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Research paper

Time- and dose-dependent development of humoral immune responses to *Ascaridia galli* in experimentally and naturally infected chickens

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ABSTRACT

Factors affecting the development of *Ascaridia galli*–specific humoral responses and their protective roles are largely unknown. We investigated the effects of time and infection dose on *A. galli*–specific IgY antibody levels following experimental infection. The acquisition and development of new infections and reinfections were also monitored by using tracer birds. Relationships between the retrospective IgY and the final worm burden of the birds were investigated to determine whether humoral immune responses generated during infection provide protection to the host animal.

Young chickens were infected (+) with either 100 or 1000 embryonated eggs of *A. galli* (100+: n = 45; 1000+: n = 45) or kept as uninfected controls (CON: n = 10). Uninfected birds were also added to each infection group as tracer (T) birds (T100+; n = 5 and T1000+; n = 5). Faecal egg counts and IgY antibody concentrations in plasma and egg yolk were determined at selected intervals. Final worm burdens were quantified at 28 weeks post infection (wpi).

The plasma antibody (PAB) and egg yolk antibody (EAB) levels of experimentally infected birds were compared to those of control and tracer birds throughout the study period, and PAB levels were found to depend initially on the infection dose but thereafter mainly on reinfections. Starting at wpi 2, 1000+ had consistently higher PAB levels than CON did ($P < 0.05$). With exceptions at wpi 0, 2 and 12, PAB levels were also higher ($P < 0.05$) or tended to be higher ($P < 0.10$) in 100+ than in CON. An earlier and higher increase was observed in the PAB levels of T1000+ than in those of T100+, implying that (re-)infection occurrence depended on the infection dose. Although 1000+ showed higher ($P < 0.05$) EAB levels than CON did at both wpi 14 and 18, EAB levels were higher in 100+ than in CON only at wpi 28 ($P < 0.05$). The total worm burdens in the initial experimentally infected birds were similar ($P = 0.257$); they were also highly comparable between experimentally and naturally infected birds, indicating that final worm burden was mainly determined by the naturally occurring infections resulting from continuous exposure. When all available information on the retrospective plasma and egg yolk IgY levels was collectively evaluated to estimate the larval or total worm burdens of the experimentally infected birds, both PAB and EAB levels at particular wpi were significantly associated with worm burden, especially with larval count. In conclusion, our data support the hypothesis that the number of larvae, rather than the number of mature worms, affects the antibody levels in both plasma and egg yolk. Despite the increased levels of *A. galli*–specific antibodies in plasma and egg yolk throughout the study period, only a weak indication was found that antibodies might be directly associated with protection.

1. Introduction

Nematode infections in laying hens on farms in Europe have re-emerged during the last decade. *Ascaridia galli, Heterakis gallinarum* and *Capillaria* spp. are the most prevalent species in this area (Kaufmann et al., 2011; Thapa et al., 2015). The prevalence of *A. galli* infections at the flock and individual levels is extremely high, particularly in hens on free-range or organic farms (Kaufmann et al., 2011; Wongrak et al. 2014; Thapa et al., 2015; Grafl et al., 2017), and worm burden also seems high (Kaufmann et al., 2011; Wongrak et al. 2014). Along with impaired host performance, which manifests as lower nutrient utilization and growth (Daş et al., 2010; 2012) and is mainly caused by larval

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damage to intestinal tissues (Luna-Olivares et al., 2015), this nematode can act as a vector for *Salmonella enterica* (Eigaard et al., 2006) and may impair humoral responses after immunization against Newcastle disease virus (Pleidrup et al., 2014a). Recent data indicate that ascarid infections are also associated with elevated mortality risk in laying hens (Hinrichsen et al., 2016).

Laying hens exposed to naturally occurring helminth infections during the laying period have extremely high numbers of *A. galli*, suggesting that protective immunity to this nematode might be weak. The acquisition of immunity to intracellular and extracellular pathogens in mammals is mediated mainly through the distinct, highly polarizable type 1 (Th1) and type 2 (Th2) pathways, respectively (Mosmann et al., 1986; Degen et al., 2005). Degen et al. (2005) demonstrated that the polarization of Th1/Th2 immunity occurs in chickens exposed to a paramyxovirus (Newcastle disease virus) or the roundworm *A. galli*. Schwarz et al. (2011) confirmed these results directly, showing that immune response to *A. galli* infection manifests as Th2 cytokine induction and local T lymphocyte infiltration as well as changes in epithelial cell secretion and absorption. Moreover, increasing levels of circulating *A. galli*–specific antibodies were quantified in the infected chickens (Marcos-Atxutegi et al., 2009; Schwarz et al., 2011; Norup et al., 2013), although they correlated only weakly with worm burdens six weeks post infection (wpi) (Schwarz et al., 2011). The latest research has mainly focused on local or systemic immune responses to *A. galli* (Pleidrup et al., 2014b; Dalgaard et al., 2015; Ruhnke et al., 2017).

Although *A. galli*–specific antibodies have been investigated in several studies (Marcos-Atxutegi et al., 2009; Schwarz et al., 2011; Norup et al., 2013; Ruhnke et al., 2017), associations with worm burden are largely unknown. Chickens produce three types of immunoglobulins, namely, IgY, the equivalent to IgG in mammals, IgA and IgM, although further antibodies equivalent to mammalian IgE and IgD have been proposed (Carlander et al., 1999; Jeurissen et al., 2000; Hamal et al., 2006). Albumen is the main storage medium of IgA and IgM, whereas IgY is exclusively found in egg yolk, which is then transferred to the circulation of the chick during *in ovo* development (Rose et al., 1974). Thirty percent of the IgY found in the dam's plasma is transferred to the chick, whereas only 1% of both IgM and IgA is transferrable (Hamal et al., 2006). The available results on the protective role of antibodies produced against *A. galli* are somewhat contradictory. Sadun (1949) performed a series of studies on passively acquired immunity and concluded that serum transfer from *A. galli*-resistant to susceptible chickens conferred protection against the nematode in the recipients. In contrast, Andersen et al. (2013) quantified no protective immune response in chickens immunized with *A. galli* antigens orally or intra-muscularly. Similarly, maternally derived *A. galli*–specific antibodies provided no protection against this nematode (Rahimian et al., 2017). Nevertheless, a recent study by Norup et al. (2013) demonstrated an inverse relationship between *A. galli*–specific antibody levels and nematode egg excretion, a potential indication of an acquired protective immunity, in two inbred chicken lines possessing different major histocompatibility haplotypes. However, the relationships between humoral immune responses and infection intensity levels (i.e., worm burden) of the infected animals were not examined in that study.

IgY produced against *A. galli* provides a reliable infection proxy for diagnostic purposes in layer chickens (Daş et al., 2017). However, the factors affecting the development of nematode-specific humoral responses are largely unknown. In addition, whether and to what extent *Ascaridia galli*–specific IgY antibodies provide a protective immune function to the host animal remains unclear. Therefore, this study determined the effects of infection dose and time on *A. galli*–specific IgY antibody levels in chickens using experimentally induced and naturally occurring infections. Furthermore, relationships between retrospective humoral immune responses and final worm burden were investigated to elucidate whether retrospective humoral immune responses provide a protective function to the host animal.

Table 1
Number of birds in the experimental groups at the beginning and end of the experiment, with corresponding cumulative mortality rates and sample sizes for *Ascaridia galli*–specific IgY in plasma (PAB) and in egg yolks (EAB).

Group	N at wpi 0	N at 28 wpi	Mortality, %	Sample size	
				PAB	EAB
CON	10	9	10.0	137	18
100+	45	41	8.9	644	81
1000+	45	31	31.1	544	62
Tracers in 100+	5	4	20.0	70	8
Tracers in 1000+	5	4	20.0	60	8
Total N	**110**	**89**	**19.1**	**1455**	**177**

2. Material and methods

2.1. Experimental setup, chickens and management

A total of 110 one-day-old female chicks of a layer genotype (Lohmann Selected Leghorn) were floor-housed in an experimental stable under helminth-free conditions until 16 wk. Then, 3 groups of birds with similar stocking densities were formed. The birds were randomly allocated to an uninfected control group (CON; n = 10) or experimentally infected (+) with either 100 (100+; n = 45) or 1000 (1000+; n = 45) embryonated/infective *A. galli* eggs. To monitor the acquisition and development of naturally occurring new infections or reinfections, 5 uninfected birds were additionally placed in each of the experimentally infected groups as tracer (T) birds (T100+; n = 5 and T1000+; n = 5). The experimental setup and sample collection schemes are presented in Supplementary Fig. 1. Table 1 shows the numbers of birds in all the groups.

The birds were fed commercial diets suitable for their age-specific nutrient requirements (e.g., 3–8 wk: grower diet, 18.4% crude protein (CP); 9-16 wk: developer diet, 16.2% CP; ≥17 wk: layer diet, 18.5% CP) *ad libitum* and had free access to fresh water. Environmental conditions (temperature, lighting schedule) in the experimental stable were in line with the recommendations of a commercial breeding company (Lohmann Tierzucht GmbH, Cuxhaven, Germany).

The birds were vaccinated against Marek's disease virus, Newcastle disease virus and Salmonella on the 1st, 14th and 18th days of life, respectively. The experimental birds were not vaccinated against coccidiosis and received no anthelmintic treatment before or during the study period. Pooled faecal samples on infection day (i.e., at 16 wk of age) indicated no gastrointestinal helminth infection during the pre-experimental period. Furthermore, parasitological examination of gastrointestinal tracts from five birds that died during the pre-experimental period also confirmed the helminth-free status of the birds.

2.2. Experimental infection

The source of infection material (mature female worms) was obtained from the small intestines of naturally infected chickens slaughtered in a local slaughterhouse. *A. galli* was identified based on morphology (Ramadan and Abou Znada, 1992). The eggs were isolated from the uteri of adult female worms as described previously (Rahimian et al., 2016) and embryonated in 0.1% $K_2Cr_2O_7$ at room temperature for 4 wk (no refrigerator storage at any stage). After incubation, approximately 40% of the eggs were classified as fully embryonated based on the developmental stage classifications by Rahimian et al. (2016). Using the same batch of embryonated eggs, two infection dosages (100 and 1000 embryonated eggs per bird) were prepared and given to the birds of the corresponding infection groups in 0.2 ml of water using a 5-cm oesophageal cannula. The uninfected controls and tracer birds received 0.2 ml of water orally as a placebo. To prevent the tracer birds from contamination with the *A. galli* eggs given to the experimentally