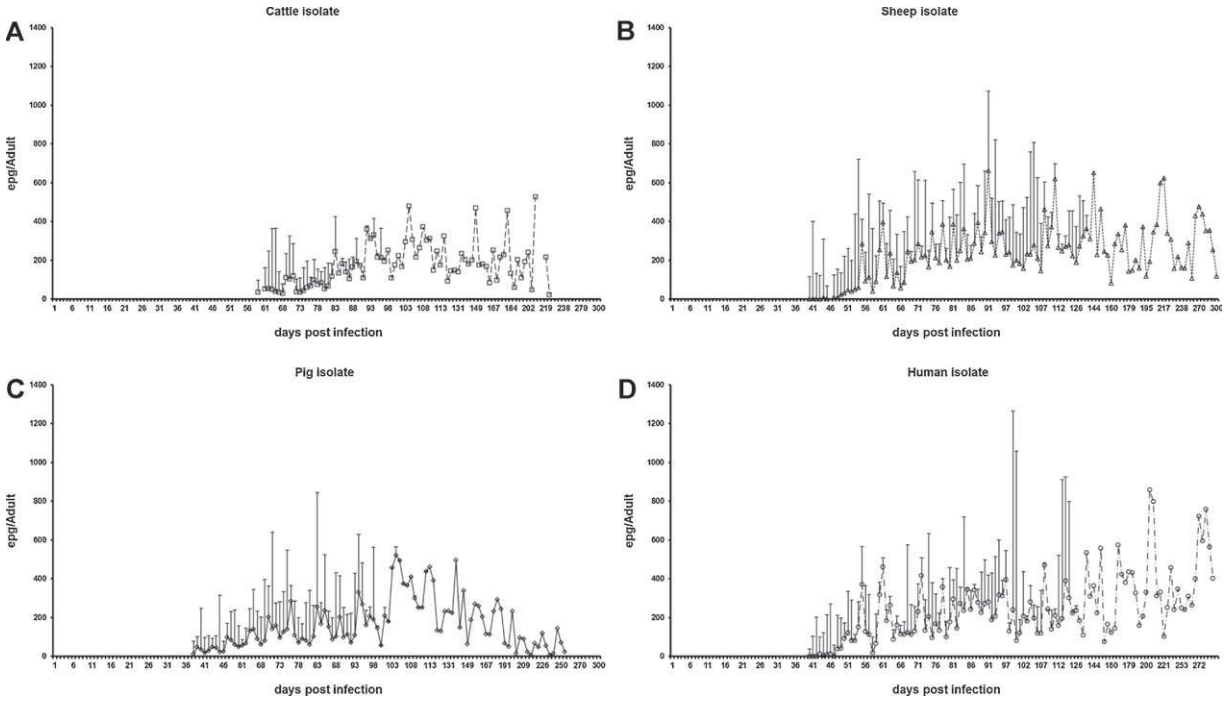

Fig. 1. Eggs per gram of faeces (epg)/adult/day along days post infection obtained in the four *Fasciola hepatica* isolates analyzed: (A) cattle isolate (□); (B) sheep isolate (△); (C) pig isolate (◊) and (D) human isolate (○). Each symbol represents the average for each day analyzed. The bars represent SD.

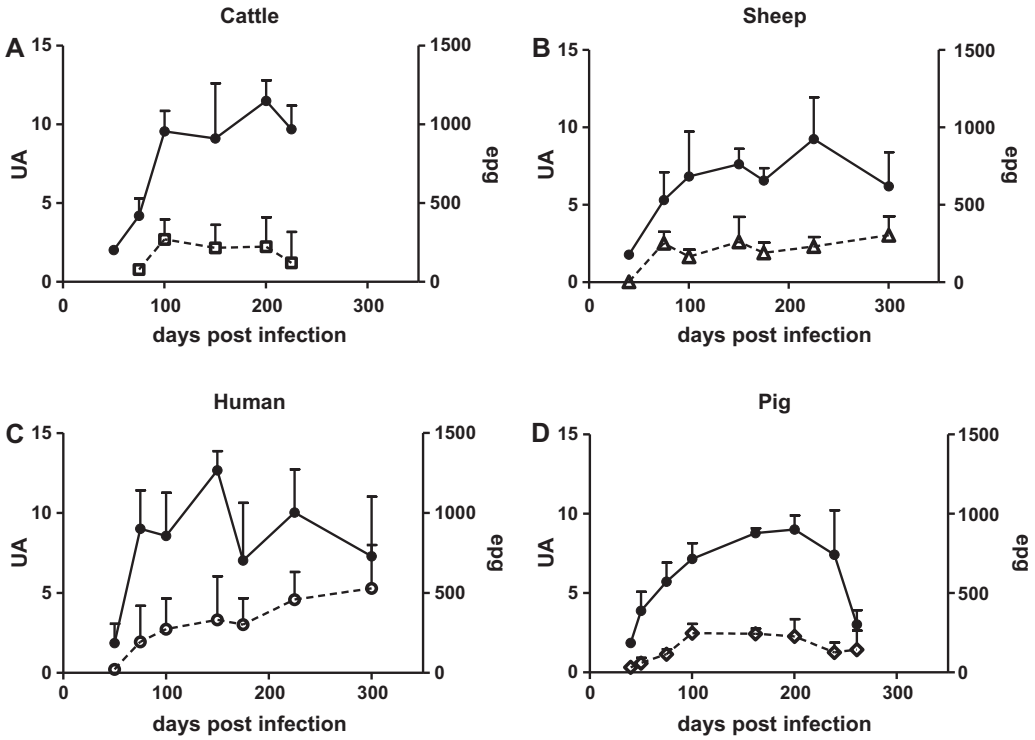

Fig. 2. Evolution of liver-fluke uterus area (UA) and eggs per gram of faeces (epg)/adult along days post infection in the four *Fasciola hepatica* isolates analyzed: (A) cattle isolate; (B) sheep isolate △; (C) human isolate ○ and (D) pig isolate ◊. Each symbol represents the average for each day analyzed. UA: each symbol represents the average UA of each liver-fluke group at different days. Epg: each symbol represents epg average shed between 10 days prior to the day when corresponding UA data was obtained; ——, UA; - - -, epg/adult.

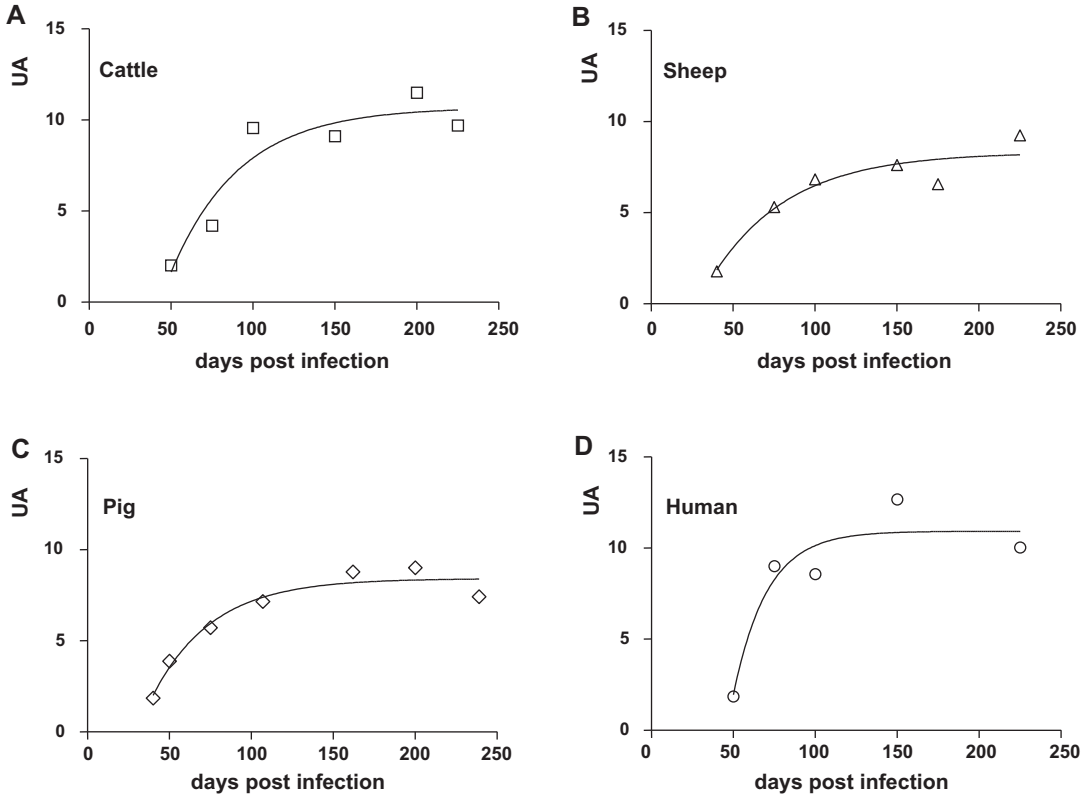

Fig. 3. Ontogenetic trajectory of uterus area (UA) (in mm^2) as a function of time (days) with the saturated model $y = y_m[(1 - z_0 \exp(-kt)]$ in adults obtained in experimentally infected Wistar rats in the four *Fasciola hepatica* isolates analyzed: (A) cattle isolate (□); (B) sheep isolate (△); (C) pig isolate (◊) and (D) human isolate (○). Each symbol represents the average UA in the corresponding day.

als at 300 dpi was observed (8%), which corresponds to the cessation of egg shedding in the advanced chronic stage. Hence, UA average development in each isolate was analyzed exclusively between 40 and 225 dpi in liverflukes. UA follows a damped model until 225 dpi, and thereafter decreases. The ontogenetic trajectories of liverfluke UA measures were studied. The results obtained are reflected in Fig. 3. Table 2 compares the fit of the two models for each biometric measure. The saturated model (Eq. (a)) fits better than its logistic counterpart.

4. Discussion

The determination of epg shed by helminths is a frequently applied approach in experimental assays. For instance, the efficacy of anthelmintic treatment is usually experimentally determined by the reduction of faecal egg counts (McConville et al., 2009). Apart from reducing fluke burdens, some vaccines can elicit a concurrent reduction in parasite egg production (Hillyer, 2005; Piedrafita et al., 2010). Practically applied studies will benefit from an increased precision, since reliable estimates of fae-

Table 2
Comparison between the saturated and logistic models applied to *Fasciola hepatica* adults experimentally obtained in Wistar rats (z_o, k constants that appear in both models, r^2 correlation coefficient, y_m maximal value of uterus area in both models, sse least-squares residual in both models).

Isolate	y_m	$z_0 \pm$ SE	$-k \pm$ SE	r^2	sse
Logistic model (b)					
Cattle	8.5	30.913 ± 2.0734	0.045 ± 0.000	0.99	0.0014
Sheep	9.8	40.587 ± 199.829	0.086 ± 0.064	0.90	0.8533
Pig	8.9	21.790 ± 16.467	0.048 ± 0.012	0.97	0.7668
Human	13.1	29.814 ± 45.167	0.046 ± 0.019	0.88	9.2548
Saturated model (a)					
Cattle	11.6	1.390 ± 0.001	0.0103 ± 0.000	0.99	0.00002
Sheep	9.8	720.424 ± 4750.640	0.100 ± 0.087	0.94	0.47784
Pig	9.0	1.937 ± 0.332	0.022 ± 0.003	0.98	0.28987
Human	13.3	3.0043 ± 1.1650	0.025 ± 0.006	0.93	5.22824

[a] $y = y_m(1 - z_0 \exp(-kt))$.
[b] $y = y_m/(1 + z_0 \exp(-kt))$

cal egg counts are essential in this context. Commonly employed microscopic techniques in quantitative diagnosis of helminth eggs are very specific but rather insensitive. Amounts of epg in faeces are often very variable (data elsewhere) and various factors have to be taken into account. However, the technique applied in the epg count is of particular importance (Ebrahim et al., 1997). The Kato–Katz technique is routinely employed to diagnose and quantify the burden in the majority of human helminthiases (Katz et al., 1972). The simplicity of the test and its ease of performance under field conditions have ensured its universal application. However, the sensitivity of a single examination may be very low due to a combination of factors such as: (i) small amounts of stool in each examination; (ii) variation in the distribution of eggs within a single stool specimen; (iii) differences in worm burdens; (iv) daily fluctuations within an individual due to faecal production and consistency; (v) daily fluctuations within an individual related to oviposition patterns of the parasite (Hall, 1982; De Vlas et al., 1992; Engels et al., 1997; Utzinger et al., 2001). Moreover, the consistency of the faecal material depends upon the parasite burden, i.e. moderate to heavy infections may cause diarrhoea (Teesdale et al., 1985; Feldmeier and Poggensee, 1993). Also, the amounts of fibre or fat in the host's diet have to be taken into account. These factors limit the value of the quantitative techniques applied when calculating epg, thus posing potential sources of inaccuracy and therefore compromise experimental assays (Ebrahim et al., 1997; Utzinger et al., 2001). Consequently, little confidence can be attached to a single epg quantification, in turn numerous samples are required for the detection of differences between groups of hosts or parasites.

Recent years have seen a dramatic resurgence of fascioliasis which has been attributed to, among other factors, climate change (Mas-Coma et al., 2009b). Moreover, resistance to commercially available fasciolicides such as triclabendazole (Fasinex) and nitroxynil (Trodax) (Overend and Bowen, 1995; Fairweather and Boray, 1999; Gaasenbeek et al., 2001) has been widely reported. Therefore, further experimental studies on drug or vaccine efficacy are pressing and are currently being carried out. In these studies, drug or vaccine efficacy is usually determined using the decrease in egg production as biomarker. Nevertheless, the emission of *F. hepatica* eggs in faeces is usually subject to oscillations along time in animals as well as humans (Valero et al., 2002; El-Morshedy et al., 2002). To avoid these inaccuracies, some authors have quantified the condition of the reproductive structures (testes, vitellaria, ovary and uterus) according to a scoring scheme (Hanna et al., 2006; McConville et al., 2009).

This work shows a relationship between liver-fluke UA and egg production, which is consistent with similar findings in other helminths. A positive correlation between worm size and egg production is commonly described in nematodes of animals (Skorping et al., 1991; Stear et al., 1997; Stear and Bishop, 1999; Richards and Lewis, 2001; Irvine et al., 2001; Dezfuli et al., 2002; Ugland et al., 2004) as well as in *Ascaris lumbricoides* infections of humans (Sinniah and Subramaniam, 1991; Walker et al., 2009). For instance, in *A. lumbricoides*, a relationship between egg production and weight, length and diameter of worms was described (Sinniah and Subramaniam, 1991). Interestingly, after female *A. lumbricoides* worms had reached their maximum growth, there was a decline in egg production, just as seen here in *F. hepatica*.

Fasciolid adults follow an established development process in their definitive hosts (Valero et al., 2001a, 2006b; Periago et al., 2008). In *F. hepatica*, the length of the uterus in different stages of development correlates with body size (Neuhaus, 1978). In populations from naturally infected cattle, sheep and pigs from the Northern Bolivian Altiplano, UA vs body area follows a damped model (Valero et al., 2001b). In this experimental study it was demonstrated that *F. hepatica* UA development along time fitted a saturated model, while body growth follows a logistic model characterized by two phases (Valero et al., 1998): the 'exponential' part of logistic growth corresponds to body development during migration through the abdominal cavity and liver parenchyma as well as to development and sexual maturation in the biliary duct system up to the onset of egg production. From this moment onwards, development follows the 'saturated' part of logistic growth with a considerable persistence of body growth after sexual maturity. Oviposition is the inflection point of the logistic growth marking the end of the 'exponential' period and the beginning of the 'saturated' period, i.e. the beginning of egg shedding to the external environment constitutes the biological factor that marks the inflection point (Valero et al., 2006b). This work shows for the first time that the UA follows a saturated model vs dpi and also agrees with the above-mentioned studies on body size development, i.e. after egg shedding liver-fluke body area and UA follow a synchronized growth model.

The variability inherent in different *F. hepatica* isolates and the relationship between these differences vs the fecundity of the parasite has been analyzed (Walker et al., 2006). These authors compared *in vitro* egg production of two distinct isolates, namely Oberon and Fairhurst, and showed that the latter produced four times more eggs. In the present study differences in egg production between the isolates analyzed were also detected. These results imply the necessity to characterize the isolates employed in fascioliasis studies in which egg production is used as a biological tag.

Conflicts of interest

The authors declare no conflicts of interest.

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Fasciola hepatica phenotypic characterization in Andean human endemic areas: Valley versus altiplanic patterns analysed in liver flukes from sheep from Cajamarca and Mantaro, Peru

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ABSTRACT

Fascioliasis is a zoonotic parasitic disease caused by *Fasciola hepatica* and *Fasciola gigantica*. Of both species, *F. hepatica* is the only one described in the Americas, mainly transmitted by lymnaeid snail vectors of the *Galba/Fossaria* group. Human fascioliasis endemic areas are mainly located in high altitude areas of Andean countries. Given the necessity to characterize *F. hepatica* populations involved, the phenotypic features of fasciolid adults infecting sheep present in human fascioliasis endemic areas were analysed in the Cajamarca Valley and Mantaro Valley (valley transmission patterns) and the northern Bolivian Altiplano (altiplanic transmission pattern). A computer image analysis system (CIAS) was applied on the basis of standardized measurements. The aforementioned highland populations were compared to standard lowland natural and experimental populations of European origin. Liver fluke size was studied by multivariate analyses. Two phenotypic patterns could be distinguished in *F. hepatica* adult size: the valley pattern (Cajamarca and Mantaro, Peru) and the altiplanic pattern (northern Altiplano, Bolivia). Results showed that the Andean valley population and European standard populations presented a phenotypic homogeneity. The Altiplano population showed a large size range with a pronouncedly lower minimum size indicating that uterus gravidity is reached at a smaller size than in valley populations. The results of this study demonstrate that there is no apparent relationship between the shape of fasciolid adults with regard to altitudinal difference or geographical origin and that allometry-free shape appears as a more stable trait than size in fasciolid species. Results are analysed in terms of intensity/crowding effect aspects and permanent/seasonal transmission characteristics.

1. Introduction

Fascioliasis is a parasitic disease of different epidemiological, pathological and control characteristics depending on the causal agents, *Fasciola hepatica* and *Fasciola gigantica*. Classically it has been accepted that *F. hepatica* is present worldwide, while the distribution of the two species overlaps in many areas of Africa and Asia (Mas-Coma et al., 2005, 2009). Their specific identification poses problems in the aforementioned overlap areas. It has been demonstrated that differentiation of both species cannot be made by traditional diagnostic methods (Marcilla et al., 2002; Valero et al., 2009) but only by adult morphometric markers (Periago et al., 2006, 2008; Ashrafi et al., 2006), molecular tools (Marcilla et al., 2002) and DNA sequences (Mas-Coma et al., 2009).

Of both species, *F. hepatica* is the only one described in the Americas (Mas-Coma et al., 2009), mainly transmitted by lymnaeid snail vectors of the *Galba/Fossaria* group (Bargues et al., 2007). Human fascioliasis cases have been reported from many Latin American countries, including high impact areas, described in the last two decades, focusing on Andean countries, above all on high and very high altitude areas where fascioliasis transmission appears to be enhanced as a consequence of the adaptation of both the liver fluke and its lymnaeid vectors to the extreme altitudinal environmental conditions (Mas-Coma et al., 2001).

In Andean countries, fascioliasis is a serious public health problem. Human fascioliasis hyperendemic areas have been described in the northern Altiplano of Bolivia (Esteban et al., 1997a,b, 1999; Mas-Coma et al., 1999) and also Peru (Esteban et al., 2002), as well as in Andean valleys such as the Peruvian valleys of Cajamarca (Ortiz et al., 2000; Hillyer et al., 2001; Espinoza et al., 2007; Gonzalez et al., 2011) and Mantaro (Stork et al., 1973; Marcos et al., 2004).

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Within the human fascioliasis high altitude transmission pattern related to F. *hepatica*, two different subpatterns of the general Andean pattern have been distinguished in South America according to physiographic and seasonal characteristics (Mas-Coma, 2005; Mas-Coma et al., 2009):

(a) The altiplanic subpattern, with very high prevalences and intensities in humans, endemicity distributed throughout an area of homogeneous altitude and transmission along the entire year due to high evapotranspiration rates leading lymnaeid vectors to concentrate in permanent water bodies (Mas-Coma et al., 1999), e.g. the northern Bolivian Altiplano (Esteban et al., 1997a,b, 1999) and the Peruvian Altiplano of Puno (Esteban et al., 2002).
(b) The valley subpattern, with very high prevalences but somewhat lower intensities in humans, endemicity distributed throughout an area of heterogeneous altitude, including prevalences correlating with altitude (Gonzalez et al., 2011), and seasonal transmission related to climate (Claxton et al., 1997, 1999),e.g. the valleys of Cajamarca and Mantaro, both in Peru.

In recent years, the availability of a very effective drug against fascioliasis, triclabendazole (Savioli et al., 1999), prompted WHO to launch a worldwide initiative against human fascioliasis (WHO, 2007, 2008). This initiative focuses on human fascioliasis endemic areas presenting different epidemiological situations and transmission patterns (Mas-Coma, 2005). Bolivia and Peru were selected for priority intervention due to the very large public health problem posed by this disease. Various pilot schemes were designed to assess the best control strategies according to the different epidemiological situations and transmission patterns. The northern Bolivian Altiplano and Cajamarca province were selected as models of the altiplanic and valley patterns of human hyperendemic areas, respectively.

The present study is a further step in the characterization of fascioliasis in human hyperendemic areas of the valley pattern, furnishing the baseline on which to design appropriate control measures for each South American disease transmission pattern. Although metacercarial infectivity does not appear to differ in isolates from different livestock species (Valero and Mas-Coma, 2000), the animal species affected pronouncedly influences the phenotype of both adult stage and egg of the liver fluke, mainly due to the different size of the liver duct microhabitat (Valero et al., 2001a, b, 2002, 2009). Therefore, a computer image analysis system (CIAS) method (Valero et al., 2005) was applied to flukes from sheep only, considering the typical host species for *F. hepatica* worldwide. Studies were performed on the basis of standardized measurements known to be useful for the differentiation of both fasciolid species (Periago et al., 2006). To complete the characterization, all these South American highland populations were compared to standard lowland populations of (i) *F. hepatica* natural infection from Valencia, Spain, and (ii) *F. hepatica* experimental adults from Bialowieza National Park, Poland.

2. Materials and methods

2.1. Parasites

Post-mortem examinations were carried out on all sheep (*Ovisaries*) immediately after death. The liver fluke in the definitive host presents parasite migration from the liver parenchyma to the adult location in the bile duct. Valero et al. (2006) modelled through a logistic model the liver fluke body growth, characterized by two phases: the 'exponential' part of logistic growth corresponding to body development during migration in the abdominal cavity and liver parenchyma as well as to development and sexual maturation in the biliary duct system up to the onset of egg production. From this moment, development follows the 'saturated' part of logistic growth, i.e. entry into the bile duct induces maturation and egg production. In this sense, bile ducts were examined and only flukes inside were analysed. In this study only fasciolids with eggs in the uteri were included ($n = 542$). The samples analysed in each of the five geographical areas included the largest possible worm variability (different stages of maturity, body size and gravid uteri). Adult worms were fixed in Bouiń s solution between slide and coverglass but without coverglass pressure to avoid distortion. The specimens were stained with Grenacheŕs borax, differentiated, dehydrated and finally mounted in Canada balsam.

2.1.1. Natural F. hepatica infections

All the sheep analysed were adults originating from areas close to the slaughterhouses where the parasite material was obtained:

(A) Material from Cajamarca Valley (Peru): *F. hepatica* adults from 8 Merino sheep (130 worms; 2,2,12,15,18,21,28,32 worms per sheep, respectively), slaughterhouse of Rodicio.
(B) Material from Mantaro Valley (Peru): 47 *F. hepatica* adults from the 5 autochthonous Junin breed (47 worms; range: 3,5,8,10,21), slaughterhouses of Huayucachi, Pachacayo and Huancayo.
(C) Material from the northern Altiplano (Bolivia): *F. hepatica* adults from 12 Merino sheep (201 worms; 6,8,9,12,15,18, 19, 22,22,26,30), slaughterhouse of Batallas (altitude around 4000 m).
(D) Material from the Mediterranean coast (Spain): *F. hepatica* adults from 5 Merino sheep (37 worms, range: 2,7,8,10,10), slaughterhouse of Massamagrell (close to sea level).

2.1.2. Experimental infections with F. hepatica

Fasciola hepatica metacercariae were experimentally obtained as previously described (Mas-Coma et al., 2001). *Fasciola hepatica* and its snail host *Galba truncatula* originated from Bialowieza, Poland and Valencia Albufera, Spain, respectively. The experimental group included four four-to-five-week-old sheep (Guirra autochthonous breed) bred and kept close to sea level. The animals were infected *per os* with 200 *F. hepatica* metacercariae, respectively. Food and water were provided *ad libitum*. Permission for animal research was obtained from the Ethics Animal Research Committee of Valencia University. Animal ethics guidelines were strictly adhered to in the care of the animals. Necropsy was carried out 40 weeks post-infection. A total of 127 (20, 28, 34 and 45 worms per sheep, respectively) *F. hepatica* adults was recovered.

2.2. Measurement techniques

All standardised measurements of adults were made according to methods proposed for Fasciolidae by Valero et al. (2005) and Periago et al. (2006, 2008) (Fig. 1):

(A) Lineal biometric characters: body length (BL), maximum body width (BW), body width at ovary level (BWOv), body perimeter (BP), body roundness (BR), cone length (CL), cone width (CW), maximum diameter of oral sucker (OS max), minimum diameter of oral sucker (OS min), maximum diameter of ventral sucker (VS max), minimum diameter of ventral sucker (VS min), distance between the anterior end of the body and the ventral sucker (A-VS), distance between the oral sucker and the ventral sucker (OS-VS), distance between the ventral sucker and the union of the vitelline glands (VS-Vit), distance between the union of the vitelline

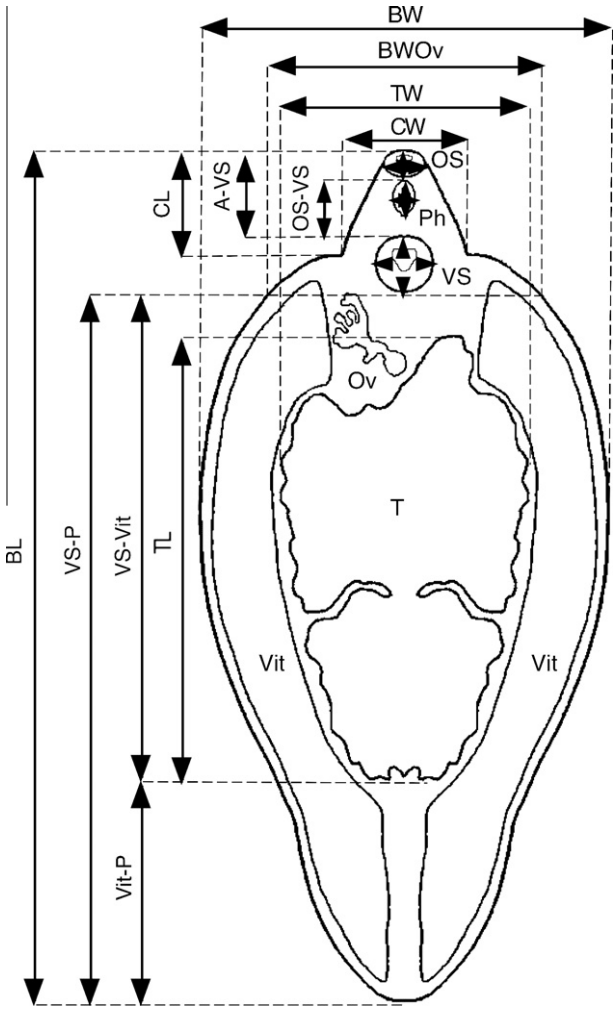

Fig. 1. Standardised measurements in gravid adults of *Fasciola hepatica*.

glands and the posterior end of the body (Vit-P), distance between the ventral sucker and the posterior end of the body (VS-P), pharynx length (PhL), pharynx width (PhW), testicular length (TL), testicular width (TW), testicular perimeter (TP).

(B) Areas: body area (BA), oral sucker area (OSA), ventral sucker area (VSA), pharynx area (PhA), and testicle area (taking both testes together, TA).

(C) Ratios: body length over body width (BL/BW), body width at ovary level over cone width (BWOv/CW), oral sucker area over ventral sucker area (OSA/VSA), and body length over the distance between the ventral sucker and the posterior end of the body (BL/VS-P).

The body roundness measurement ($BR = BP^2/4\pi BA$) was used to quantify the body shape. It is a measurement of how circular an object is (the expected perimeter of a circular object divided by the actual perimeter). A circular object will have a roundness of 1.0, while more irregular objects will have larger values (Anonymous, 2001; Ohnuma et al., 2006).

Measurements were made with a microscope and images captured by a digital camera (Nikon Coolpix) and analysed by image analysis software (ImagePro plus version 5.0 for Windows, Media Cybernetics, Silver Spring, USA).

2.3. Numerical methods

Morphological variation is quantified by geometrical morphometrics (Rohlf and Marcus, 1993), a technique offering an estimate of size by which different axes of growth are integrated into a single variable (the "centroid size"; Bookstein, 1989). The estimate of size is contained in a single variable reflecting variation in many directions, as many as there are landmarks under study, and shape is defined as their relative positions after correction for size, position and orientation. With these informative data, and the corresponding software freely available to conduct complex analyses, significant biological and epidemiological features can be quantified more accurately (Dujardin, 2008). Current statistical techniques in morphometrics make it possible to test the null hypothesis of conspecific populations being simply the allometric extension of each other, provided a common allometric trend is identifiable (Rohlf and Marcus, 1993; Klingenberg, 1996; Dujardin and Le Pont, 2004). Morphometric data was explored using multivariate analysis. Size-free canonical discriminant analysis was used on the covariance matrix of log-transformed measurements to assess morphometric variation between samples. This technique consists of removing the effect of within-group ontogenetic variation, regressing each character separately on the within-group first principal component, which is a multivariate estimate of size (Dos Reis et al., 1990). Analyses were carried out using BAC v.2 software and the PAD (Permutaciones, Analisis Discriminante) module of the CLIC program (http://www.npl.ird.fr/morphometrics) (Dujardin, 2002; Dujardin and Le Pont, 2004; Dujardin et al., 2010). Values were considered statistically significant when

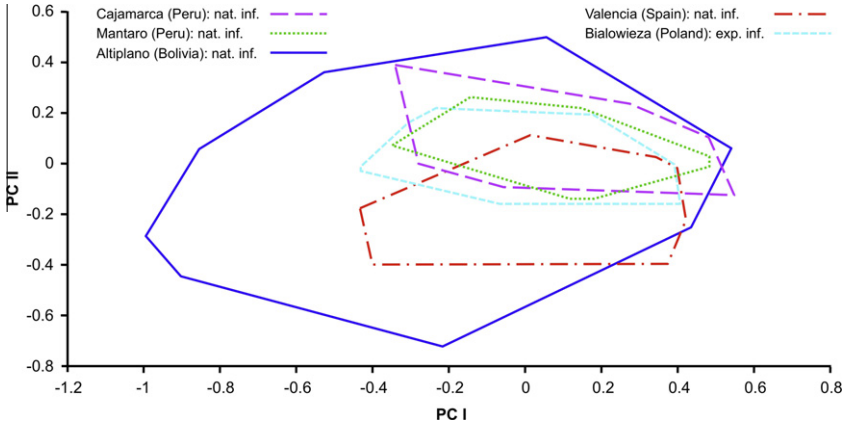

Fig. 2. Factor map corresponding to *Fasciola hepatica* specimens from naturally infected sheep from the Cajamarca and Mantaro valleys (Peru), northern Altiplano (Bolivia), Valencia (Spain) and experimentally infected sheep (*Fasciola hepatica* = Polish isolate). Samples are projected onto the first (PC1, 59%) and second (PC2, 17%) principal components. Each group is represented by its perimeter.

Table 1
Comparative morphometric data (extreme values, mean and standard deviation) of natural *Fasciola hepatica* adults infecting highland sheep from Cajamarca valley, Mantaro valley and the Northern Bolivian Altiplano, and natural and experimental *F. hepatica* from lowland sheep of Europe (n = number of individuals).

Adult measurements (mm)	Natural infection				Experimental infection
	Cajamarca Peru n = 130	Mantaro Peru n = 47	N Altiplano Bolivia n = 201	Valencia Spain n = 37	Bialowieza Poland n = 127
Body area, BA	47.34–283.95	79.89–250.58	31.11–236.14	75.09–239.13	68.09–227.11
	135.87 ± 3.73	149.30 ± 5.64	106.39 ± 3.35	142.75 ± 6.22	129.78 ± 3.17
Body length, BL	13.48–30.97	13.41–27.75	9.64–31.04	14.21–31.17	12.45–26.68
	18.86 ± 0.31	19.70 ± 0.40	18.08 ± 0.31	20.82 ± 0.64	18.52 ± 0.29
Body width, BW	5.06–14.23	7.60–13.93	4.23–13.41	7.49–12.76	6.88–12.74
	10.25 ± 0.14	10.88 ± 0.23	8.26 ± 0.14	9.75 ± 0.16	10.19 ± 0.10
BL/BW ratio	1.31–3.73	1.30–2.46	1.41–3.74	1.70–2.89	1.30–2.62
	1.86 ± 0.03	1.83 ± 0.04	2.22 ± 0.03	2.14 ± 0.05	1.82 ± 0.02
BW at ovary level, BWOv	4.23–11.97	5.48–11.31	3.51–11.71	6.45–10.57	5.69–10.17
	8.30 ± 0.11	8.50 ± 0.19	6.71 ± 0.12	8.13 ± 0.17	7.98 ± 0.08
Body perimeter, BP	30.20–71.11	33.71–64.51	26.89–68.35	39.85–71.41	33.22–66.06
	45.70 ± 0.66	48.15 ± 0.98	47.51 ± 0.72	52.90 ± 1.33	47.91 ± 0.64
Body roundness, BR	1.09–1.94	1.13–1.48	1.11–2.12	1.31–1.76	1.23–1.73
	1.25 ± 0.01	1.26 ± 0.01	1.53 ± 0.01	1.46 ± 0.02	1.43 ± 0.01
Cone length, CL	1.32–2.98	1.36–2.59	1.32–3.04	1.55–2.98	1.10–3.07
	2.11 ± 0.02	1.89 ± 0.04	2.12 ± 0.02	2.10 ± 0.06	2.01 ± 0.03
Cone width, CW	2.12–4.41	2.30–4.21	1.78–3.92	2.46–4.12	2.08–4.19
	3.17 ± 0.04	3.04 ± 0.06	2.65 ± 0.03	3.25 ± 0.06	3.27 ± 0.03
BWOv/CW ratio	1.79–3.72	1.86–3.94	1.53–3.83	2.03–3.90	1.86–3.81
	2.64 ± 0.03	2.82 ± 0.07	2.53 ± 0.03	2.51 ± 0.05	2.46 ± 0.03
Oral sucker area, OSA	0.20–0.67	0.19–0.72	0.21–0.66	0.33–0.66	0.24–0.53
	0.46 ± 0.01	0.42 ± 0.01	0.38 ± 0.01	0.44 ± 0.01	0.39 ± 0.01
Maximum diameter of the oral sucker, OS_{max}	0.63–1.14	0.61–1.02	0.53–1.06	0.63–1.00	0.70–1.01
	0.84 ± 0.01	0.81 ± 0.01	0.76 ± 0.01	0.84 ± 0.01	0.86 ± 0.01
Minimum diameter of the oral sucker, OS_{min}	0.36–0.85	0.29–0.90	0.42–0.86	0.52–0.86	0.33–0.74
	0.66 ± 0.01	0.62 ± 0.02	0.63 ± 0.01	0.67 ± 0.01	0.57 ± 0.1
Ventral sucker area, VSA	0.53–1.22	0.49–1.26	0.44–1.23	0.95–1.35	0.56–1.31
	0.89 ± 0.01	0.82 ± 0.02	0.78 ± 0.01	1.13 ± 0.01	0.98 ± 0.01
Maximum diameter of the ventral sucker, VS_{max}	0.89–1.29	0.83–1.31	0.75–1.25	0.92–1.40	0.89–1.40
	1.12 ± 0.01	1.08 ± 0.01	1.00 ± 0.01	1.19 ± 0.02	1.19 ± 0.01
Minimum diameter of the ventral sucker, VS_{min}	0.77–1.21	0.72–1.22	0.67–1.29	0.68–1.48	0.79–1.23
	0.99 ± 0.01	0.95 ± 0.01	0.98 ± 0.01	1.06 ± 0.03	1.04 ± 0.01
OSA/VSA ratio	0.23–0.76	0.31–0.69	0.31–0.71	0.27–0.51	0.24–0.58
	0.52 ± 0.01	0.51 ± 0.01	0.49 ± 0.01	0.39 ± 0.01	0.41 ± 0.01
Distance between the anterior end of the body and the ventral sucker, A-VS	1.61–3.26	1.75–2.88	1.52–3.35	1.66–3.15	1.16–3.09
	2.48 ± 0.02	2.29 ± 0.04	2.24 ± 0.02	2.32 ± 0.05	2.13 ± 0.03
Distance between the oral sucker and the ventral sucker, OS-VS	1.19–2.40	1.16–2.15	0.87–2.56	1.13–2.49	0.64–2.50
	1.81 ± 0.02	1.66 ± 0.03	1.60 ± 0.02	1.65 ± 0.05	1.56 ± 0.03
Distance between the ventral sucker and the union of the vitelline glands, VS-Vit	6.78–20.60	8.11–17.36	4.49–19.82	9.65–22.97	7.71–19.75
	11.56 ± 0.21	12.30 ± 0.30	11.32 ± 0.22	13.84 ± 0.47	12.33 ± 0.23
Distance between the union of the vitelline glands and the posterior end of the body, Vit-P	2.69–9.47	2.64–8.09	0.49–9.83	1.12–6.89	1.99–7.02
	4.91 ± 0.12	5.20 ± 0.19	3.76 ± 0.14	3.45 ± 0.20	4.35 ± 0.09
Distance between the ventral sucker and the posterior end of the body, VS-P	11.39–28.37	11.27–25.36	7.11–27.39	11.21–26.84	10.43–24.66
	16.47 ± 0.30	17.50 ± 0.43	15.07 ± 0.31	17.29 ± 0.58	16.68 ± 0.29
BL/VS-P ratio	1.08–1.22	1.09–1.19	0.88–1.42	1.15–1.27	1.04–1.20
	1.15 ± 0.03	1.13 ± 0.00	1.22 ± 0.01	1.21 ± 0.00	1.11 ± 0.00
Pharynx area, PhA	0.12–0.35	0.13–0.31	0.05–0.34	0.15–0.31	0.11–0.34
	0.21 ± 0.00	0.20 ± 0.01	0.17 ± 0.003	0.22 ± 0.01	0.20 ± 0.00
Pharynx length, PhL	0.55–0.96	0.58–0.91	0.37–0.93	0.59–0.89	0.51–1.07
	0.78 ± 0.01	0.75 ± 0.01	0.68 ± 0.01	0.74 ± 0.01	0.77 ± 0.01

Pharynx width, PhW	0.24–0.55	0.25–0.48	0.18–0.50	0.30–0.51	0.26–0.56
	0.36 ± 0.00	0.36 ± 0.01	0.34 ± 0.00	0.40 ± 0.01	0.36 ± 0.00
Testicular area, TA	13.10–112.85	22.54–71.71	9.31–89.48	29.90–87.37	24.12–89.10
	46.25 ± 1.32	48.33 ± 1.92	37.43 ± 1.35	46.27 ± 2.35	50.99 ± 1.29
Testicular length, TL	4.54–23.53	5.56–12.70	3.12–14.62	6.03–14.53	5.33–14.50
	8.86 ± 0.20	9.05 ± 0.26	8.12 ± 0.17	9.39 ± 0.32	8.92 ± 0.17
Testicular width, TW	3.12–9.07	4.84–9.87	2.84–8.97	5.08–9.38	4.66–9.16
	6.84 ± 0.09	7.01 ± 0.16	5.69 ± 0.10	6.42 ± 0.16	7.37 ± 0.07
Testicular perimeter, TP	16.76–48.75	20.08–41.44	13.06–48.53	20.81–44.36	22.88–47.93
	28.71 ± 0.44	30.68 ± 0.75	26.94 ± 0.56	30.93 ± 0.82	33.13 ± 0.49

$P < 0.05$. Non-redundant measurements were used (i.e. one is not included in another one): BL, BW, BP, OS max, OS min, VS max, VS min, A-VS, VS-Vit, Vit-P, PhL, PhW and TL, where at least one dimension of the most important morphological structures was included. These remaining variables were all significantly correlated with the first principal component (PC1), which contributed 59% to overall variation. PC1 could therefore be accepted as a general indicator of size (Bookstein, 1989), so that the resulting factor maps (Fig. 2) clearly illustrate global size differences in the populations analysed. The influence of within-group allometries was then removed by using variables equivalent to an orthogonal projection of the data onto the first common principal component (i.e. all the common principal components except the first one). The resulting "allometry-free", or size-free, variables were submitted to a canonical variate analysis (CVA), and Mahalanobis distances were derived (Mahalanobis, 1936; Dujardin and Le Pont, 2004). Size variation was based on one variable only, i.e. the first common principal component, thus the univariate equivalent of Mahalanobis distance known as Pearson's "Coefficient of Racial Likeliness" (Dujardin and Le Pont, 2004) was used to estimate its variation. The statistical significance of relationships of these distances with altitudinal variation and geographic distances (km) was explored by a non-parametric test. Additionally, the statistical significance of relationships of average of PC1 of liver flukes from each sheep and fluke burden was explored by a non-parametric test.

3. Results

The morphometric values of *F. hepatica* from the highlands of the Cajamarca valley, Mantaro valley and Northern Bolivian Altiplano, as well as from the lowlands of Valencia and *F. hepatica* experimental material are shown in Table 1. The results shown in Table 1 are the first phenotypic description of the *F. hepatica* adult from Peru. Furthermore, the present study shows, for the first time, the results of body roundness (BR) of *F. hepatica* populations from sheep. Within-comparison of the values of the *F. hepatica* populations shows a general overlap between them, regardless of the geographical area of origin.

The size of liver fluke adults was studied by multivariate analyses. A scatter plot of the first two principal components (PC) is shown in Fig 2. The first common principal component (CP1) of the five populations can be interpreted as a measure of overall size. The results show that *F. hepatica* populations of the Cajamarca and Mantaro valleys (Peru), Spain as well as the experimental *F. hepatica* standard population have a similar maximum size and similar minimum size. On the contrary, the northern Bolivian Altiplano population shares its maximum size with the aforementioned *F. hepatica* populations but presents a lower minimum size (Fig. 2). Our results indicate that liver flukes from Peru and Europe (natural and experimental) have a common minimum size from which the parasites begin to be gravid, however, this minimum size is smaller in Bolivian liver flukes.

There was no consistent relationship between the size-free pattern of variation and altitudinal differences (Fig. 3). No significant correlation between Mahalanobis distances and geographic distances (in km) was detected (data not shown). No significant correlation between CP1 liver fluke average and worm burden was detected (data not shown).

4. Discussion

Quantitative morphological variation informs about both genetic variation and external influences (Dujardin et al., 2009). In the case of endoparasites, it is necessary to distinguish between macro habitat (external environment according to geography) and

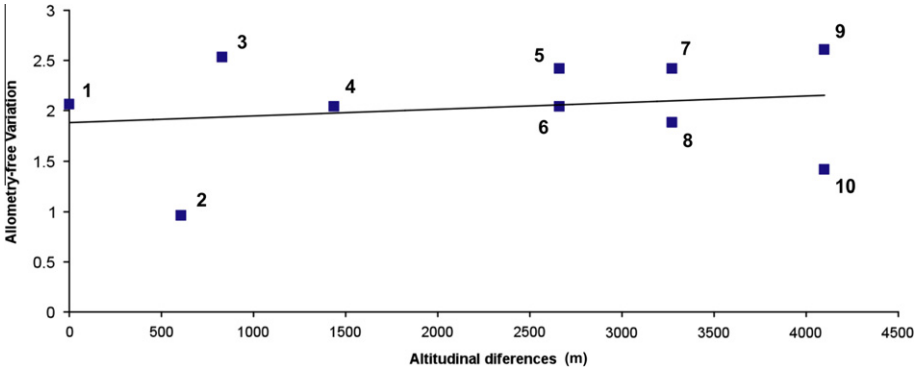

Fig. 3. Plots of the pairwise Mahalanobis distances derived from allometry-free variables (vertical axis) against the corresponding altitudinal differences (horizontal axis in m). The straight line represents the linear regression prediction. 1 = naturally infected sheep from Valencia (Spain) – experimentally infected sheep (Polish isolate); 2 = naturally infected sheep from the Cajamarca – Mantaro valleys (Peru); 3 = naturally infected sheep from the Mantaro valley (Peru) – northern Altiplano (Bolivia); 4 = naturally infected sheep from the Cajamarca valley (Peru) – northern Altiplano (Bolivia); 5 = naturally infected sheep from the Cajamarca valley (Peru) – Valencia (Spain); 6 = naturally infected sheep from the Cajamarca valley (Peru) – experimentally infected sheep (Polish isolate); 7 = naturally infected sheep from the Mantaro valley (Peru) – Valencia (Spain); 8 = naturally infected sheep from the Mantaro valley (Peru) – experimentally infected sheep (Polish isolate); 9 = naturally infected sheep from the northern Altiplano (Bolivia) – experimentally infected sheep (Polish isolate); 10 = naturally infected sheep from the northern Altiplano (Bolivia) – Valencia (Spain).

microhabitat (parasitized organ inside the host). The geographical environments analysed in this study included highland and lowlands. It is worth mentioning that in a very high altitude environment, oxygen and air density decrease, temperature and humidity are low, and there is an increase in radiation. These environmental factors exert an influence on mammals, so that animals born and living at very high altitude show morphological and physiological characteristics different from those shown by mammals inhabiting low altitudes (Jackson et al., 1987; Frisancho and Frisancho, 1992).

The results of this study show that there is no apparent relationship between the shape of fasciolid adults with regard to altitudinal difference or geographical origin. However, results obtained in the principal component analysis show that Andean valley populations and European *F. hepatica* natural and experimental lowland populations present a phenotypic homogeneity. The results show that corporal minimal size of gravid adults is similar in the material proceeding from Peruvian Valley and lowland Europe. On the other hand, the Altiplano population shows a lower minimum size when the adult reaches gravidity. This means that *F. hepatica* from the Altiplano reaches uterus gravidity earlier in its growth within the liver microhabitat (Fig. 2).

Additionally, uterus size and body size have been demonstrated to be proportional (Valero et al., 2001b). It may be concluded that flukes from the Cajamarca and Mantaro valleys present a minimum uterus size larger than that in flukes from the Altiplano population.

Thus, the present results demonstrate that flukes from Andean endemic areas may also be catalogued according to two phenotypic patterns:

- *The* valley pattern: Andean human endemic areas of high altitude (Cajamarca: 2500–3100 m; Mantaro: 3400–3500 m) where *F. hepatica* egg production by the fluke oogenotop and minimum body size when reaching gravidity are similar to those in lowland populations of Europe (Spain, Poland).
- *The altiplanic* pattern: Andean human endemic areas of very high altitude (northern Altiplano: 3800–4100 m) where *F. hepatica* egg production by the fluke oogenotop begins earlier and minimum body size when reaching gravidity is smaller than in lowland populations of Europe (Spain, Poland) as well as in Andean valleys (Cajamarca, Mantaro).

In other organisms such as insects, allometry-free shape may require important external changes to be significantly modified. For instance, in sandflies Dujardin and Le Pont (2004) detected that allometry-free variation was closely associated with altitudinal differences indicating its possible link with temperatures and/or vegetation cover. This finding is in contrast with the results obtained in our study, in which allometry-free shape appears as a more stable trait than size in fasciolid species in conditions of different altitudes and latitudes.

Several mammals may serve as definitive hosts for *F. hepatica* although there is considerable variation in the susceptibility and pathology according to species (Mas-Coma et al., 2009). By using an allometric model, it was shown that the definitive host species (sheep, cattle, pig) decisively influences the size of *F. hepatica* adults, although these influences disappear in a rodent definitive host model when experimentally infected with the different host isolates (Valero et al., 2001a). However, the existence of certain morphological features (allometric differences in BW vs. BL and BL vs. VS-P) characterizing *F. hepatica* adults in different host species was demonstrated (Valero et al., 2001a). Similarly, PC analysis of *F. hepatica* egg size shows characteristic morphometric traits in each definitive host species (Valero et al., 2009).

Parasite burden is another environmental factor that affects size variation in fasciolids. The crowding effect in *F. hepatica* adults is reflected in a reduction of the maximum morphometric value (ym) of the ontogenetic trajectories of body area, BP, BL and BW due to a reduction in the growth rate (Valero et al., 2006). Thus, in rats, worm size appears to be dependent on the level of infection and worm size decreases when the burden increases. This phenomenon is not present in light infection in sheep, with burdens equal or below 45 adults/sheep.

In natural populations, only slight differences could be found in allometric models (BL, BW, P vs. BA or BL) between *F. hepatica* adults from Bolivian highland and Spanish lowland fluke populations infecting sheep (Valero et al., 1999). Nevertheless, Bolivian Altiplano sheep and cattle liver fluke populations proved to have a smaller uterus area than European fluke populations in the same host species (Valero et al., 2001b).

The life cycle of *F. hepatica* occurs in alternate aquatic and terrestrial ecosystems. Eggs are emitted by a definitive terrestrial host, but egg development occurs in fresh water and larval stage development then occurs in aquatic and amphibious lymnaeid snails (Mas-Coma, 2005). The emission of *F. hepatica* eggs in faeces is usually subject to oscillations along time in animals as well as humans. An example of the adaptation of the chronobiology of liver-fluke egg shedding to seasonality was given by Valero et al. (2002). Thus, the black rat naturally infected with the liver fluke in the Mediterranean island of Corsica shows continuous egg shedding with minimal values in summer, when egg development is

most difficult (in the Mediterranean area in the summer months, evapotranspiration exceeds rainfall), and maximum level shedding peaks in spring and the autumn months, when there is substantial rainfall and a sufficiently wet environment (Valero et al., 2002). European populations of *G. truncatula* are markedly amphibious (Euzeby, 1971). On the contrary, altiplanic *G. truncatula* specimens are more aquatic and only rarely encountered in mud, out of water. They mainly inhabit permanent water bodies, never temporary water bodies such as those originating from rain during the rainy season (October–March). Temporary water bodies originating from rain do not remain long enough to enable colonization by lymnaeids and even in rainy years the dry season is too long to allow the survival of buried and lethargic lymnaeids. This is related to the high evapotranspiration rates proper to the high altitude, as well as to the long duration of the dry season coincident with the lowest temperatures of the year (April–September). Thus, lymnaeids scattered throughout patchily flooded pastures, which constitute the most important transmission foci in central Europe, are almost never found in the Altiplano. Similarly, the relationship of fascioliasis with rainy periods, well known in the northern hemisphere both in animals and humans (Chen and Mott, 1990), cannot be applied to the northern Altiplano. In the Altiplano, the existence of lymnaeid populations in the transmission foci throughout the whole year enables parasite transmission during all seasons. In the northern Bolivian Altiplano, seasonal differences in mean temperature are very low, only 5.0–6.5 °C. Great temperature differences, from 10.3 to 13.0 °C (on the shores of Lake Titicaca), respectively, from 13.4 to 22.4 °C (far from the Lake) occur within a day. Moreover, the lack of trees and shrubs implies no shade and hence direct intense sunshine, which increases the temperature of water bodies at midday (daily temperature range in water bodies inhabited by lymnaeids: from 3.0–10.0 °C at night to 15.0–28.9 °C during the day) (Mas-Coma et al., 1999; Fuentes et al., 1999).

Studies performed in the northern Bolivian Altiplano showed that there is no phase of the life cycle in which liver fluke development appears to be modified in such a way that transmission is reduced. However, given aspects seem to favour transmission, such as the longer cercarial shedding period and the higher cercarial production. These two aspects appear to be related to a longer survival of infected lymnaeid snails. When compared to lowlands, these differences may be interpreted as adaptation strategies to high altitude conditions (Mas-Coma et al., 2001). Our results indicate that the liver-fluke adult populations from Peru and Europe (natural and experimental) show a common minimum size from which the parasites begin to be gravid, while this minimum size is smaller in Bolivian highland populations. This difference may be attributable to intraspecific variability or may be related to the influence of high altitude. The divergence detected between the altiplanic population and the rest of populations analysed is difficult to understand, as evolutionary advantages cannot be easily envisaged. It is known that oxygen is required for egg production in *F. hepatica* (Mansour, 1958; Bjorkman and Thorsell, 1963; McGonigle and Dalton, 1995) (the transit from high altitude to very high altitude is usually noted to be at 3200 m). Thus, high altitude hypoxia may be the origin for a reduced egg production by the flukes. Moreover, the uterus in digeneans has traditionally not been considered a storage organ but mainly an organ adapted to the developmental time of the eggs (in fasciolids, eggs are laid unembryonated and the miracidium begins its development in eggs once in freshwater). Valero et al. (2011) have demonstrated that there is a direct relation between *F. hepatica* uterus size and the number of eggs shed per gram of faeces. In the valley transmission pattern and European lowland pattern, a larger storage capacity of the uterus becomes more useful due to the typical seasonal transmission, with continuous egg shedding but with oscillations, with minimum values in unfavourable seasons, and maximum shedding peaks in favourable seasons. As above-mentioned, in the northern Bolivian Altiplano, the climatic conditions, freshwater characteristics and lymnaeid ecology enable fascioliasis transmission to take place throughout the year (Fuentes et al. 1999; Mas-Coma et al. 1999), earlier egg shedding and smaller uterus storage space may be related to permanent transmission including snail vector population availability throughout the year. Further research is presently being carried out to ascertain whether the morphometric differences detected in the minimum size of gravid Bolivian *F. hepatica* adults are associated with transmission pattern differences.

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Assessing the validity of an ELISA test for the serological diagnosis of human fascioliasis in different epidemiological situations

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Abstract

OBJECTIVES To improve the diagnosis of human fascioliasis caused by *Fasciola hepatica* and *Fasciola gigantica*, we evaluated the diagnostic accuracy of an enzyme-linked immunosorbent assay (ELISA), with *Fasciola* antigen from the adult liver fluke, for the detection of IgG against fascioliasis in human sera.

METHODS The sera of 54 fascioliasis cases, originating from three endemic areas, were used in this evaluation: (i) a hyperendemic *F. hepatica* area where humans usually shed a great number of parasite eggs in faeces (11 sera); (ii) an epidemic *F. hepatica* area where humans usually shed small amounts of parasite eggs (24 sera) and (iii) an overlap area of both *Fasciola* species and where human shedding of parasite eggs in faeces is usually scarce or non-existent (19 sera). One hundred and sixty-eight patients with other parasitic infections and 89 healthy controls were also analysed.

RESULTS The respective sensitivity and specificity of this assay were 95.3% (95% confidence intervals, 82.9–99.2%) and 95.7% (95% confidence intervals, 92.3–97.5%). No correlation between egg output and the OD450 values of the *F. hepatica* IgG ELISA test was observed.

CONCLUSIONS This test could be used both as an individual serodiagnostic test for human fascioliasis when backed up by a compatible clinical history together with a second diagnostic technique for other cross-reactive helminth infections, and in large-scale epidemiological studies of human fascioliasis worldwide.

keywords sensitivity and specificity, *Fasciola hepatica*, *Fasciola gigantica*, IgG, ELISA, serodiagnosis

Introduction

Fascioliasis is a parasitic disease caused by liver fluke species of the genus *Fasciola*: *Fasciola hepatica* and *Fasciola gigantica*. While *F. hepatica* is found on a global scale, *F. gigantica* can be found in humans and animals in tropical regions of Africa and Asia (Mas-Coma *et al.* 2009a). However, in some parts of Africa and Asia, the two species overlap (Ashrafi *et al.* 2006; Periago *et al.* 2008; Mas-Coma *et al.* 2009a). Today, fascioliasis is considered an important human disease and several geographic areas have been described as endemic for the disease in humans, including hypoendemic (prevalence < 1%), mesoendemic (prevalence between 1% and 10%) and hyperendemic (> 10%) situations, with intensities ranging from low to very high, and estimates of up to 17 million people infected worldwide. This may even be an underestimate if the total lack of data from numerous Asian and African countries is considered (Mas-Coma *et al.* 1999).

Moreover, in the last two decades, human fascioliasis has been emerging in many regions, a phenomenon that has partly been related to climate change (Mas-Coma *et al.* 2009b). Recent studies have shown that the high pathogenicity of this disease is not only restricted to the acute phase, but also to long-term liver fluke infection (Valero *et al.* 2000, 2003, 2006, 2008), the immune-modulation of fasciolids in the acute phase (Brady *et al.* 1999), and their immune suppression effect in chronic and advanced chronic phases (Gironès *et al.* 2007). All this appears to be in the background of usual co-infections with other parasitic and infectious diseases (Mas-Coma *et al.* 2005). Considering this scenario of increasing concern, WHO decided to launch a worldwide initiative against this

disease (WHO 2007, 2008). Therefore, the capacity to diagnose human fascioliasis correctly becomes imperative.

The present study assesses a qualitative microtiter strip-based enzyme-linked immunosorbent assay (ELISA) for the detection of IgG class antibodies against *F. hepatica* in human serum, a solid phase enzyme immunoassay based on the sandwich principle, known as 'DRG *Fasciola hepatica* IgG (human) ELISA', in the following situations: (i) a hyperendemic *F. hepatica* area where humans usually shed large amounts of parasite eggs in faeces, (ii) an epidemic *F. hepatica* area where humans usually shed small amounts of parasite eggs and (iii) an overlap area of both *Fasciola* species where human shedding of parasite eggs in faeces is usually scarce or non-existent. Fascioliasis positivity of the samples used had been verified through egg detection (Kato–Katz technique) or the ELISA Cathepsin L1 (CL1) protease test. ELISA CL1 is used here as the serological gold standard method in samples from areas where humans usually shed small amounts of parasite eggs or where parasite eggs in faeces are usually scarce or non-existent.

ELISA CL1 was previously successfully employed for the diagnosis of human fascioliasis in the northern Bolivian Altiplano (O'Neill *et al.* 1998) and in Iran (Rokni *et al.* 2002), showing it to be highly specific (100%) and sensitive (100%).

Material and methods

Clinical samples

Sera were obtained from the serum bank of the Department of Parasitology, Faculty of Pharmacy, University of Valencia, Spain (2004–2008). Each serum was aliquoted and stored at −80 °C until used. The sera were classified in three groups: (i) 54 positive fascioliasis sera through ELISA CL1 and eggs in faeces quantified through the Kato–Katz technique, originating from three different epidemiological situations; (ii) 168 serum samples from individuals with parasitic/fungal diseases other than fascioliasis (to evaluate potential cross-reactivity) were obtained from clinically, serologically and sometimes parasitologically confirmed cases and (iii) 89 sera from parasitologically negative cases were used from serum collections stored at Centro Nacional de Microbiología (ISCIII, Madrid, Spain) and Servicio de Microbiologia, Hospital Universitario y Politecnico 'La Fe' (Valencia, Spain). The age and gender characteristics of the individuals are detailed in Table 1.

ELISA Cathepsin L1

Cathepsin L1 (CL1) was obtained from the Molecular Parasitology Laboratory, School of Biotechnology, Faculty of Health and Science, Dublin City University, Dublin, Ireland. Application of the ELISA CL1 test was carried out as previously described (O'Neill *et al.* 1999). Briefly, 100 μl of CL1 antigen (5 μg/ml) were incubated overnight at 37 °C in wells of microtiter plates (Nuclon, Kamstrup, Roskilde, Denmark). The wells were blocked with a solution of 2% bovine serum albumin/0.1% Tween 20 in phosphate-buffered saline for 30 min at 37 °C, and human sera (1:100 dilution) were then added into duplicate wells. Bound human antibodies were detected using biotin-conjugated anti-human IgG4 (1:2,000 dilution), avidin-conjugated alkaline phosphatase, and the substrate 2,2-azino-bis(3-ethylbenzthiazoline-6-suflonic acid) (all from Sigma Chemical Co., Poole, Dorset, UK). Plates were read

Table 1 Gender and age characteristics of individuals whose serum samples were used in the validation

	Total		Male		Female		Ind.	
	N	Average SD (min–max)	N	Age Average SD (min–max)	*N*	Age Average SD (min–max)	*N*	Age Average SD (min–max)
Individuals positive fascioliasis	54	(36.30 ± 1.09) (7–75)	25	(34.25 ± 1.21) (7–74)	29	(38.00 ± 1.00) (10–75)	–	–
Individuals with parasitic/fungal diseases other than fascioliasis	168*	(38.15 ± 1.07) (1–89)	82	(40.42 ± 1.11) (1–89)	83	(36.05 ± 1.03) (1–89)	3	31.50 ± 0.67 (23–40)
Individuals parasitologically negative	89	(40.28 ± 0.81) (1–73)	55	(42.68 ± 0.68) (22–73)	33	(36.33 ± 0.95) (1–73)	1	Unknown Unknown
Total								

*Amoebiasis 12; leishmaniasis 3; malaria 12; toxoplasmosis 10; schistosomiasis 15; hydatidosis 15; hymenolepiasis 1; taeniasis/cysticercosis 15; trichinellosis 15; strongyloidiasis 18; ascariasis 1; toxocariasis 8; anisakiasis 25; loasis 2; gnathostomiasis 10; trichuriasis and ascariasis coinfection 1; malaria, trichuriasis and ascariasis coinfection 2; *Entamoeba coli*, trichuriasis, ascariasis and ancylostomiasis coinfection 1; trichuriasis, ancylostomiasis and enterobiasis coinfection 1; histoplasmosis 1.

after 20 min on a multiscan ELISA plate reader at an absorbance of 405 nm (Bio-Rad, Model 680, Hercules, CA, USA). The cut-off point between the clusters was set at 3.09 standard deviations from the mean of the control sera absorbance and was 0.098.

DRG kit

DRG Instruments GmbH, Germany, provided all commercial kits used in this study. The test is based on Excretion/Secretion antigens of *F. hepatica* predominantly containing fluke cysteine proteases (Salimi-Bejestani *et al.* 2005). All steps were carried out according to the manufacturer's instructions. Human sera, negative and positive controls and cut-off control (CO) (1:100 dilution) were added into triplicate wells. Plates were read after 15 minutes on a multiscan ELISA plate reader at an absorbance of 450 nm (Bio-Rad, Model 680, Hercules, CA, USA). The test run may be considered valid if substrate blank has an absorbance value below 0.100, negative control below 0.200, CO control between 0.250 and 0.750 and positive control above 0.600. The results are given as the mean of the optical density (OD) obtained from triplicate samples expressed as a percentage of the CO, using the following formula:

$$Percent\ positive(PP) = ((Mean\ OD\ of\ test\ sample)/ Mean\ OD\ of\ C+) \times 100.$$

A serum is considered positive when its absorbance value is above 10% of CO.

The results in DRG Units (DU) were calculated according to the following formula:

$$DU = (sample\ (mean)\ absorbance\ value \times 10)/CO.$$

The results were negative if DU < 9, and positive if DU > 11.

To avoid any bias, all sera when being processed were blinded with regard to the adscription of the aforementioned three groups (fascioliasis positive, other parasitoses or negative sera).

Ethical aspects

The present study was approved by University of Valencia Ethics Committee.

Data analysis

The diagnostic sensitivity and specificity values were calculated (Fletcher & Fletcher 2005) with its 95% confidence interval (95% CI; EPIINFO). The positive predictive value (PPV) was calculated using the following formula:

$$PPV = (\text{sensitivity} \times \text{prevalence})/(\text{sensitivity} \times \text{prevalence}) + ((1 - \text{specificity}) \times (1 - \text{prevalence})).$$

The negative predictive value (NPV) was calculated using the following formula:

$$NPV = (\text{specificity} \times (1 - \text{prevalence}))/(((1 - \text{sensitivity}) \times \text{prevalence}) + ((\text{specificity}) \times (1 - \text{prevalence}))).$$

Student's *t*-test was calculated using SPSS software.

Results

The mean of DU values for all serum groups is shown in Figure 1A,B. DU values were significantly higher in the proven fascioliasis group than in the groups of parasitic diseases other than fascioliasis and the healthy controls, respectively ($P < 0.001$).

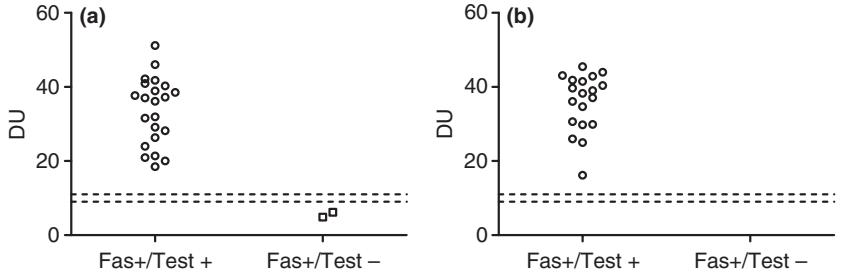

Figure 1 Mean DRG Units (DU) obtained with the DRG *F. hepatica* IgG ELISA test in fascioliasis patients from different epidemiological situations. (A) Endemic *F. hepatica* area where humans usually shed small amounts of parasite eggs; (B) an overlap area of both *Fasciola* species where human shedding of parasite eggs in faeces is usually scarce or non-existent. Data points are the mean optical density (OD) obtained from triplicate samples expressed as a percentage of the cut-off (CO), using the following formula:
Percent positive(PP) = ((Mean OD of test sample)/Mean OD*of*C+) × 100. A serum is considered positive when its absorbance value is above 10% of CO. The results in DRG Units (DU) were calculated according to the following formula:
DU = (sample (mean) absorbance value × 10)/CO. The results were negative if DU <9, and positive if DU > 11 (dotted line).

The numbers of true positive and false negative test results in fascioliasis cases of humans with and without detection of eggs in their faecal samples are provided in Table 2.

In the samples from a hyperendemic *F. hepatica* area egg detection in faeces through the Kato-Katz technique was used as reference standard, and the sensitivity of the *F. hepatica* IgG ELISA test was 100% (67.9–100%). No correlation between egg output – a measure of infection intensity – and the OD450 values of the *F. hepatica* IgG ELISA test was observed in this study area (Figure 2).

In the samples from an epidemic *F. hepatica* area CL1 ELISA was used as reference standard. Sensitivity was 91.7% (95% confidence intervals, 71.5–98.5%), detecting 2 false negative cases of which one was egg positive in faeces, while the other was not.

In the samples from an overlap area of both *Fasciola* species CL1 ELISA was also used as reference standard. Sensitivity was 100% (95.9–100%). Populations of the two latter endemic areas were grouped as result of the ELISA CL1 and the *F. hepatica* IgG ELISA test evaluation as follows: fascioliasis positive cases by CL1 (Fas+)/seropositive by *F. hepatica* IgG ELISA test (test+) and fascioliasis positive cases by CL1 (Fas+)/seronegative by *F. hepatica* IgG ELISA test (test-) (Figure 1A,B). Statistical differences between the results for sensitivity values from the two latter situations were not found, and true positive cases (41) and false negative cases (2) for these two areas together were used for the calculation of sensitivity, presenting a value of 95.3% (82.9–99.2%).

Specificity was calculated from the results in Table 2 providing the numbers of false negative and true positive test results in infected sera other than fascioliasis and negative cases. These results can be grouped as follows:

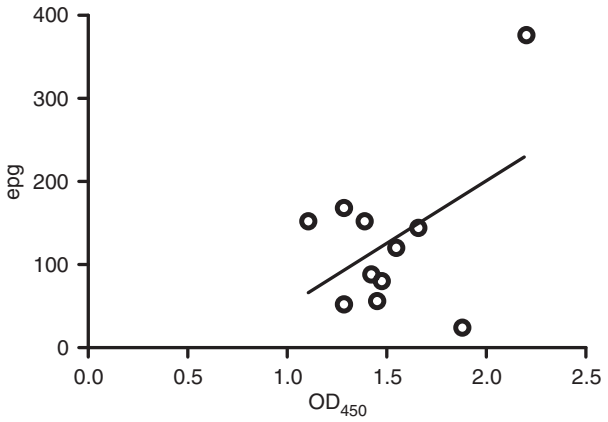

Figure 2 DRG *F. hepatica* IgG ELISA test and intensity of *F. hepatica* infection. Data points represent the mean absorbance at 450 nm from copropositive patients from a hyperendemic *F. hepatica* area where humans usually shed a great deal of parasite eggs in faeces. epg = egg count per gram of faeces.

infected positive cases other than fascioliasis (infected+)/seropositive by *F. hepatica* IgG ELISA test (test+) and infected positive cases other than fascioliasis (infected+)/seronegative by *F. hepatica* IgG ELISA test (test-) (Figure 3A). Table 2 also shows the total number of sera without fascioliasis, and these values were used to calculate specificity, presenting a value of 95.7% (92.3–97.5%). Ten serum samples from a heterologous infection cross-reacted with the kit, mainly originating from patients with helminthiases, namely schistosomiasis (4 samples), hydatidosis (2 samples), taeniasis/cysticercosis (1 sample), trichinosis (1 sample), strongyloidiasis (1 sample), and histoplasmosis (1 sample) (see Figure 3B). Theoretical PPVs and NPVs vs fascioliasis prevalence are represented in

Table 2 Diagnostic characteristics of the *F. hepatica* IgG ELISA test applied to fascioliasis patients' sera from different disease endemic areas, sera from patients with helminthiases other than fascioliasis and negative sera from healthy subjects

	True positive	False negative	True negative	False positive
Hyperendemic *F. hepatica* area where humans usually shed a great deal of parasite eggs in faeces*	11	0	–	–
Epidemic *F. hepatica* area where humans usually shed small amounts of parasite eggs†	22	2	–	–
Overlap area of both *Fasciola* species where human shedding of parasite eggs in faeces is usually scarce or non-existent†	19	0	–	–
Total sera with fascioliasis	52	2	–	–
Individuals with parasitic/fungal diseases other than fascioliasis	–	–	158	10
Individuals parasitologically negative	–	–	88	1
Total sera without fascioliasis	–	–	246	11

*Gold standard determined according to the Kato–Katz technique.
†Gold standard determined according to the ELISA CL1 test.

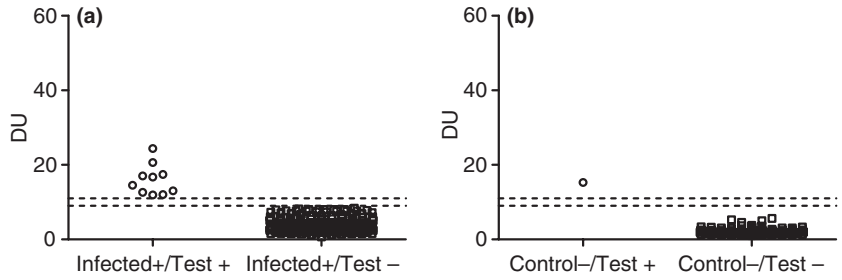

Figure 3 Mean DRG Units (DU) obtained with the DRG *F. hepatica* IgG ELISA test in (A) patients with parasitoses other than fascioliasis (B) and negative control sera. Data points represent the mean absorbance at 450 nm obtained from three replicates of each serum tested. The dotted line represents the cut-off value of OD at 450 nm. Data points are the mean optical density (OD) obtained from triplicate samples expressed as a percentage of the cut-off (CO), using the following formula: Percent positive (PP) = ((Mean OD of test sample)/Mean OD of C+) × 100. A serum is considered positive when its absorbance value is above 10% of CO. The results in DRG Units (DU) were calculated according to the following formula: DU = (sample (mean) absorbance value × 10)/CO. The results were negative if DU < 9, and positive if DU > 11 (dotted line).

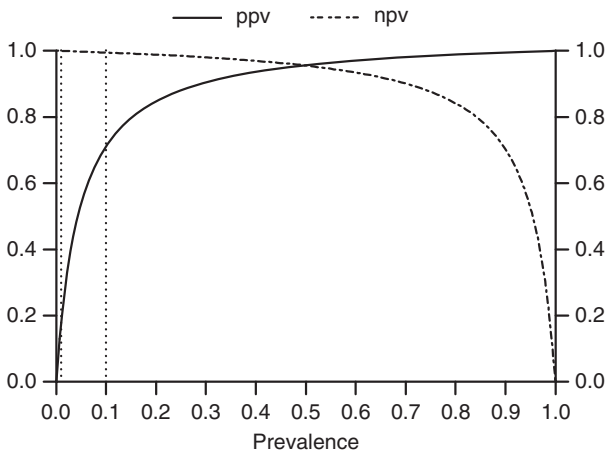

Figure 4 Theoretical PPVs and NPV values vs fascioliasis prevalence, showing the expected PPVs and NPV values if the test were used in low (below 1%), medium (between 1% and 10%) or high (above 10%) prevalence scenarios (expressed in vertical lines).

Figure 4, showing the expected PPVs and NPVs depending on whether the test was used in low, medium or high prevalence scenarios.

Discussion

Coprological analysis, based on the identification of eggs found in stools, duodenal contents or bile analysis is still commonly employed to diagnose human fascioliasis (Valero *et al.* 2009a), despite the overwhelming consensus that this method is not wholly reliable (Hillyer 1999) for several reasons. Eggs are not detected until the latent period of infection when much of the liver damage has already occurred, and similarly patients may not shed eggs in faeces in ectopic cases known in both infection by *F. hepatica* and *F. gigantica* (Le *et al.* 2007). Additionally, eggs are released sporadically from the bile ducts and hence stool samples of infected patients may not necessarily contain eggs (Mas-Coma *et al.* 1999).

However, coprological methods of egg detection are still usually considered the gold standard, although unfortunately they are not applicable in endemic areas where: (i) humans do not shed eggs in faeces, (ii) the shedding is often very low and difficult to detect and also (iii) in cases of communities that do not supply stool samples due to ethnic/cultural customs. Hence, it is essential to have serological techniques also available when diagnosing the disease. Therefore, in this study, besides Kato–Katz, another serological test, the ELISA CL1 test, was also used as gold standard.

Serodiagnosis of fascioliasis in human and animal species has been successfully carried out employing several antigenic fractions of *Fasciola* (Mezo *et al.* 2003; Sánchez-Andrade *et al.* 2008; Demerdash *et al.* 2011), purified antigens (O'Neill *et al.* 1998; Rokni *et al.* 2002), and recombinant antigens (O'Neill *et al.* 1999; Carnevale *et al.* 2001). Cathepsins L are the most frequently used target antigens for detecting anti-*Fasciola* antibodies (Carnevale *et al.* 2001; Rokni *et al.* 2002; Mezo *et al.* 2004, 2007, 2010; Intapan *et al.* 2005; Wongkham *et al.* 2005; Valero *et al.* 2009b; Muiño *et al.* 2011), as circulating antibodies to these molecules remain at high levels for long periods (Valero *et al.* 2009b).

The commercial DRG test was evaluated in cattle, obtaining a sensitivity and specificity of 98% (96–100%) and 96% (93–98%) respectively at a cut-off value of 15% positivity (Salimi-Bejestani *et al.* 2005). The sensitivity and specificity values of the DRG test herein agree with the results obtained by other authors for *F. hepatica* IgG

ELISA in-house assays: 100% and 100% (O'Neill *et al.* 1998; Rokni *et al.* 2002), 92.4%, and 83.6% (Espinoza *et al.* 2007), 97.2% and 100% (Rahimi *et al.* 2011), 97.0% and 96.6% (Cornejo *et al.* 2010) and 100 and 95.6% (Figueroa-Santiago *et al.* 2011). Commercial kits offer advantages over in-house assays: they save time and provide quality control reagents for better reproducibility within and between laboratories, which hampers the comparison between in-house and commercial tests. Furthermore, only very few commercial kits, such as the DRG *F. hepatica* IgG ELISA test evaluated here, are presently available for the diagnosis of human fascioliasis. Comparing DRG test results only with commercial assays results in sensitivity and specificity below the 100% sensitivity, respectively, specificity obtained by SeroFluke strips (Martínez-Sernández *et al.* 2011).

The results obtained show the test to be highly sensitive and specific. The sensitivity of this assay did not detect differences in positive samples from different epidemiological situations. Nevertheless, PPVs calculated for diverse epidemiological situations are very different. Thus, PPVs in hypoendemic areas were below 2.2%, in mesoendemic situations PPVs oscillated between 2.2% and 71.2%, while in hyperendemic situations PPVs were above 71.17%, making this test recommendable in such situations. Contrarily, NPVs calculated for diverse epidemiological situations are similar. NPVs for hypoendemic areas reached values above 99.9%, in mesoendemic situations NPVs oscillated between 99.5% and 99.9%, while in hyperendemic situations NPVs were below 99.5%.

In conclusion, the DRG test could be used both as (i) an individual serodiagnostic test for human fascioliasis when backed up by a clinical history, together with a second diagnostic technique in certain situations of possible cross-reactions (patients co-infected with other helminths) and (ii) in future large-scale epidemiological studies of human fascioliasis worldwide (recommended for mass screening, especially when considering its convenient handling).

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Field Evaluation of a Coproantigen Detection Test for Fascioliasis Diagnosis and Surveillance in Human Hyperendemic Areas of Andean Countries

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Abstract

Background: Emergence of human fascioliasis prompted a worldwide control initiative including a pilot study in a few countries. Two hyperendemic areas were chosen: Huacullani, Northern Altiplano, Bolivia, representing the Altiplanic transmission pattern with high prevalences and intensities; Cajamarca valley, Peru, representing the valley pattern with high prevalences but low intensities. Coprological sample collection, transport and study procedures were analyzed to improve individual diagnosis and subsequent treatments and surveillance activities. Therefore, a coproantigen-detection technique (MM3-COPRO ELISA) was evaluated, using classical techniques for egg detection for comparison.

Methodology and Findings: A total of 436 and 362 stool samples from schoolchildren of Huacullani and Cajamarca, respectively, were used. Positive samples from Huacullani were 24.77% using the MM3-COPRO technique, and 21.56% using Kato-Katz. Positive samples from Cajamarca were 11.05% using MM3-COPRO, and 5.24% using rapid sedimentation and Kato-Katz. In Huacullani, using Kato-Katz as gold standard, sensitivity and specificity were 94.68% and 98.48%, respectively, and using Kato-Katz and COPRO-ELISA test together, they were 95.68% and 100%. In Cajamarca, using rapid sedimentation and Kato-Katz together, results were 94.73% and 93.58%, and using rapid sedimentation, Kato-Katz and copro-ELISA together, they were 97.56% and 100%, respectively. There was no correlation between coproantigen detection by optical density (OD) and infection intensity by eggs per gram of feces (epg) in Cajamarca low burden cases ($<$400 epg), nor in Huacullani high burden cases ($\geq$400 epg), although there was in Huacullani low burden cases ($<$400 epg). Six cases of egg emission appeared negative by MM3-COPRO, including one with a high egg count (1248 epg).

Conclusions: The coproantigen-detection test allows for high sensitivity and specificity, fast large mass screening capacity, detection in the chronic phase, early detection of treatment failure or reinfection in post-treated subjects, and usefulness in surveillance programs. However, this technique falls short when evaluating the fluke burden on its own.

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Introduction

Fascioliasis is an important human and animal disease caused by the trematode species *Fasciola hepatica* and *F. gigantica*. At present, fascioliasis is emerging or re-emerging in numerous regions of Latin-America, Africa, Europe and Asia, both in humans and animals, a phenomenon which has partly been related to climate change [1]. Major human health problems are encountered in Andean countries (Bolivia, Peru, Chile and Ecuador), the Caribbean (Cuba), northern Africa (Egypt), western

Author Summary

A coproantigen-detection technique (MM3-COPRO ELISA) was evaluated in 436 and 362 schoolchildren of Huacullani, Bolivia, and Cajamarca valley, Peru, respectively. Classical techniques for egg detection were used for comparison. In Huacullani, using Kato-Katz as gold standard, sensitivity and specificity were 94.68% and 98.48%, respectively, and using Kato-Katz and COPRO-ELISA test together, they were 95.68% and 100%, respectively. In Cajamarca, using rapid sedimentation and Kato-Katz together, these results were 94.73% and 93.58%, respectively, and using rapid sedimentation, Kato-Katz and copro-ELISA together, results were 97.56% and 100%, respectively. There was no correlation between coproantigen detection by optical density (OD) and infection intensity by eggs per gram of feces (epg) in Cajamarca low burden cases (<400 epg), nor in Huacullani high burden cases (≥400 epg), although there was in Huacullani low burden cases (<400 epg). Six cases of egg emission appeared negative by MM3-COPRO, including one with a high egg count (1248 epg). The coproantigen-detection test allows for high sensitivity and specificity, fast large mass screening capacity, detection in the chronic phase, early detection of treatment failure or reinfection in post-treated subjects, and usefulness for surveillance programs. However, this technique falls short when evaluating the fluke burden on its own.

Europe (Portugal, France and Spain) and the Caspian area (Iran and neighbouring countries) [1]. Emergence, long-term pathogenicity [2–4] and immunological interactions [5,6] prompted the WHO to include this disease among the so-called neglected tropical diseases (NTDs), which are chronic, debilitating, poverty-promoting and among the most common causes of illness in developing countries. Their control and elimination is now recognized as a priority to achieve the United Nations Millennium Development Goals and targets for sustainable poverty reduction [7,8].

Among Andean countries, the highest human fascioliasis prevalences and intensities are encountered in the Northern Altiplano of both Bolivia [9,10] and Peru [11], as well as in the Cajamarca valley (Peru) [12], where *F. hepatica* is the only fasciolid species present [13] and children and females are the subjects most affected [1]. Within the human fascioliasis high altitude transmission pattern related to *F. hepatica* transmitted by lymnaeid vectors of the *Galba/Fossaria* group in Andean countries, two different subpatterns have been distinguished according to physiographic and seasonal characteristics [1,13]: a) the Altiplanic pattern, with endemicity distributed throughout an area of homogeneous altitude and transmission throughout the whole year caused by high evapotranspiration rates leading lymnaeid vectors to concentrate in permanent water bodies, e.g. the Northern Bolivian Altiplano [14]; b) the valley pattern, with endemicity distributed throughout an area of heterogeneous altitude and seasonal transmission related to climate, e.g. the Cajamarca valley in Peru [12,15,16].

In recent years, the availability of a very effective drug against fascioliasis, namely triclabendazole [17], prompted the WHO to launch a worldwide initiative against human fascioliasis [18,19]. This initiative includes interventions in human fascioliasis endemic areas presenting different epidemiological situations and transmission patterns [1]. Bolivia and Peru are two of the countries selected for priority intervention due to the very large public health problem posed by this disease. Different pilot schemes were designed to assess the best control strategies according to the different epidemiological situations and transmission patterns. The Northern Bolivian Altiplano was chosen as an example of the Altiplanic pattern, while the Cajamarca valley was chosen as an example of the valley pattern.

An alternative to coprological egg detection is the use of immunodiagnostic tests based on the detection of anti-*Fasciola* antibodies and/or coproantigens released by the parasite. In the last decades, several ELISA methods based on the use of polyclonal and monoclonal antibodies have been reported to be useful for detection of *F. hepatica* and *F. gigantica* in the feces of sheep and cattle [20–23] and also rat feces [24]. Nevertheless, surveys on human fascioliasis have usually been made through various coprological techniques verifying the presence of eggs in stools [25] and antibody detection tests to confirm the diagnosis of human fascioliasis [26]. Among these techniques, classical coprological egg detection methods are the most frequently used [27]. However, so far, the use of coproantigen detection was applied to diagnose *F. hepatica* infection in patients in Cuba only [28,29].

Enzyme-linked immunosorbent assay (ELISA) methods developed for determination of *Fasciola* coproantigens in stool samples from animals and humans provide an alternative to coprological examination [30,31]. One of these methods is the MM3 capture ELISA (MM3-COPRO) test for fascioliasis diagnosis detection of fecal excretory-secretory antigens (ESAs) using a monoclonal antibody (mAb), whose usefulness for detection of *F. hepatica* and *F. gigantica* coproantigens in experimental and natural *Fasciola* infections of sheep and cattle has already been demonstrated [22,32]. This test proved to be highly sensitive (confirmed by necropsy) and specific (no cross reaction was observed with antigens from other helminths), and allowed for the detection of *Fasciola* infections 1–5 weeks before patency in cattle. Furthermore, other researchers recently tested a commercial version of the test, and its appropriateness for the detection of *F. hepatica* infections in cattle was confirmed under field conditions [33]. The suitability of the MM3-COPRO method for detection of *Fasciola* coproantigens in both fresh and preserved stools from hospital patients has been demonstrated [34], but its applicability for detection of *F. hepatica* infections in humans under field conditions has not been proved.

An efficient coproantigen test for human fascioliasis diagnosis represents a valuable tool to facilitate population screening and post-treatment surveillance in control campaigns, above all in communities where people are reluctant to furnish blood samples due to ethnic/religious aspects. The aim of the present study is to evaluate the coproantigen technique MM3-COPRO ELISA under field conditions for human fascioliasis diagnosis in human hyperendemic areas of Andean countries, using classical coprological techniques for egg detection for comparison purposes (rapid sedimentation and Kato-Katz). Thus, two endemic areas were chosen: Huacullani (Bolivia) representing the Altiplanic pattern with high prevalences and intensities, and the rural areas of Cajamarca (Peru), representing the valley pattern with high prevalences but with low intensities. Results of the pilot intervention implemented in Huacullani to assess the feasibility of a strategy of large-scale administration of triclabendazole, with a focus on safety and efficacy, are included in another article [35].

Materials and Methods

Ethics statement

In Bolivia, the study was approved by the Comisión de Etica de la Investigación of the Comité Nacional de Bioética. In Peru, it was approved by the Comité Institucional de Etica of the

Universidad Peruana Cayetano Heredia and the Comité de Ética of the Instituto Nacional de Salud.

All subjects involved provided written informed consent. Samples from children were obtained after consent from the children's parents, following the principles expressed in the Declaration of Helsinki. Consent was also obtained from the local authorities of the communities and heads and teachers of the school.

In Huacullani, activities were performed in collaboration with the Servicio Departamental de Salud La Paz and the Unidad de Epidemiología of the Bolivian Ministry of Health and Sports (MSyD). In Cajamarca, the study was done in cooperation with the Dirección Regional de Salud of Cajamarca, and the Estrategia Nacional de Zoonosis, Dirección General de Salud de las Personas, Ministerio de Salud (MINSA), Lima.

Stool samples and coprological techniques

Coprological studies were carried out in the locality of Huacullani, which belongs to the municipality of Tiwanaku, third section of the province of Ingavi of the Departamento de La Paz, Bolivia. This locality is situated 85 km from the capital La Paz, at the western end of the so-called Tambillo-Aygachi corridor of the Northern Bolivian Altiplano. Huacullani has 2525 inhabitants, according to the last 2005 census of the Bolivian Instituto Nacional de Estadística. Stool collection was performed in the school of the locality and samples were obtained from a total of 436 children. Previous surveys in that locality showed very high prevalence rates of 38.2% in the year 1992, 31.2% in 1993, and 34.8% in 1996 in children, and 18.4% in 1996 and 11.8% in 1997 in total community surveys (children plus adults) [10].

Stool samples were also obtained in the Departmento de Cajamarca, Peru, which covers an area of around 35,400 km^2 in the northern Andean part of Peru and is inhabited by 1,416,000 people. This Department comprises 13 provinces and the province of Cajamarca in turn includes 12 districts [12]. A total of 362 fecal samples were obtained from children of the schools of Escuela de Varones del Distrito (Jesus district), Santa Rosa de Chaquil (La Encañada district), and Andres Avelino Caceres (Baños del Inca district). Previous surveys showed very high prevalences in that endemic area, with a mean of 24.4% and the maximum prevalence of 47.7% in Santa Rosa de Chaquil, the hitherto highest local prevalence detected in Peru [12].

Classical coprological techniques for egg detection were used for qualitative (rapid sedimentation and Kato-Katz) and quantitative (Kato-Katz) diagnosis. The combined use of highly specific techniques has been reported as a means of compensating the low sensitivity of the Kato-Katz technique [36]. Thus, identification of true positive and true negative cases was carried out by using two criteria: i) finding of *F. hepatica* eggs in feces; ii) egg finding plus COPRO ELISA test results. Applying the Kato-Katz technique, eggs were detected in fresh stools after analysis of three Kato-Katz slides (Helm-Test, AK test, AK Industria e Comércio Ltda, Belo Horizonte, Brasil) per sample, depending on the concentration of *Fasciola* eggs following WHO recommendations, using a template delivering about 41.7 mg of feces [37]. The average egg output was calculated as eggs per grams of feces (epg). Parasitological analysis was done microscopically by a trained parasitologist. Intensity of infection, measured as eggs per gram (epg), was used as an indicator of *F. hepatica* burden in infected subjects. Kato-Katz was used in both study areas and rapid sedimentation was an additional test done in Cajamarca. In the case of the Huacullani samples, a single Kato-Katz slide was used for each sample. In the case of the Cajamarca samples, the rapid sedimentation procedure was applied and those fecal samples positive for the MM3-COPRO ELISA were also quantitatively analyzed by three Kato-Katz slides.

Fecal sample procedures and analyses

Children were not included in the study if they presented any chronic or acute hepatic disease, pregnancy, breast-feeding, any acute infection within a week of enrolment, or receiving treatment for any other disease or condition. In Huacullani, at the time of the baseline survey (April 2008), the school population consisted of 459 children aged 5 to 14 years, who were all considered eligible for an interventional treatment study. A total of 447 children returned the plastic container. From these, 437 fecal samples from an equivalent number of children were examined (four children returned an empty plastic container, and six other children provided insufficient stool quantities to apply both Kato-Katz slide and COPRO ELISA). Thus, fecal samples were obtained from 437 children (220 males and 217 females) of 5–16 years of age (mean $\pm$ SD = 8.8$\pm$2.3). A clean, plastic, wide-mouthed, numbered container with a snap-on lid was given to every participant. All subjects were then asked to try to fill the container with their own feces and to return it immediately. One stool sample per subject was collected and personal data (name, sex, and age) were noted on delivery of the container. Samples were transported to the parasitological laboratory of the Faculty of Pharmacy, Universidad Mayor de San Andrés (UMSA), La Paz, within 1–3 h after collection. One aliquot was used to carry out the MM3-COPRO ELISA and another was preserved at 4°C to make the Kato-Katz slides. All Kato-Katz slides were made at the Laboratory of Parasitology of the Faculty of Medicine, UMSA, and were initially examined within 1 h of preparation to avoid overclarification of some helminth eggs.

In Cajamarca, at the time of the baseline survey (December 2007), the target population was 616 school children (age range 6–15 years old), with a coverage of 4.25% of the school children population and 0.86% of the overall population from the three aforementioned districts. Thus, in the present study, fecal samples were obtained from 362 children (264 males and 98 females), 7–15 years of age (mean $\pm$ S.D. = 9.9$\pm$2.2), by similar procedures. Samples were transported to Cajamarca city within 1–3 h after collection and stored at 4°C until being sent to the Laboratory of Parasitology at the Instituto de Medicina Tropical Alexander von Humboldt, Lima, where coproparasitological analyses were carried out. Both ELISA and Kato-Katz slides were applied to two aliquots of the material preserved at 4°C. A third aliquot was preserved in 10% formalin solution (1:3) for subsequent egg detection by means of the rapid sedimentation technique [38].

To assure quality standards and possible handling differences, procedures in the two laboratories were implemented by the same personnel of the Valencia team in addition to the respective local personnel of each laboratory. In Cajamarca, stool samples were distributed into two groups according to the 400-epg threshold used for identifying high intensity infections [18]: a high burden group ($\geq$400 epg) and a low burden group ($<$400 epg). However, in Huacullani, as precautionary measure, a lower threshold (300 epg) was requested to be applied by Bolivian health responsibles to distinguish between samples whose respective infected children were in need to be hospitalized for prevention follow up of potential post-treatment colics, and samples whose respective infected children were not hospitalized and were treated on an out-patient basis [35].

Statistical analyses

Statistical analyses were done using PASW 17 software. For the evaluation of categorical variables, the chi-square test or Fisher's

exact test was used. Bivariant correlations (Pearson's correlation) were calculated to assess the relationship between optical density (OD) and epg of *F. hepatica*. A P value below 0.05 was considered significant.

Theoretical positive predictive values (PPV) and negative predictive values (NPV) were calculated from sensitivity and specificity values obtained using only classical coprological tests for the identification of *F. hepatica* eggs in feces as "gold standard". The following formulas were used for their calculation:

$$PPV = (\text{sensitivity} \times \text{prevalence}) / (\text{sensitivity} \times \text{prevalence}) + ((1 - \text{specificity}) \times (1 - \text{prevalence}))$$

$$NPV = (\text{specificity} \times (1 - \text{prevalence})) / (((1 - \text{sensitivity}) \times \text{prevalence}) + ((\text{specificity}) \times (1 - \text{prevalence})))$$

MM3-COPRO ELISA detection of *Fasciola hepatica* coproantigens in fecal samples

The MM3-COPRO ELISA kits were prepared and tests performed as previously described [22,32,34]. Kits were provided by Dr. F.M. Ubeira (University of Santiago de Compostela, Spain). Briefly, polystyrene microtiter 1×8 F strip plates (Greiner Bio-One GmbH, Frickenhausen, Germany) were coated overnight with 100 µL/well of a solution containing 10 µg/mL of rabbit anti-*Fasciola* polyclonal IgG antibody in phosphate buffered saline (PBS) (wells from odd-numbered rows), or with 100 µL/well of a solution containing 10 µg/mL of IgG polyclonal antibodies from non-immunized rabbits (wells from even-numbered rows). Uncoated sites were blocked with 1.5% of sodium caseinate in PBS for 1 h at RT, and each fecal supernatant (100 µL) was then added in quadruplicate (2 odd-numbered wells plus 2 even-numbered wells), and incubated overnight at 4°C. After washing 6 times with PBS containing 0.2% Tween-20 (PBS-T), 100 µL of a solution containing 0.3 µg of biotinylated MM3 antibodies in PBS-T plus 1% bovine serum albumin (PBS-T-BSA) was added to each well and incubated for 1 hr at 37°C. After washing as above, bound MM3 antibody was detected by incubation, first with peroxidase-conjugated neutravidin (Pierce, Rockford, Illinois; dilution 1:2000 in PBS-T-BSA) for 1 hr at 37°C, and then with the substrate (buffered H_2O_2 and o–phenylenediamine [OPD], Sigma-Aldrich, Madrid, Spain). After incubation for 20 min at RT, the reaction was stopped by addition of 3N H_2SO_4. Finally, OD was measured at 492 nm. The OD value for each sample was calculated as OD1–OD2, where OD1 is the mean for the 2 even-numbered wells (coated with anti-*Fasciola* polyclonal antibodies), and OD2 is the mean for the 2 odd-numbered wells (coated with irrelevant polyclonal antibodies). The OD value for each sample was calculated by subtracting the OD of the blank well from the OD of the test well using the cut-off point 0.097 [34].

Results

Diagnostic parameters of the MM3-COPRO ELISA were estimated by (i) only choosing coprology as the "gold standard" assay to detect *F. hepatica* infection in humans, and also by (ii) considering results of both coprology and COPRO ELISA together. Positive cases of the MM3-COPRO ELISA and egg detection techniques of *F. hepatica* infection and performance characteristics of the MM3-COPRO ELISA according to study site are summarized in Table 1.

Table 1. Performance characteristics of MM3-COPRO ELISA by study site.

Endemic area	positive cases by MM3-COPRO		positive cases by egg detection	MM3-COPRO +	MM3-COPRO +	MM3-COPRO −	MM3-COPRO −	Sensitivity	Specificity	KATO-KATZ			
	%	%		Egg detection +	Egg detection −	Egg detection +	Egg detection −	%	%	N[a]	AM ± SD	Range	GM
Huacullani (N[b] = 436)	24.77	21.56		89	19	5	324	94.68 (*)	98.48 (*)	94	334.98±92.56	24–8088	142.17
								95.68 (**)	100 (**)				
Cajamarca (N[b] = 362)	11.05	5.24		18	22	1	321	94.73 (*)	93.58 (*)	17	116.47±84.80	16–376	89.80
								97.56 (**)	100 (**)				

Positive cases (%) of MM3-COPRO ELISA and egg detection techniques of *Fasciola hepatica* infection.
N[a] = total number of positive children with *Fasciola* infection by Kato–Katz.
N[b] = number of children analyzed.
AM = arithmetic mean; GM = geometric mean.
Identification of true positive and true negative cases was carried out by using two criteria:
i) finding of *F. hepatica* eggs in feces (*); ii) egg finding plus COPRO ELISA test results (**).
doi:10.1371/journal.pntd.0001812.t001

Studies in Huacullani, Bolivia

Huacullani positive cases were globally 24.77% using MM3-COPRO ELISA and 21.56% applying an egg detection technique (Kato-Katz). No significant differences were encountered between either % (P = 0.093).

In this Bolivian locality, using Kato-Katz as gold standard, sensitivity and specificity were 94.68% and 98.48%, respectively, and using Kato-Katz and COPRO-ELISA test together as gold standard, sensitivity and specificity were 95.68% and 100%, respectively.

Of 436 samples assayed, 94 showed the presence of eggs through the Kato-Katz technique (21.56%). MM3-COPRO ELISA was positive in 108 samples (24.77%), which included samples with *Fasciola* eggs (89) and without *Fasciola* eggs (19), i.e. 82.40% of the children who were positive for the MM3-COPRO ELISA were also positive through the Kato-Katz procedure. It should be emphasized that there were five children shedding eggs with emissions of 48, 72, 96, 120 and 1248 epg, whose MM3-COPRO ELISA results were negative (1.14%). The stool sample showing 1248 epg was repeatedly re-analyzed and a negative result was always obtained with the MM3-COPRO ELISA test.

The geometric mean egg content in *F. hepatica* positive samples was 142.17 epg, and the arithmetic mean was 334.98 (with SD of ±92.56), with a range of 24 to 8088 epg (Table 1). In these samples from Huacullani, epg data were distributed into two groups: a high burden group (≥400 epg) and a low burden group (<400 epg) of samples.

The OD values obtained for individual *F. hepatica* positive and negative fecal samples from Huacullani are shown in Figure 1. Positive samples with *F. hepatica* eggs showed OD values above the cut-off value except in five cases (determined by the Kato-Katz technique).

In children who were positive in egg emission, the bivariant correlation between OD and epg data from low and high burden groups was carried out separately. A significant positive correlation was detected only between OD and low burden ($r^2 = 0.20$) (Figure 2), but no significant positive correlation was detected when considering OD and high burden ($r^2 = 0.01$) (Figure 3).

Theoretical PPVs and NPVs vs fascioliasis prevalence are represented in Figure 4A, showing the expected PPVs and NPVs depending on whether the test was used in low, medium or high prevalence scenarios in this Altiplanic highly endemic locality.

Studies in Cajamarca, Peru

Cajamarca positive cases were globally 11.05% using MM3-COPRO ELISA and 5.60% employing egg detection techniques (rapid sedimentation and Kato-Katz). Significant differences were detected between both % (P = 0.007).

Differences between the two local patterns were detected, i.e. significant differences were found when comparing MM3-COPRO ELISA positive cases % from Huacullani and Cajamarca (P = 0.0025), and also when comparing egg detection positive cases % from Huacullani and Cajamarca (P = 0.001).

In Cajamarca, using rapid sedimentation and Kato-Katz together as gold standard, sensitivity and specificity were 94.73% and 93.58%, respectively, and using rapid sedimentation, Kato-Katz and copro-ELISA together as gold standard, results were 97.56% and 100%, respectively.

In this Peruvian locality, of 362 samples assayed, 19 showed the presence of eggs through the rapid sedimentation and Kato-Katz techniques (5.24%), whereas MM3-COPRO ELISA was positive in 40 samples, which included the samples with *Fasciola* eggs (18) and without *Fasciola* eggs (22), i.e. 45.0% of the children who were positive by MM3-COPRO ELISA were also positive through coprological egg detection procedures. Interestingly, one child shed eggs (by the rapid sedimentation technique) but was negative by MM3-COPRO ELISA. The remaining 321 MM3-COPRO ELISA negative samples, however, included 237 negative samples,

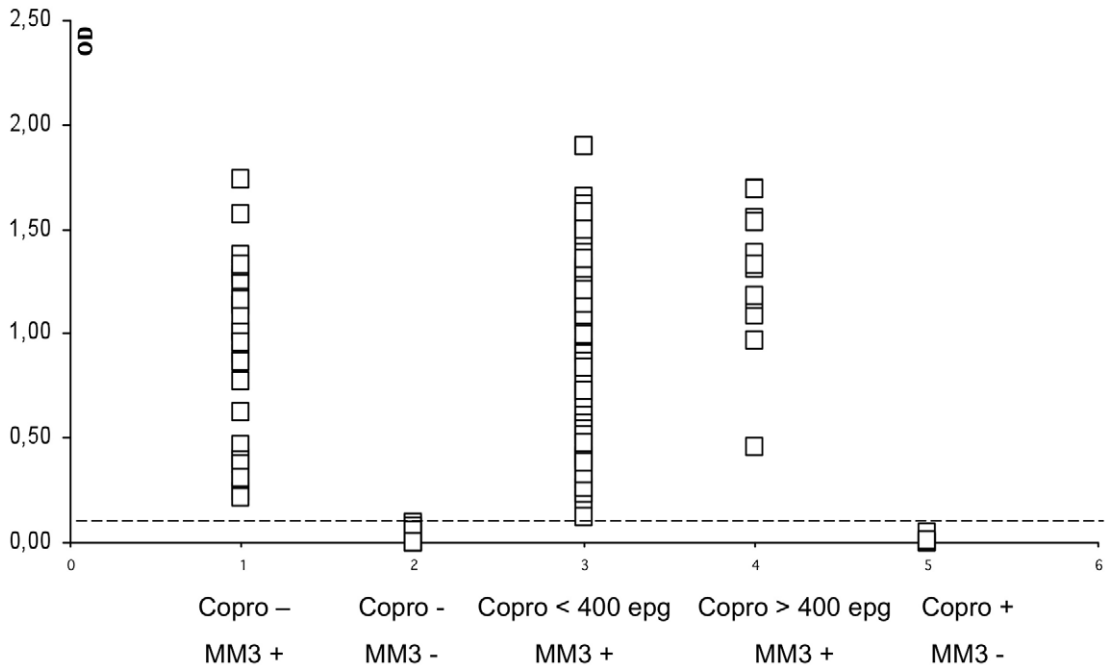

Figure 1. MM3-COPRO ELISA in feces from children (n = 436) from Huacullani (Northern Bolivian Altiplano). Data points represent the mean absorbance at 492 nm obtained from three replicates of each sample tested. The dotted line represents the cut-off value 0.097 units of OD at 492 nm.
doi:10.1371/journal.pntd.0001812.g001

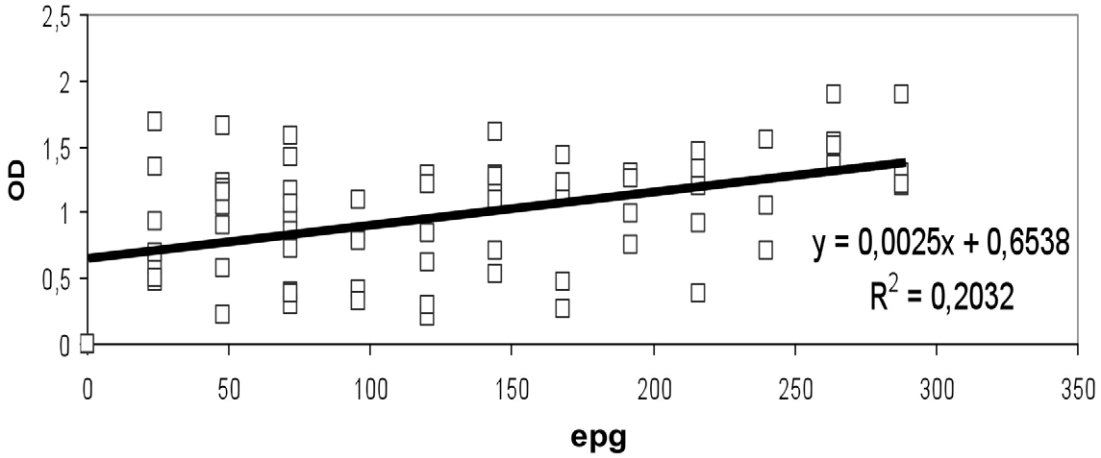

Figure 2. MM3-COPRO ELISA and intensity of *Fasciola hepatica* infection in the low burden group of Huacullani. Data points represent the mean absorbance at 492 nm from egg positive children from Huacullani. epg represents the egg count per gram of feces. The dotted line represents the cut-off value 0.097 units of OD at 492 nm.
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and 84 positive samples for parasitic infections other than *Fasciola*. They involved one or more parasitic protozoans (*Blastocystis hominis*, *Chilomastix mensnilii*, *Giardia intestinalis*, *Entamoeba histolytica/E. dispar/E. moshkovskii*, *E. coli*, *Endolimax nana*, *Iodamoeba buetschlii*) and helminth species (*Strongyloides stercoralis*, *Ascaris lumbricoides*, *Trichuris trichiura*, *Enterobius vermicularis* and *Hymenolepis nana*).

The geometric mean egg content in *F. hepatica* positive samples from Cajamarca was 89.80 epg, and the arithmetic mean was 116.47 (with SD of ±84.80), with a range of 16 to 376 epg (Table 1), i.e. samples were all considered as belonging to the low burden group as their epg counts were <400.

The OD values obtained for individual *F. hepatica* positive and negative fecal samples from Cajamarca are shown in Figure 5. Positive samples with *F. hepatica* eggs showed OD values above the cut-off value except in one case (determined by the rapid sedimentation technique).

In children who were positive in egg emission, the bivariant correlation between OD and epg data (low burden) was carried out. No significant positive correlation between OD and low burden ($r^2 = 0.05$) was detected (Figure 6).

Theoretical PPVs and NPVs vs fascioliasis prevalence are represented in Figure 4B, showing the expected PPVs and NPVs depending on whether the test was used in low, medium or high prevalence scenarios in this Peruvian highly endemic locality.

Discussion

Sensitivity is defined as the proportion of people with the disease who have a positive test for the disease. A sensitive test will rarely miss people with the disease. Specificity is the proportion of people without the disease who have a negative test. A specific test will rarely misclassify people as having the disease when they do not [39]. Knowing true positive and true negative cases is essential when calculating sensitivity and specificity, respectively. The identification of true positive and true negative cases was carried out using classical coprological tests for the identification of *F. hepatica* eggs in feces. Nevertheless, in the case of human fascioliasis, the application of the rapid sedimentation or Kato-Katz techniques may result in false negative cases. The ethiological diagnosis based on egg detection in stools is complicated because

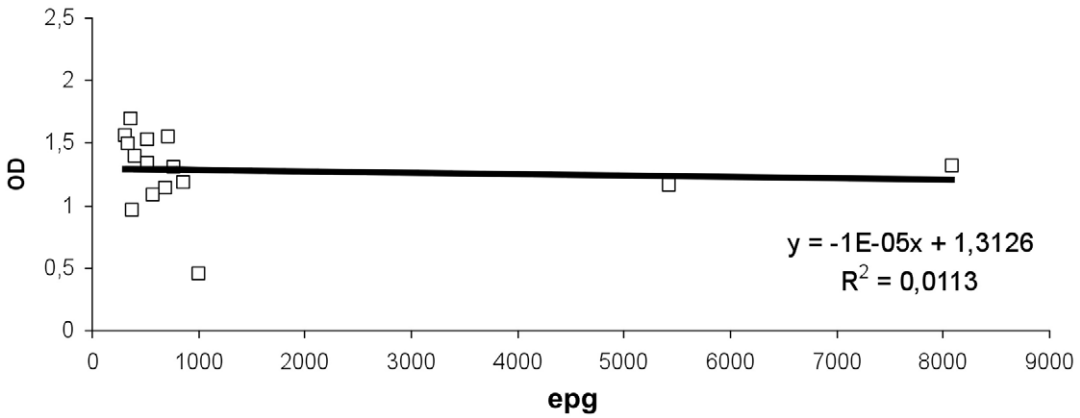

Figure 3. MM3-COPRO ELISA and intensity of *Fasciola hepatica* infection in the high burden group of Huacullani. Data points represent the mean absorbance at 492 nm from egg positive children from Huacullani. epg represents the egg count per gram of feces. The dotted line represents the cut-off value 0.097 units of OD at 492 nm.
doi:10.1371/journal.pntd.0001812.g003

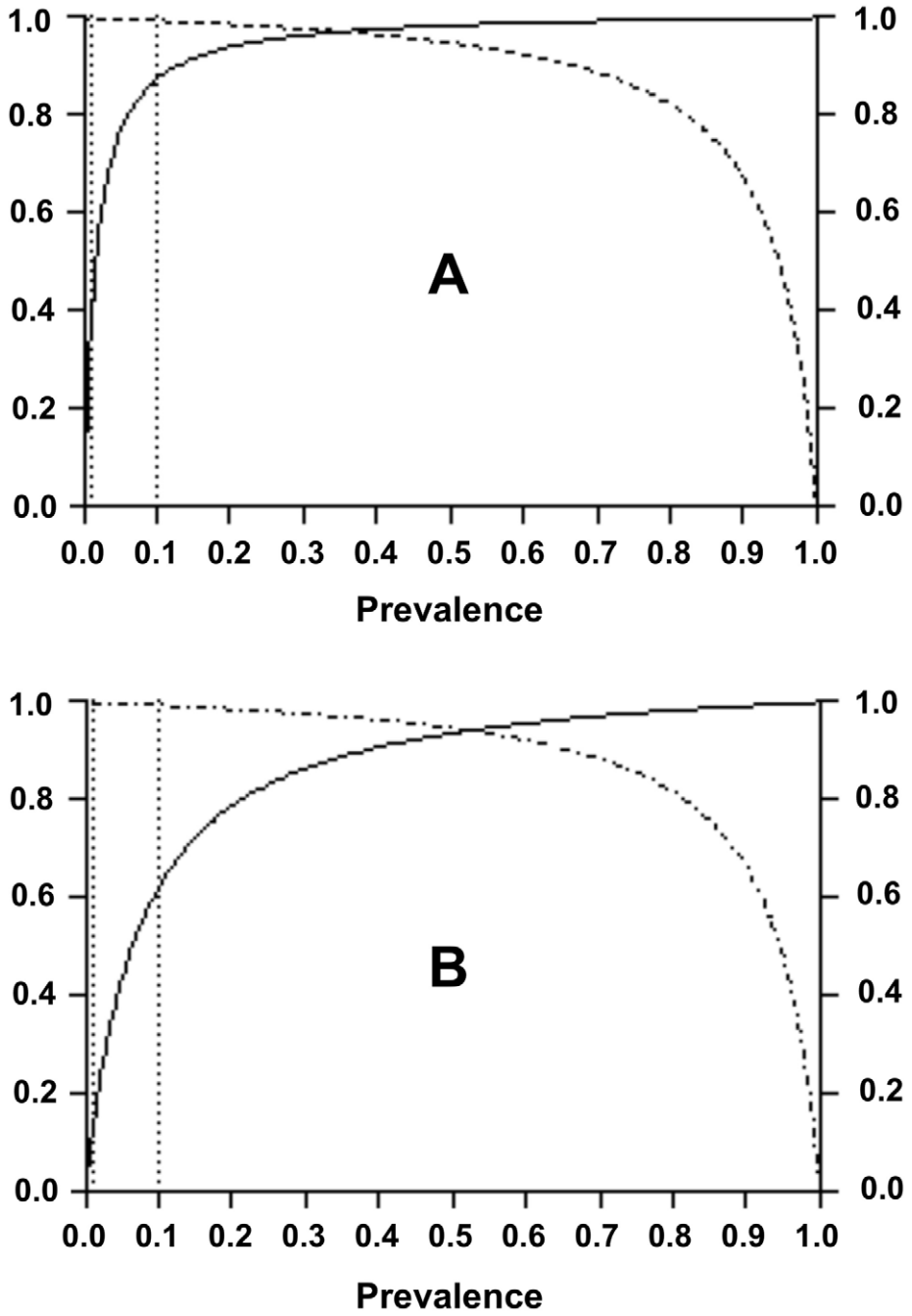

Figure 4. Theoretical PPVs and NPV values vs fascioliasis prevalence. Curves show the expected PPVs (continuous line) and NPV (dotted line) values in low (below 1%), medium (between 1% and 10%) or high (above 10%) prevalence scenarios (expressed in vertical lines) in Huacullani (A) and Cajamarca (B).
doi:10.1371/journal.pntd.0001812.g004

parasite eggs are not found during the prepatent period [27,40], when juvenile worms migrate through the intestinal wall to the peritoneal cavity (at one week), penetrate the liver parenchyma (at five to seven weeks), and pass into the biliary tract where they ultimately reach maturity (at two months or more). Previous studies have even estimated a period of at least three to four months to be necessary for *F. hepatica* flukes to attain sexual maturity in humans [27,41]. Once the worms have matured, diagnosis still remains difficult because commonly employed microscopic techniques for quantitative diagnosis of *Fasciola* eggs are very specific but rather insensitive. In addition, in some cases diagnosis is also difficult during the biliary stage, due to the intermittent excretion of parasite eggs. Fecal egg counts are known to follow inter- and intraindividual variations in fascioliasis [42,43]. In our case, we used the Kato-Katz technique as a "gold standard" because it is considered the best available for quantitative analysis, although taking into account that it is admittedly rather imperfect. Therefore, results from the rapid sedimentation were also considered to improve the first gold standard in Cajamarca, and the combination of results from both

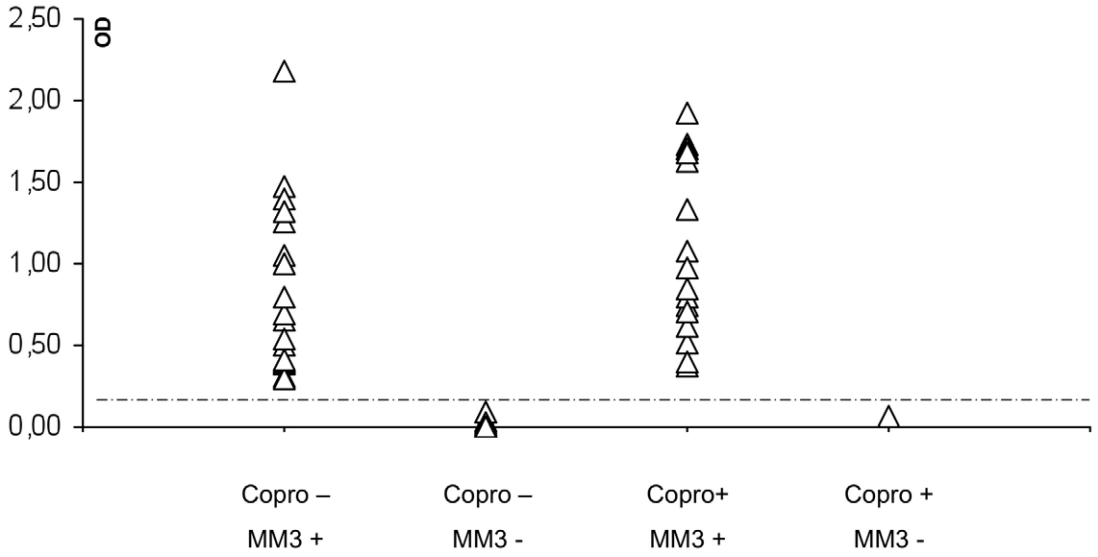

Figure 5. MM3-COPRO ELISA in feces from children (n = 362) from Cajamarca valley (Peru). Data points represent the mean absorbance at 492 nm obtained from three replicates of each sample tested. The dotted line represents the cut-off value 0.097 units of OD at 492 nm.
doi:10.1371/journal.pntd.0001812.g005

coprological methods and COPRO ELISA were used for both study areas.

When liver-flukes are located in the bile ducts, excretory-secretory (ES) products are released, being eliminated via feces. The detection of these products by means of a sandwich-ELISA reflects the installation of flukes in the bile ducts and the presence of the biliary stage of the disease [30]. No statistically-significant differences were detected between prevalence results obtained using egg detection techniques and the MM3-COPRO ELISA in Huacullani, where egg intensities are higher according to the typical feature of the Altiplanic pattern. On the contrary, such differences were detected in Cajamarca, probably as a consequence of the low egg intensities characteristic of the valley pattern, i.e. in Cajamarca low burdens are common and therefore the higher probability of infected subjects intermittently shedding very few eggs is higher, with the consequence that such cases go unnoticed.

Five and one cases of egg emission were negative using the MM3-COPRO ELISA in Huacullani and Cajamarca, respectively, including one case of very high egg count (1248 epg) in the Bolivian locality. Such cases pose a question mark. This result raises the question as to whether these false negative cases may be interpreted as being inherent to the kit design, or due to external factors not attributable to the ELISA kit. However, considering that there is broad experience in detecting *Fasciola* coproantigens in ovine and bovine samples, and that animals with egg emission were not found to be negative with the MM3 ELISA, it is unlikely that these false negative results were due to kit construction. Furthermore, a recent study has shown that the monoclonal antibody used in the MM3 assay recognizes L1 and L2 cathepsins [44], as does the ES78 in the FasciDig test [20], and there were no observations of false negative results with this test either. Three possible explanations include (i) an intermittent release of the ES products from the liver to the intestine through the bile ducts, (ii) spurious infections, and (iii) the existence of food remains in the intestine masking or interfering with the detection of the fluke ES antigens. The first does not seem to be the case, as previous studies using this and other kits do not indicate the emission of cathepsins L1 and L2 in either human or animal species

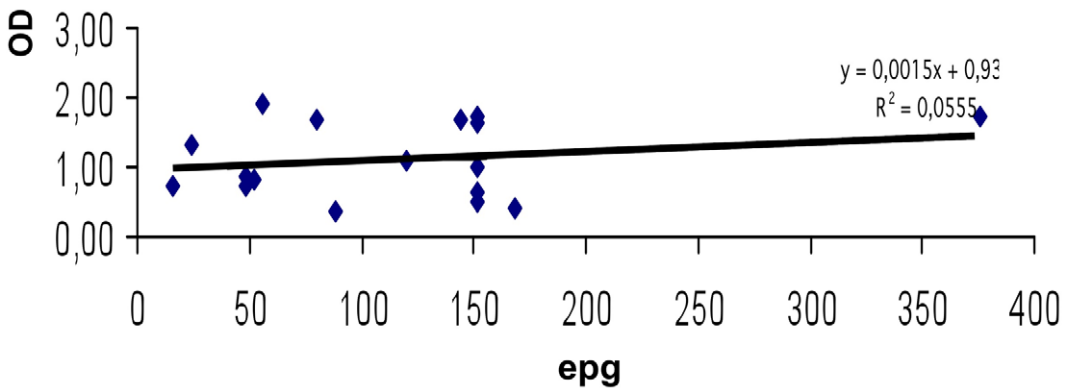

Figure 6. MM3-COPRO ELISA and intensity of *Fasciola hepatica* infection in the low burden group of Cajamarca. Data points represent the mean absorbance at 492 nm from egg positive children from Cajamarca. epg represents the egg count per gram of feces. The dotted line represents the cut-off value 0.097 units of OD at 492 nm.
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to be intermittent (unlike with eggs). A spurious infection was excluded after meticuluous study of the aspect of eggs in each one of these children. A potential negative influence of high temperatures during the transport or/and an inadequate handling of the samples at a given moment throughout the whole procedure cannot be ruled out, although the relatively short storage period does not suggest a considerable denaturation of the L-cathepsins to have occurred.

In both Huacullani (representing the fascioliasis Altiplanic pattern) and Cajamarca (representing the fascioliasis valley pattern), cases of coproantigens present in the feces of humans without *F. hepatica* eggs in stools were detected. Previous time-course studies in animals on the detection of *F. hepatica* coproantigens by ELISA indicated that coproantigens were detectable prior to patency [45]. Furthermore, a marked increase in the levels of these coproantigens at the beginning of fecal egg output was observed [32]. Considering the positivity/negativity of MM3-COPRO ELISA and the presence/absence of eggs in feces, two situations were established in the current study: the Altiplanic pattern with a correlation between positivity of MM3-COPRO ELISA and the presence of eggs, and the valley pattern with a larger number of positive cases applying MM3-COPRO ELISA but without presence of eggs in feces.

The Altiplanic pattern, characterized by higher prevalences and intensities, showed no statistical differences between the percentage of children who were positive in coproantigens and eggs in feces. Thus, it may be concluded that the majority of children with liver-flukes in the biliary ducts shed eggs. Nevertheless, in the valley pattern, characterized by high prevalences but low intensities, differences were detected between the percentage of children who were positive to coproantigens and the reduced number of these children that shed eggs in feces. This suggests that children did not shed eggs or only a much reduced, undetectable number even despite presenting parasites in their biliary canals.

In Europe, for instance, the diagnosis of human fascioliasis is frequently established using serological tests, because the detection of *F. hepatica* eggs in stools is not always possible. Thus, in an epidemiological survey from 1970 to 1999 to record cases of human fascioliasis detected in the Limousin region (central France), egg detection in stools was positive in only 27.6% of a total of 711 persons with fascioliasis [46]. Future studies are needed in Cajamarca (and other endemic areas in valleys of the Andean countries) to verify whether in these cases the non-detection of eggs implies that the parasite (i) has not reached the biliary ducts or is located in the bile-ducts but oviposition has not yet started (suggesting a more or less recent infection) or (ii) oviposition is taking already place but with only very low egg numbers or with intermittent shedding (indicating that subjects present only one or a very few flukes in the chronic stage).

Negative results by the MM3-COPRO ELISA after treatment, which occurs approximately one to three weeks in animals, is usually accepted when determining the efficacy of anthelminthic treatment of biliary fascioliasis [22]. Contrarily, serological methods have limitations when determining the efficacy of anthelminthic treatment because the presence of antibodies indicates previous exposure to the parasite rather than the existence of a current infection. Additionally, after successful anthelminthic treatment, several months have to pass for serological antibody-detection tests to become negative. Hence, the detection of specific antigens in feces allows for the confirmation of a current infection, whereas antibody detection tests need to be complemented by another technique to confirm the results obtained in treated subjects. Future studies should be carried out to determine the time required for negativization of MM3-COPRO ELISA results after effective treatment in humans.

The MM3-COPRO ELISA is also a reliable method for detecting *F. gigantica* coproantigens in fecal samples from experimentally infected sheep [32]. Although most reported cases of human fascioliasis are caused by *F. hepatica*, infections by *F. gigantica* have also been reported [25]. The fact that the MM3-COPRO ELISA can detect infections by both species may be of great value to ensure diagnosis of human and animal fascioliasis in countries where *F. gigantica* predominates, or where both species of *Fasciola* are present [25,34].

Determining a patient's parasitic burden is crucial given the necessity to monitor drug treatment in order to prevent a hepatic colic as the consequence of the massive expulsion of liver-flukes [18], similar to other helminth diseases [47]. The Kato-Katz is usually employed as a coprological quantitative technique. Nevertheless, this technique has a low sensitivity, and the elaboration of several slides from the same individual stool sample is indispensable. The application of the Kato-Katz technique in community surveys becomes problematic because (i) it is pronouncedly time consuming when the number of samples is high, (ii) microscopic egg count is also time consuming in cases of heavy egg burdens, and (iii) it requires an additional technique to increase the sensitivity in areas where subjects shed a very low number of eggs in an intermittent way.

Results obtained in the samples from Huacullani showed that the concentration of coproantigens in feces is correlated with epg in the low burden group ($<$300 epg). This result agrees with a previous study using the MM3-COPRO ELISA in cattle, which showed that the concentration of coproantigens in feces is also correlated with the number of flukes found in the livers of animals collected after slaughter [22], as well as with the results of positive correlation found with another coproantigen test in fascioliasis infected patients in Cuba [30]. Nevertheless, our findings in the high burden group ($\geq$300 epg) showed that the concentration of coproantigens in feces is not correlated with epg. This result agrees with the absence of any correlation between egg shedding in human samples from Hospital patients, measured by the Kato-Katz technique, and coproantigen concentration, measured by the MM3-COPRO ELISA [34]. One possible explanation for this discrepancy may be that the positive cases analyzed in Cuba [30] probably corresponded to recent infections with less than a year of age (early chronic stage), whereas our samples were from patients with chronic infections, in which egg excretion is probably more erratic. It must be kept in mind that fasciolid flukes may survive for up to 13.5 years in humans, and the pattern of egg shedding is not linear but fluctuates between maximum and minimum values [43]. By comparison, the kinetics of coproantigen release versus the kinetics of egg shedding showed a similar pattern but with a two-week time lag in epg [32].

In Cajamarca, chronic fascioliasis in valley samples, coproantigen levels did not show a good correlation with epg. Therefore, the use of only one coproproantigen technique appears to be insufficient to evaluate the fluke burden.

In these hyperendemic areas, the number of subjects who participate in surveys of this kind is very large, which implies the problem of transporting and preserving the fecal samples, as the coproantigen degrades at ambient temperature within a few days and the fecal material cannot be treated with any classical fixative. The monoclonal antibody MM3 recognizes a single conformational epitope, located in *Fasciola* cathepsins L1 and L2, which are the main cysteine proteases produced by adult flukes gut [44]. The stability of the antigen was observed during a period of 5 weeks, except for samples preserved in CoproGuard, which were observed for 17 weeks. Comparison of the different preservation conditions revealed that even when maintained at 37°C, only the

antigenicity of coproantigens in the samples diluted with CoproGuard did not vary throughout the observation period. In contrast, biocides such as sodium azide and thimerosal did not preserve the antigenicity, as the start signal decreased to approximately 30% by the end of the observation period. When the samples were maintained at 4°C, the *F. hepatica* coproantigens retained about 70% of their initial antigenicity after 5 weeks. However, the antigens are relatively stable in some stools. This suggests that degradation of MM3-recognized *Fasciola* coproantigens depends on the presence of particular protease species, or other factors, which differ for each patient [34].

Other studies have also referred to the stability of *Fasciola* coproantigens. The monoclonal antibody F10 recognizes a 26–28 kDa antigen which is a monomeric proteoglycan secreted and excreted from the tegument and the gut of the flukes. The antigenicity of that coproantigen was noted to be stable or even enhanced by the action of proteolytic enzymes found in the digestive tract and under a variety of standard laboratory storage conditions. Storage at various temperatures resulted in some break down of the protein. The storage of the purified protein at room temperature overnight gave rise to several new bands ranging from 8 kDa to 20 kDa. Incubation of the purified coproantigen at 4°C for two months resulted in a major band at 8 kDa and a minor band at 20 kDa which decreased in size with longer incubation. Storage of ES for more than three years resulted in a major band at 8 kDa not seen in fresh ES. All these bands were recognized by the monoclonal antibody. The 26–28 kDa band was always detectable and the smaller bands are lower in intensity, suggesting that the coproantigen is relatively stable during storage. Thus, that degradation probably represents a loss of carbohydrate, since antigenicity is maintained [48,49].

One possible alternative would be freezing the samples at −20°C, but this poses the additional problems of (i) difficult and expensive transport of frozen samples to the laboratory where determination is to take place, and (ii) the non-appropriateness of frozen samples for the diagnosis of other parasite species present in coinfections. Another solution is the use of Coproguard, which has been demonstrated to be convenient for sample preservation in this kind of surveys including the application of a coproantigen-detection test [34].

In Huacullani and Cajamarca, the PPVs calculated for diverse epidemiological situations are very different. PPVs in hyperendemic situations were very high, making this test recommendable for such situations. Contrarily, NPVs calculated for diverse epidemiological situations are similar.

Current efforts for the control of human fascioliasis need diagnostic techniques which allow for high sensitivity and specificity, large mass screening, detection in the chronic phase, early detection of treatment failure or reinfection in post-treated subjects, and usefulness in surveillance programs. Our results indicate that a coproantigen-detection test such as MM3-COPRO ELISA fulfils all these aspects. It provides a good tool to detect biliary fascioliasis in humans under field conditions in Andean hyperendemic countries, including a higher sensitivity than egg detection techniques, especially in areas where burdens are usually low, such as in areas of the valley transmission pattern. Hence, the MM3-COPRO ELISA appears to be not only useful for individual diagnosis in hospitals, but also in human surveys in fascioliasis endemic areas characterized by low to high parasitic burdens.

The present MM3-COPRO ELISA validation is expected to facilitate the improvement of human fascioliasis diagnosis in endemic areas (a commercial version of the MM3-COPRO ELISA is today available). The practical application of this sensitive and convenient method for large scale surveillance in the control programs in the Northern Bolivian Altiplano and Cajamarca could improve screening of human fascioliasis in these endemic areas by detecting infected humans in the biliary stage of the disease, as a large number of samples can easily be processed. Keeping in mind that most affected subjects are usually children, the attainment of fecal samples is easier and faster than taking blood samples, which is considered invasive. The former does not pose difficulties for community elders, school head teachers and parents who usually give their consent. Moreover, to many of these indigenous communities, blood extraction is culturally not acceptable.

Furthermore, our experience in Huacullani and Cajamarca indicates that MM3-COPRO ELISA offers the easiest and fastest way to adequately face large mass screenings, by initially applying the coproantigen technique to all the coprological samples obtained in the community survey and thereafter applying the Kato-Katz technique only or first in coproantigen-positive samples. It is recommended to treat subjects with coproantigen-positive samples but with negative egg detection. This allows for a quick selected treatment action, lending the positive effects of (i) fast response in the communities surveyed that verify that infected subjects are treated within a few days after the survey, and (ii) reducing the probability of drug resistance appearance. The remaining coproantigen-negative samples may finally be analyzed for the eventual detection and subsequent selected treatment of very few subjects shedding eggs, although this last step will unavoidably be time-consuming.

Given the aforementioned advantages a coproantigen-detection test offers, one wonders why there are only relatively few of such tests for parasitic diseases affecting the digestive system available: amebiasis [50], giardiasis [51], opisthorchiasis [52], taeniasis [53], trichinelliasis [54], strongyloidiasis [55], hookworm infection [56]. Developing and/or improving highly specific coproantigen-detection test for diseases in which coprological diagnosis requires specialized personnel and time-consuming microscope work would evidently be welcome.

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Author Contributions

Conceived and designed the experiments: MAV SM-C. Performed the experiments: MAV MVP IP-C CA WS PH AT. Analyzed the data: MAV RA FV JRE HT DE AFG SM-C. Contributed reagents/materials/analysis tools: RA FV JRE PH. Wrote the paper: MAV SM-C.

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