Veterinary Parasitology xxx (2009) xxx–xxx

Contents lists available at ScienceDirect

Veterinary Parasitology

journal homepage: www.elsevier.com/locate/vetpar

Antibody and inflammatory responses in laying hens with experimental primary infections of *Ascaridia galli*

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ARTICLE INFO

Article history:
Received 14 May 2008
Received in revised form 25 November 2008
Accepted 3 December 2008

Keywords:
Ascaridia galli
Antibody
Blood
Yolk
Inflammatory response
Intestinal mucous

ABSTRACT

Ascaridia galli, an intestinal nematode that affects hens and other domestic and wild birds, causes economic losses in avian exploitations. The present work shows that *A. galli* stimulates a strong antibody response as well as an intense inflammatory reaction, in the intestinal mucous of experimentally infected Lohmann Brown laying hens. IgG antibodies against soluble extracts of *A. galli* embrionated eggs and adult worms, were detected in both blood and yolks eggs from infected hens during a period of 105 days after the infection. This indicates that hens transfer to their offspring a part of the IgG antibodies produced when they become infected. The antigens responsible for the stimulation of specific IgG were molecules of 30–34, 44–54 and 58–90 kDa, while in the yolk eggs of infected hens a reactivity directed against antigens of molecular weight (M_w) lower than 50 kDa was detected. Histology revealed traumatic lesions with leukocyte infiltration, and inflammation of the intestinal wall of the infected hens after 105 days of initial infection.

The possible influence of the immune and inflammatory response on the population dynamics of the parasite is discussed.

1. Introduction

In recent years, the changes in the consumer's demands have resulted in an increase of the number of laying hens kept in alternative production systems (Gauly et al., 2002). The environmental characteristics extant in these systems have resulted in a re-emergence of some helminthic infections, like ascaridiasis, caused by *Ascaridia galli* (Permin et al., 1997). This worldwide-distributed parasitic nematode locates itself in the small intestine of different domestic and wild birds (Chadfield et al., 2001). It is responsible for economic losses due to the growth and weight reductions it causes in its hosts (Gauly et al., 2005). In traditional exploitations, that maintain birds in soil and in alternative systems, prevalence of ascaridiasis is high. For example, in traditional free-range systems of central Spain we have recently observed a mean seroprevalence of 21.8% (ranging from 7.6% to 95%) (Martín-Pacho et al., 2005). Prevalence is also high in other countries, such as Austria, where the 64.1% of 609 laying hens analyzed eliminate eggs of *A. galli* in their feces (Hohenberger, 2000). In Denmark, the prevalence of *A. galli* in chickens raised in free-range systems was 63.8%, 41.9% in deep litter systems, 37.5% in backyard system, and 55% in battery cage systems (Permin et al., 1999).

A. galli has a direct life cycle. Infection occurs when hosts ingest embrionated eggs. Afterward, L3 invade the intestine wall where they moult to L4. Adult worms

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doi:10.1016/j.vetpar.2008.12.011

mature in the intestinal lumen where fertilized females produced unembrionated eggs that are excreted in the feces of infected birds (Ackert, 1931; Todd and Crowdus, 1952; Araujo and Bressan, 1977; Chadfield et al., 2001). The immune response developed by hosts against ascarid worms and its impact on the regulation of intestinal helminth populations have been extensively studied in mammals (Cooper et al., 2000; Miquel et al., 2005). In avian ascaridiasis, studies on the population dynamics of *A. galli* have been conducted to investigate the possible existence of host genetic or age related resistance to the parasite. Some studies indicate that the age of birds has a limited role in resistance to the infection (Idi et al., 2004), while hormonal and immune status related to laying activity seems to have a negative impact on resistance (Gauly et al., 2005). Recently, it has been demonstrated that chickens experimentally infected with *A. galli* eggs, develop a typical Th2-type cytokine pattern, 14 days post-infection (p.i.) (Degen et al., 2005). Nevertheless, as far as we know, no studies have been conducted to identify the specific antibodies and cells involved in the response against *A. galli*.

The objective of this study is to determine the dynamics of the IgG antibody response against larval and adult *A. galli* antigens in primary infections of Lohmann Brown laying hens, and to correlate this response to the parasitologic characteristics of the infection, as well as to provide initial data on the inflammatory alterations caused by the parasite in the intestine of infected birds.

2. Materials and methods

2.1. Parasites

A. galli eggs were recovered from the uteri of gravid female worms, obtained from naturally infected hens. Eggs were incubated at 20 °C in a 4% potassium–bichromate solution until they became infective, according to the procedure of Gauly et al. (2002).

2.2. Experimental infections

Twelve 18 weeks old, Lohmann Brown laying hens (procured from Ibertec, Parque Tecnologico de Boecillo, Valladolid), born and raised in helminth free conditions, were employed. The absence of helminth parasites was confirmed by faecal analysis. The hens were orally infected using a plastic Pasteur pipette as described by Permin et al. (1997) with individual doses of 250 eggs of *A. galli*. Six hens were maintained uninfected as negative control. All animals received water and food "ad libitum". They were followed on a daily basis and examined clinically for signs of the disease. Individual fecal and serum samples were collected before the infection (day 0), and weekly until the end of the experiment, 105 days p.i.

Eggs produced by hens were also collected during the experiment. Yolks were separated, mixed 1:2 in a 0.1 M PBS pH 7.2 solution and stored at −20 °C, until used.

On day 105 of the experiment, all hens were slaughtered and the gastrointestinal tracts were removed, opened in a longitudinal section, and washed with tap water. The contents were poured onto a sieve with a mesh aperture of 100 μm and then washed. The remains of the screen were examined for the presence of adult and immature *A. galli* using a stereomicroscope. Worms were identified, sexed, counted and weighed.

2.3. Antigen preparation

Soluble antigenic extracts from embrionated eggs and from *A. galli* adult worms were prepared as follows: embrionated eggs and adult worms were washed, macerated and sonicated (three cycles of 70 kHz, 30 s) in sterile saline solution. The homogenate was centrifuged at 16,000 × *g* for 30 min. The supernatant was dialyzed against 0.01 M PBS, pH 7.2. The protein concentration was measured (Bradford, 1976) and adjusted to a 4 μg/μl final solution. All procedures were carried out at 4 °C. Both antigens were stored at −20 °C, until used.

2.4. Faecal egg counts (FEC)

Individual fecal samples were analyzed using a modified McMaster technique (MAFF, 1986) with saturated sodium chloride solution and the MSD counting chamber.

2.5. ELISA for the detection of anti-A. galli IgG antibodies

Anti-*A. galli* IgG antibodies were analyzed on serum samples and egg yolks from experimentally infected hens and 6 non-infected hens by an enzyme immunoassay tests (ELISA) performed, with some modifications, as described by Marcos-Atxutegi et al. (2003). Briefly, polystyrene microplates were coated with 0.8 μg/well of each antigen, overnight at 4 °C. Serum samples were examined at day 0 and weekly until the end of the experiment (day 105 p.i.). They were tested at 1/200 and 1/400 dilutions, and an anti-IgG anti-chicken antibody conjugated to horseradish peroxidase (HRP) (Sigma) was employed as secondary antibody at 1/10,000 and 1/800 dilutions, respectively, to test reactivity against both antigens. Optical densities (OD) were measured at 492 nm in an Easy-Reader, Bio-Rad. To test the IgG response against antigens of *A. galli* adult worms in yolk samples, these were analyzed at 1/40 dilution and the anti-chicken IgG was employed at 1/4000.

2.6. Western blot

Western blot (WB) was carried out as described by Tsang et al. (1985). Proteins of the soluble antigen extract from embrionated eggs and from *A. galli* adult worms were separated on 12% gel slabs, in accordance to Laemli's method (1970) in a Miniprotean II (Bio-Rad Laboratories Inc., USA). All samples were treated with 0.15 dithiothreitol, 2% SDS, 1 M Tris–HCl (pH 6.8), 10% glycerol and 0.2% bromophenol blue. Samples were heated in a 100 °C water bath for 3 min and then transferred to nitrocellulose.

Serum samples were tested at 1/25 and 1/200 dilutions for each antigen and the anti-chicken IgG-HRP was employed at 1/2500 and 1/4000 dilution, respectively.

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Yolks samples were tested at 1/40 dilutions and the anti-chicken IgG-HRP was employed at 1/4000 dilution.

Taking into account the antibody response revealed by ELISA, serum samples were examined at days 0, 14, 21 and 42 p.i. against soluble antigen extract from embrionated eggs and at days 0, 14 and 21 against soluble antigen extract from *A. galli* adult worms. Days 0, 30, 60 and 90 p.i. were chosen in the case of yolks.

2.7. Histology

On day 105 p.i., the gastrointestinal tracts were removed, and different pieces were trimmed and fixed by immersion in 4% buffered formalin for 24 h. The blocks, obtained were dehydrated in a graded series of ethanol, and embedded in paraffin. Three micrometers thick sections were cut, mounted on glass slides and counter-stained with hematoxylin–eosin for light microscopy analysis.

2.8. Statistical analysis

Statistical analysis was performed to assess differences in IgG antibody response, measured by the ELISA test, among the hens. The non-parametric Kruskal–Wallis test and the multiple-comparison Dunn test were used to identify differences in the antibody levels between post-infectious days. Significant differences were defined when $P < 0.01$ and $P < 0.05$, respectively.

3. Results

Parasitological analysis—Two parameters were studied to confirm the infection: the excretion of parasite eggs in hen's feces (Fig. 1) and the number of adult worms in the gastrointestinal tract at the end of the experiment (Table 1). Parasite eggs in hen's feces were detected for the first time, on day 42 p.i. Between this day and day 84 p.i., an increase in the number of eggs was detected. Following this, the number of eggs decreased until the end of the experiment. The number of parasite eggs was significantly higher on days 77 and 84 p.i. than on days 49 p.i. ($P < 0.01$) and 98 ($P < 0.05$). There were no significant differences between days 77 p.i. and 84 p.i. or between days 56 p.i. and 98 p.i. At the end of the study (day 105 p.i.) the birds were slaughtered.

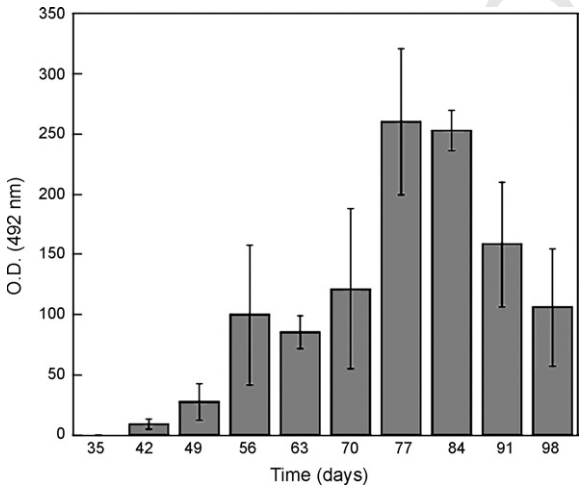

Fig. 1. Mean fecal parasite egg count in the 12 experimentally infected hens. The Bars indicate standard deviations.

Table 1
Parasitological parameters (mean ± standard deviation) in infected hens slaughtered on day 105 p.i.

PARA Mil TCR	Mean ± standard deviation
Worm burden (total)	5 ± 3.36
Female worm burden	2.4 ± 1.76
Male worm burden	2 ± 1.92
Female worm length (cm)	6.9 ± 1.71
Male worm length (cm)	5.3 ± 1.00
Female worm weight (mg)	0.124 ± 0.059
Male worm weight (mg)	0.058 ± 0.029

The mean of total worm burden was 5 ± 3.36, being similar the number of male and female worms (Table 1).

IgG antibody response in serum samples from experimentally infected laying hens. The IgG response against both antigens was analyzed weekly until the end of the experiment (Fig. 2). The mean OD and standard deviations (SD) of the IgG antibody response against antigens of embrionated eggs are shown in Fig. 2A. An increase in the mean OD was detected from day 0 until day 42 p.i., with a non-significative and transient decrease on day 28 p.i. between days 42 and 105 p.i. mean ODs showed periodical fluctuations. There were statistical differences between days 14 and 21 p.i. and between days 14 and 42 p.i. ($P < 0.01$ in both cases). There were no significant differences between the mean ODs observed after day 42.

The IgG response against adult worm antigen are shown in Fig. 2B. A rise in the mean ODs between day 0 and 21 p.i. is observed, followed by slight decrease on day 28 p.i. Between day 28 and day 84 p.i. some non-significant fluctuations were detected. Mean ODs fall on day 91, reaching similar levels to those observed on days 63–70 p.i. afterwards. There were significant differences between mean ODs observed on days 14 and 21 p.i. ($P < 0.01$), but not between those obtained on days 21 and 28 p.i. Significant differences were also observed in mean ODs obtained on day 91 p.i. when compared with those observed on days 84 and 98 p.i.

Antibody response in yolks from infected hens. The presence of IgG antibodies against *A. galli* was analyzed in yolks from laying hens eggs until day 105 p.i. (Fig. 3). IgG antibody response against antigens of embrionated eggs is shown in Fig. 3A. The highest mean ODs were observed on days 28 and 35 p.i.

Afterwards reactivity showed a tendency to decrease until the end of the experiment. However, there was a slight increase in mean OD between days 49 and 84 p.i. There were significant differences between mean ODs obtained on days 28 and 35 p.i. when compared to days 7, 14 and 21 p.i. ($P < 0.01$), 49–77 p.i. ($P < 0.05$), and 84–105 p.i. ($P < 0.01$).

IgG antibody response against antigens from *A. galli* adult worms appears in Fig. 3B. In this case, IgG increased, with some fluctuation, from the beginning to the end of the

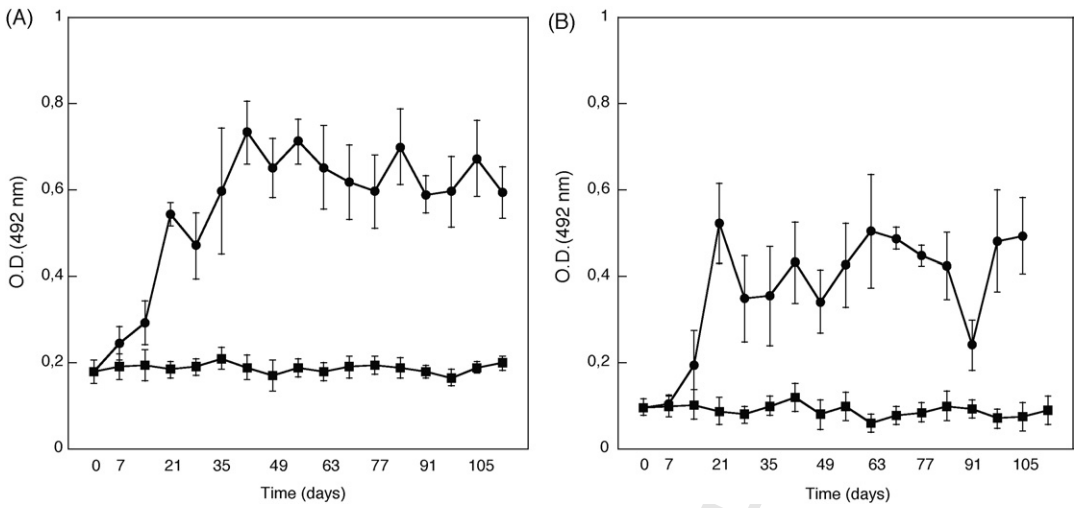

Fig. 2. Time evolution of the specific IgG antibody response against *A. galli* antigens in blood samples from the experimentally infected and control hens. (A) Antibody response against embrionated egg antigen. (●) Infected hens. (■) Non-infected hens. (B) Antibody response against adult somatic antigen. (●) Infected hens. (■) Non-infected hens. The antibody response was measured by ELISA. Each point corresponds to the mean OD obtained from 12 individual experimentally infected hens. The Bars indicate standard deviations.

experiment. Significant differences were only observed between days 7 and 105 p.i. ($P < 0.05$).

Identification of molecules involved in reactivity against *A. galli*. Molecules from *A. galli* embrionated eggs and adult worms antigens involved in the antibody stimulus at the blood level were identified by Western blot (Fig. 4A and B respectively). Molecules from antigens of *A. galli* embrionated eggs. Fourteen days p.i., there was a small increase in the reactivity stimulated by groups of antigens with M_w of approximately 28–30 kDa and a molecule of 11 kDa. This response is still present in day 21 p.i. but a clear increase in the number of molecules recognized is detected 42 p.i. At this time reactivity around 107, 100, 90, 50, 28–30 and 11 kDa antigens was observed (Fig. 4A). Molecules form antigens of *A. galli* adult worms. Fourteen days p.i., there was an obvious but moderate increase in the reactivity stimulated by three groups of antigens with M_w of approximately 30–34, 44–54 and 58–90 kDa, respectively, and a molecule of 98 kDa. Twenty-one days p.i. reactivity increase, being specially intense against the antigens detailed before. Furthermore, antigens of M_w lower than 29 kDa were recognized intensely, at this time (Fig. 4B).

Antigens responsible for the antibody stimulus, detected on yolk eggs from infected laying hens, are shown in Fig. 5. The first bands were precipitated 30 days

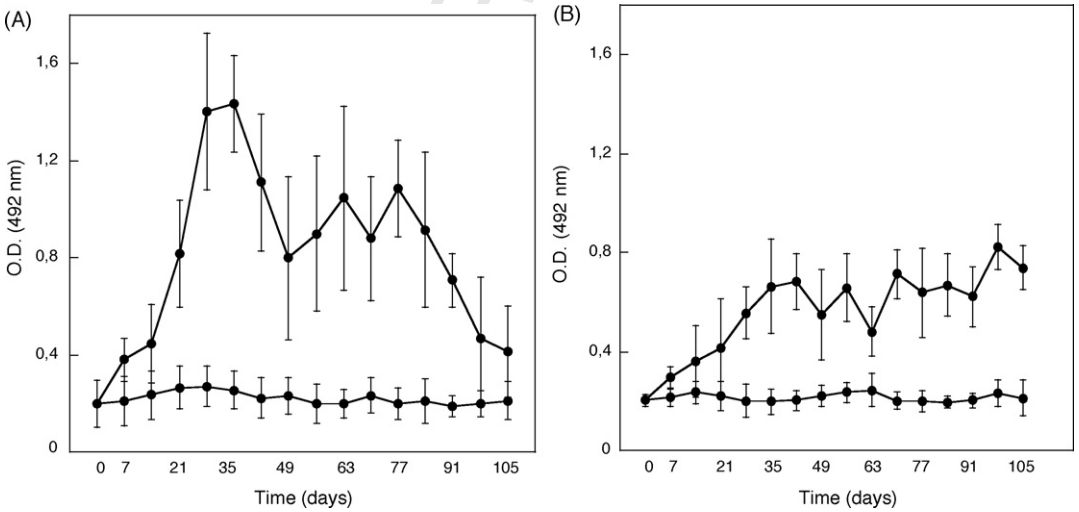

Fig. 3. Time evolution of the specific IgG antibody response against *A. galli* antigens in egg yolk samples from the experimentally infected hens. (A) Antibody response against embrionated egg antigen. (●) Infected hens. (■) Non-infected hens. (B) Antibody response against adult somatic antigen. (●) Infected hens. (■) Non-infected hens. The antibody response was measured by ELISA. Each point corresponds to the mean OD obtained from 12 individual experimentally infected hens. The Bars indicate standard deviations.

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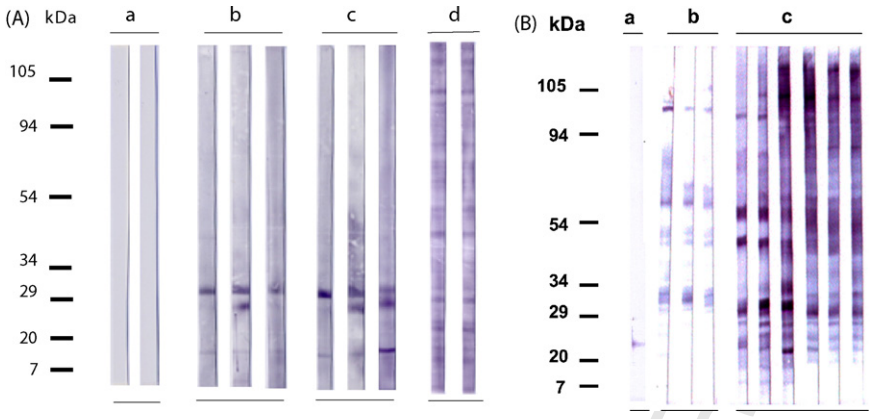

Fig. 4. Western blot analysis showing the recognition pattern of polypeptides of the *A. galli* antigens. (A) Response against embrionated eggs antigens. (a) Negative control serum sample. (b) Serum samples taken 14 days after infection. (c) Serum samples taken 21 days after infection. (d) Serum samples taken 42 days after infection. (B) Response against adult worms antigens. (a) Negative control serum sample. (b) Serum samples taken 14 days after infection. (c) Serum samples taken 21 days after infection.

p.i. A moderate reactivity produced by a group of antigens of approximately 42–50 kDa and by the 98 kDa molecule, was observed. Bands between 7 and 50 kDa were recognized 60 days pi.; the band of 30–32 kDa was specially intense. The recognition pattern did not change in yolks from eggs laid 90 days p.i. in comparison to those laid 60 days p.i. Only one of the analyzed samples showed recognition of antigen bands over 54 kDa.

Histology—The histological study of the gastrointestinal tract of infected laying hens is shown in Fig. 6. Fig. 6A shows a macroscopic longitudinal section of the small intestine from an infected laying hen containing some *A. galli* adult worms. No morphological lesions were observed on the wall and intestinal villi of healthy hens (Fig. 6B). On the contrary, small intestines of infected hens showed intense anatomic alterations consistent of traumatic lesions in the wall similar to stretch produced by a strange migrant body, that continue in the mucous layer (Fig. 6C and D). In some parts, these stretches appear infiltrated by inflammatory cells (Fig. 6E). The mucous layer was completely altered; the villi disappeared and it showed hemorrhagic areas indicating vascular lesions (Fig. 6F). Moreover, an intense inflammatory cell infiltration in the basal zone of some villi was observed (Fig. 6G). The most common leukocytes observed were lymphocytes and macrophages.

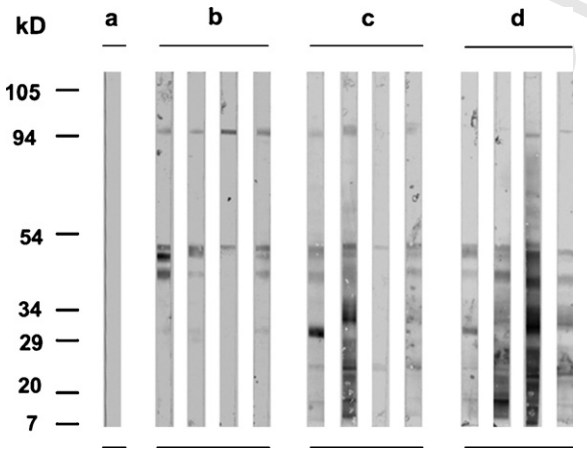

Fig. 5. Western blot analysis showing the recognition pattern of polypeptides of the *A. galli* somatic antigen by representative samples of egg yolks laid by the infected hens. (A) Negative control serum sample. (B) Yolk samples taken 30 days p.i. (C) Yolk samples taken 60 days p.i. (D) Yolk samples taken 90 days p.i.

4. Discussion

The results presented here demonstrate that *A. galli* stimulates a strong immune response in their hosts. In fact high concentrations of specific IgG anti-*A. galli* antibodies were detected in both blood and egg yolks from infected laying hens. Moreover, an inflammatory reaction at the level of the intestinal wall, with the appearance of an intense cellular infiltration in the mucous and submucous membrane, was observed.

Different studies suggest that the resistance to *A. galli* infection increases with the age of the infected birds (Ackert et al., 1935; Ikeme, 1973; Idi et al., 2004). This fact is probably due, among other causes, to the increase in the capacity of immune response of the infected hens. Thus, we have selected for our study 18 weeks old Lohmann Brown laying hens, to have a reasonable confidence that the experimental infections stimulate an accurate and measurable immune response. All hens were infected and all excreted parasite eggs in their feces. The parasitological parameters obtained (rate of establishment, size of the worms and egg number) were slightly lower than those observed by other authors in comparable conditions (Permin and Ranuig, 2001a; Gauly et al., 2005). This could be due, at least in part, to the length of the experiment and the dose of infection (Idi et al., 2004).

Specific IgG antibodies against antigens of embrionated eggs as well as adult worms antigens have been detected. Interestingly, in both cases the first significant increase in the antibody level occurs between 14 and 21 days p.i. This data is consistent with that obtained by Degen et al. (2005), which observed the expression of the Th2-related

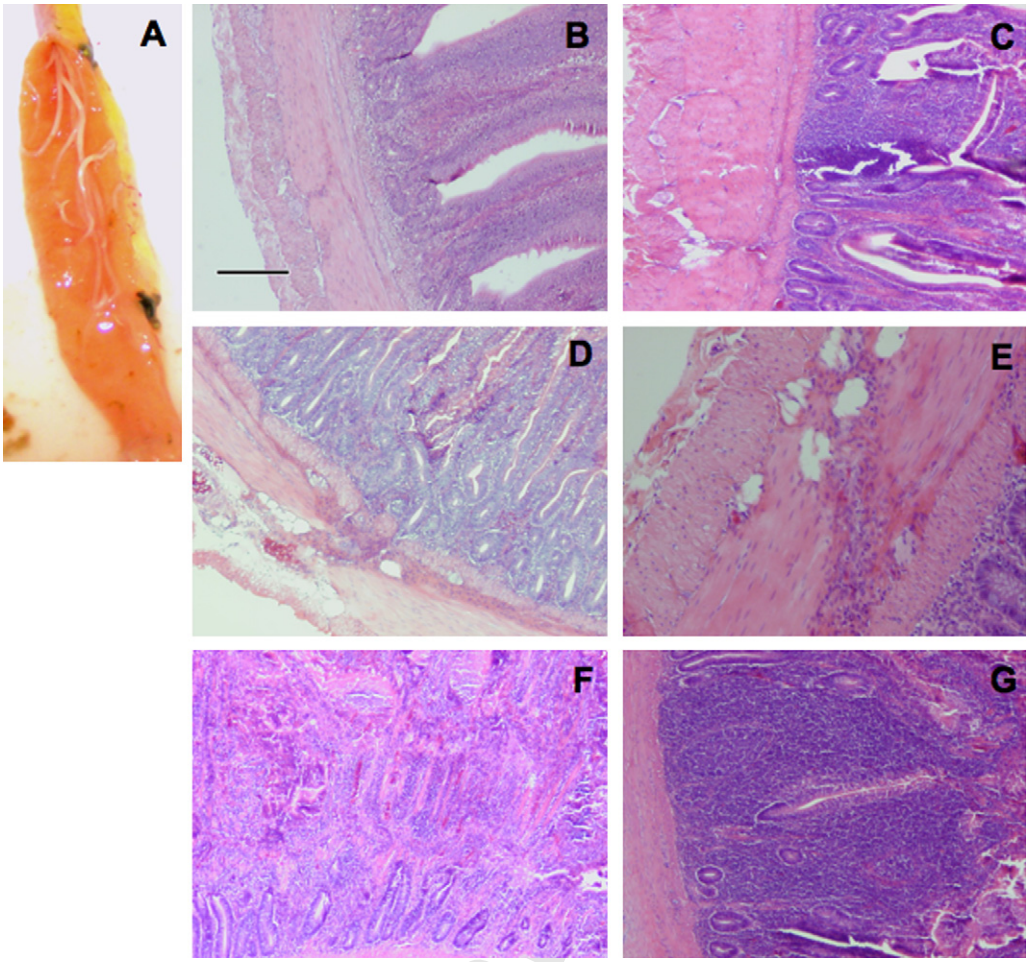

Fig. 6. Small intestines histology of infected hens. (A) Macroscopic section of intestine containing *A. galli* adult worms. (B) Intestinal wall and villi of a healthy hen. (C and D) Traumatic lesions in the small intestines of infected hens similar to those that can be caused by a strange migrant body. (E) Inflammatory cell infiltrate in the traumatic lesions. (F) Completely altered mucous layer showing hemorrhagic areas and absence of villi. (G) Intense inflammatory infiltrate in the basal zone of the intestinal mucous layer. Figure E Bar is 80 mm length while figures B, C, D, F and G is 200 mm length.

cytokines in *A. galli* infected hens, 14 days after the infection. The IgG antibody response developed by hens was slightly stronger against embrionated eggs than adult antigens. This could be attributed to the different behavior and location of the larvae and adult worms during the development of the endogenous life cycle in the host. Larvae invade the intestinal wall, thus they may produce a stronger stimulation of IgG, while the adult worms locate themselves in the intestinal lumen, which probably stimulate a predominant antibody response of the IgA isotype, not studied in this work. The highest excretion of parasite eggs coincides with a significant fall of the antibody level against adult antigens detected between 70 and 91 days p.i. We do not know the reason of this finding, but the inverse relationship between the antibody level and the intensity of egg production by the parasites is evident. This is consistent with the direct effect of the host immunity on the reduction of helminth fecundity (Urquhart et al., 1996).

Another important fact for the correct understanding of the influence of the immune response on the parasite population dynamics is the accumulation of anti-*A. galli* IgG in the egg yolks of the infected hens. This indicates that mothers transfer to their offspring a part of the anti-*A. galli* antibodies produced when they became infected, in a similar way to mammals (Carlier and Truyens, 1995). This fact is not in contradiction to the data demonstrating a high susceptibility to the infection in 1-day-old chickens when compared to that observed in other ages (Ackert et al., 1935; Kerr, 1955). Experimental infections of chickens from *A. galli* infected and non-infected hens that will allow us to identify establishment rate, worm size and fecundity in both groups, are necessary to demonstrate the protective effect of the transferred antibodies and its influence in the parasite population dynamics. On the other hand, Western blot revels a very limited molecules recognition until 21 days p.i. in embrionated eggs antigens and until 14 days p.i. in adult somatic antigen. After 42 and 21 days p.i., respectively, reactivity increase and many antigens are recognized in the entire M_w range. Only specific antibodies against antigens of medium/low M_w pass into the yolk eggs of infected hens. Clearly, these

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antigens could be candidates to be part of a hypothetic vaccine against *A. galli*, if their protective activity is confirmed.

Histology reveals severe traumatic lesions in the small intestinal wall, together with an intense cellular infiltration by lymphocytes and macrophages. In some areas the normal structure of the mucous membrane became completely altered, the villi and crypts disappearing. Nevertheless, considering the time elapsed from the invasion of the intestine wall by the larvae to the histological study, the lesions observed can be interpreted as residual alterations caused by the migrating larvae and/or damages caused by the adult worms located in the intestine. An interesting question is whether this cellular as well as the antibody response detected, could play a role in the control of larvae that complete their development into adults. Moreover, despite these alterations, the infected birds manifested neither symptoms nor signs nor weight loss or decrease in their egg output rates (data not shown), during the time of the experiment. It is probable that, as indicated in other studies, a low number of adult worms in the intestine together with an appropriate feeding keep the hosts away from the appearance of illness signs, stressing the importance of a good feeding in the resistance against *A. galli* (Permin and Ranuig, 2001a). Nevertheless, the existence of infected asymptomatic hens in commercial farms can be extremely dangerous for other members of the avian community, as they are a source of parasite eggs.

In conclusion, for the first time, data demonstrating the development of both an antibody and cellular inflammatory response against *A. galli* infective eggs and adult worms, have been obtained. Moreover, transference of specific IgG antibodies to the yolk eggs of infected hens is shown. The role of these antibodies, as wells as the inflammatory reactions, on events like the arrest of larval development in the intestine wall and the parasite population control on an infected bird population, must be investigated in the future for an accurate understanding of the protective mechanisms developed by the birds against *A. galli*.

Acknowledgments

This work has been supported by a research contract between Ibérica de Tecnología Avícola (IBERTEC) S.L. and the University of Salamanca through a proyect granted by the Agencia de Desarrollo Económico de Castilla y León (FEDER-founds, 2005).

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