TÜBİTAK

Turkish Journal of Veterinary and Animal Sciences

http://journals.tubitak.gov.tr/veterinary/

Research Article

Turk J Vet Anim Sci
(2016) 40: 764-768
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doi:10.3906/vet-1602-4

Selecting Eimeria *tenella*-resistance markers by comparing resistant and susceptible groups of Jinghai Yellow chickens

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Received: 01.02.2016 • **Accepted/Published Online:** 02.05.2016 • **Final Version:** 15.12.2016

Abstract: Improving the genetic resistance of chickens to parasitic diseases is an alternative way to protect the poultry industry. To establish valid measurable in vivo parameters indicating coccidiosis resistance, 232 Jinghai Yellow chickens were randomly selected and orally infected with *Eimeria tenella* (1.5×10^4 sporulated oocysts per chicken). The resistant and susceptible chickens were classified based on their cecal lesion scores 8 days after infection. Ten plasma components, including antioxidant enzymes, interleukins (ILs), nitric oxide (NO), interferon-γ (IFN-γ), and β-carotene, were compared among the resistant, susceptible, and control chickens (uninfected with *E. tenella*). The NO, superoxide dismutase (SOD), IL17, and IFN-γ concentrations were significantly higher in the resistant group than in the susceptible group ($P < 0.05$), and malondialdehyde (MDA) was significantly lower in the resistant group than in the susceptible group ($P < 0.05$). These results suggest that plasma NO, IFN-γ, SOD, MDA, and IL17 can be used as markers of resistance or susceptibility to *E. tenella* in the selection of *E. tenella*-resistant chickens.

Key words: Coccidiosis-resistance markers, *E. tenella* infection, plasma, chickens

1. Introduction

Avian coccidiosis, caused by seven protozoan parasite species of the genus *Eimeria*, can significantly reduce the feed utilization and growth of chickens, causing more than $3 billion in economic losses annually worldwide (1,2). The conventional measures used to prevent coccidiosis with anticoccidial drugs have several limitations, including drug resistance, and food safety (3–5). Although vaccination can be effective, it is costly to use in the meat industry and no single vaccine can protect against all species of *Eimeria* in the field (6). Therefore, identifying chickens with genetic resistance to coccidiosis would be extremely valuable to the poultry industry in combating this very costly disease (7).

The measurement of resistance phenotypes is the first step in selecting an animal for disease resistance (8). Interferon-γ (IFN-γ) can activate macrophages to produce nitric oxide (NO), which inhibits *E. tenella* replication within host cells (9). NO is important in the process of coccidiosis infection and the host's immune response, not only because it participates in killing the parasite, but also because it regulates the synthesis and secretion of interleukins (ILs) and IFN (10). Plasma components, such as β-carotene (β-C), NO, and IFN-γ, have also been used to evaluate coccidiosis resistance in chickens infected with *E. maxima* (11–13). Wang et al. (14) found that a eukaryotic plasmid expressing chicken interleukin-2 (IL-2) enhanced protective immunity against coccidiosis. IL-17 may play a role in the immune regulation of birds infected with *Eimeria* (15), and is involved in a broad range of cellular activities against infection-induced inflammation by inducing the production of proinflammatory cytokines and chemokines. Plasma antioxidative enzymes, such as malondialdehyde (MDA), catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GSH-Px), have been used to assess the potential oxidative damage induced by *Cryptosporidium parvum* infection (16–18). However, few studies have comprehensively evaluated the correspondence between different plasma parameters and resistance to *E. tenella* in chickens (19,20). Based on previous results, 10 plasma components (β-C, NO, IFN-γ, MDA, CAT, SOD, GSH-Px, IL2, IL6, and IL17) were compared between resistant and susceptible groups of Jinghai Yellow chickens to identify appropriate markers for the selection of *E. tenella*-resistant chickens.

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2. Materials and methods

2.1. Experimental chickens and pathogens

Two hundred and thirty-two 1-day-old Jinghai Yellow chickens (obtained from the Jinghai Yellow Chicken Resource Farm, Haimen, Jiangsu Province, China) were raised in a specific-pathogen-free housing facility and were allowed access to feed and water ad libitum. The chickens had not been vaccinated and were fed an antibiotic-free diet during the experiment. Fecal samples from each chicken were regularly checked for *Eimeria* oocysts to ensure that all the experimental chickens were uninfected with the parasite.

The pure *Eimeria tenella* oocyst was originally isolated using single oocyst infected techniques (21) in the field in Yangzhou, China, and maintained in the Department of Parasitology, College of Veterinary Medicine, Yangzhou University, Jiangsu Province, China. The parasite oocysts were harvested, sporulated, and stored as previously described (19).

2.2. Grouping experimental chickens

When the chickens were 30 days old, they were transferred to individual wire cages (one bird/cage), and 16 birds (eight cockerels and eight pullets) were randomly selected as the control group (uninfected with *E. tenella*). The remaining 216 birds were orally infected with 1.5×10^4 sporulated oocysts of *E. tenella* per chick. Based on the cecal lesion scores at 8 days postinfection (PI) (20), 16 birds (nine cockerels and seven pullets) with cecal lesion scores of <1 and 16 birds (eight cockerels and eight pullets) with lesion scores of >3 were selected as the resistant and susceptible groups, respectively. Because the remaining 184 birds were rated between resistant and sensitive, they were unsuitable for evaluating coccidiosis resistance.

The experiment was conducted according to the regulations of the Administration of Affairs Concerning Experimental Animals (Ministry of Science and Technology, China, revised in June 2012) and approved by the Institution Review Board of the Yangzhou University (permit no. SYXK [Su] 2012-0029).

2.3. Method of marker detection

The survival ratio (%), relative bodyweight gain (%), lesion index, oocyst index, and anticoccidial index (ACI) were determined as described by Pablos (22): ACI = (survival ratio + relative bodyweight gain) – (lesion index + oocyst index). Blood samples were collected from the wing vein of each bird at 8 days PI. Ethylenediaminetetraacetic acid (EDTA) was used as the anticoagulant. The plasma was separated by centrifugation (2000–3000 × *g* for 10 min) and stored at –20 °C before the detection of the plasma markers targeted. In this context, NO, CAT, SOD, GSH-Px, MDA, IL-2, IL-16, IL-17, IFN-γ, and β-C, were detected with Procarta Immunoassay Kits (Affymetrix Inc., Santa Clara, CA, USA), according to the manufacturer's instructions.

2.4. Statistical analysis

Statistical analyses were performed with SPSS (SPSS 15.0 for Windows; SPSS, Chicago, IL, USA). The linear model for two-way analysis of variance (ANOVA) was $y_{ijk} = \mu + \alpha_i + \beta_j + (\alpha \times \beta)\ i_j + e_{ijk}$, , where y_{ijk} is the value of the indicator, μ is the overall mean, α_i is the fixed effect of different groups, β_j is the fixed effect of different sexes, $(\alpha \times \beta)\ i_j$ is the interaction effect between sex and group, and e_{ijk} is the random error term. The differences in mean values between groups or sexes were analyzed for significance with a least significant difference (LSD) test. The sample means and standard deviations for all markers are presented in the form $\overline{x} \pm s$.

3. Results

3.1. Comparison of ACI in resistant and susceptible chickens infected with *E. tenella* oocysts

The marker values for the resistant and susceptible chickens are presented in Table 1. The survival rate in each group (resistant, susceptible, and control) was 100%. The relative bodyweight gain (%) of the resistant group was higher than that of the susceptible group. The lesion index and oocyst index were lower in the resistant group than in the susceptible group. The ACIs of the resistant and susceptible groups were 168.37 and 117.46, respectively, indicating that the groupings of resistant and susceptible chickens were reasonable and reliable, because chickens lack anticoccidian activity when the ACI value is less than 120, and have very effective anticoccidian activity when the ACI value is higher than 160 (22).

Table 1. Comparison of the anticoccidial indices (ACIs) of resistant and susceptible chickens.

Group	Survival ratio (%)	Relative body weight gain (%)	Lesion index [a]	Oocyst index	ACI [b]
Resistant group	100	84.6	6.23	10	168.37
Susceptible group	100	68.7	31.24	20	117.46
Control group	100	100.0	0.00	0	200.00

a: lesion index = 10 × lesion score. b: ACI = (survival ratio + relative bodyweight gain) – (10 × lesion score + oocyst value).

3.2. Marker levels in differently coccidiosis-resistant groups

A comparison of the marker values for the resistant, susceptible, and control groups is presented in Table 2. According to two-way ANOVA, there was no significant interaction effect between sex and group. However, the NO, SOD, IL17, and IFN-γ concentrations were significantly higher in the resistant group than in the susceptible or control group ($P < 0.05$). The CAT and GSH-Px concentrations were significantly higher in the resistant and susceptible groups than in the control group. The MDA concentration was significantly lower in the resistant group than in the susceptible group ($P < 0.05$). There were no significant differences in the remaining three indicators among the resistant, susceptible, and control groups.

3.3. Comparison of markers between sexes

In the analysis of the marker levels according to sex (Table 3), only the IFN-γ concentration differed significantly between cockerels and pullets ($P < 0.05$), and was significantly higher in the pullets than in the cockerels. There were no differences between the cockerels and pullets in the other nine markers ($P > 0.05$).

4. Discussion

In this study, the NO concentration was significantly higher in the resistant group than in the susceptible group at 8 days PI, suggesting that the resistant birds had a stronger ability to regulate their immune responses and could produce more NO to kill or inhibit the coccidian parasites than the susceptible birds. The concentration of IFN-γ was also significantly higher in the resistant group than in the susceptible group, suggesting that the plasma concentration of IFN-γ could be useful for estimating genetic resistance.

When *E. tenella* infects the body, the balance of the antioxidant defense system is altered. SOD is the most important enzyme in the inactivation of CAT and peroxidases, and CAT and GSH-Px protect SOD (23), and so these three enzymes form a mutual protection group in the antioxidative defense system. The results of this study show that the concentrations of CAT and GSH-Px were significantly higher in the resistant and susceptible groups than in the control group, indicating that CAT and GSH-Px play important roles in oxidation resistance during the process of coccidia infection. The SOD concentration was significantly higher in the resistant group than in the susceptible group ($P < 0.05$). MDA is used as a biomarker for radical-induced damage. The MDA concentration was significantly lower in the resistant group than in the susceptible group ($P < 0.05$), indicating that the radical-induced damage was more severe in the susceptible birds than in the resistant birds. Therefore, SOD and MDA should be useful as markers of *E. tenella* resistance in poultry breeding.

Lowenthal (24) found that birds injected with cytokines such as IL-2 displayed increased secretion of immunoglobulin A (IgA) antibodies. The peak IL-2 concentration in the serum coincided with the time of maximum intestinal lesions, measured with the cecum lesion score (25). IL-16 is a proinflammatory cytokine secreted by $CD8^+$ T lymphocytes. It is a chemokine produced by a variety of immune cells, is involved in the inflammatory response, and plays an important

Table 2. Comparisons of resistance markers among different coccidiosis-resistant groups.

Parameter	Group			F test probability
	Resistant (n = 16)	Susceptible (n = 16)	Control (n = 16)	
NO (μmol/L)	56.49 ± 4.13 [a]	53.56 ± 3.94 [b]	52.67 ± 3.73 [b]	0.043
IFN-γ (ng/L)	40.06 ± 4.54[a]	37.01 ± 3.81 [b]	36.12 ± 3.87[b]	0.048
CAT (U/L)	62.11 ± 6.97[a]	60.75 ± 5.03[a]	55.97 ± 5.94[b]	0.038
SOD (U/L)	125.14 ± 13.15[a]	115.56 ± 12.97[b]	129.15 ± 15.87[a]	0.046
GSH-Px (U/L)	382.81 ± 19.57[a]	378.97 ± 18.03[a]	364.32 ± 22.07[b]	0.026
MDA (mmol/L)	5.30 ± 0.77[a]	5.89 ± 0.86[b]	5.14 ± 0.72[a]	0.022
IL-2 (ng/L)	36.14 ± 4.57[a]	35.09 ± 4.36[a]	32.96 ± 5.20[a]	0.457
IL-16 (ng/L)	53.13 ± 5.28[a]	52.12 ± 4.36[a]	49.95 ± 3.47[a]	0.145
IL-17 (pg/mL)	40.39 ± 3.57[a]	37.45 ± 4.10 [b]	36.97 ± 3.70[b]	0.039
β-C (μmol/L)	66.75 ± 5.85[a]	64.72 ± 5.53[a]	68.29 ± 6.73[a]	0.112

Note: Values with different letters in each row indicate a significant difference ($P < 0.05$) and the same letters indicate no significant difference ($P > 0.05$).

Table 3. Comparisons of coccidiosis-resistance markers in the two sexes.

Parameter	Group		F test probability
	Cockerel (n = 25)	Pullet (n = 23)	
NO (μmol/L)	54.49 ± 4.05[a]	53.96 ± 3.69[a]	0.676
CAT (U/L)	60.12 ± 7.18[a]	59.06 ± 7.50[a]	0.649
SOD (U/L)	122.45 ± 19.26[a]	124.19 ± 12.41[a]	0.713
GSH-Px (U/L)	378.84 ± 19.78[a]	371.59 ± 23.61[a]	0.401
MDA (mmol/L)	5.53 ± 0.98[a]	5.51 ± 0.94[a]	0.248
IL2 (ng/L)	34.85 ± 3.92[a]	34.59 ± 3.42[a]	0.621
IL16 (ng/L)	52.13 ± 4.48[a]	51.30 ± 4.36[a]	0.187
IL17 (pg/ml)	39.32 ± 3.82[a]	37.12 ± 4.16[a]	0.208
IFN-γ (ng/L)	37.11 ± 4.06[a]	38.40 ± 4.69[b]	0.037
β-C (μmol/L)	67.35 ± 6.54[a]	65.76 ± 4.32[a]	0.260

Note: Values with different letters in each row indicate a significant difference ($P < 0.05$) and the same letters indicate no significant difference ($P > 0.05$).

role in regulating the inflammatory process. Although there were no significant differences in the IL-2 or IL-16 concentrations in the resistant and susceptible group, they were higher in the resistant group than in the susceptible group. Considering the importance of IL-2 and IL-16, their levels should be compared between resistant and susceptible chickens on different days PI in a future study. IL-17 promotes the formation of tight junctions between the epithelial cells of the small intestine, is involved in the regulation of the intestinal barrier function, and also assists in the release of a number of proinflammatory factors and in the recruitment of neutrophils (26). In the present study, the IL-17 concentration was significantly higher in the resistant group than in the susceptible group. Therefore, the IL-17 concentration may be useful in evaluating the genetic resistance of chickens to *E. tenella* coccidiosis at 8 days PI.

In summary, because the plasma concentrations of NO, SOD, IL-17, IFN-γ, and MDA differed significantly in the resistant and susceptible chickens, their plasma levels could be used as markers for the selection of coccidiosis-resistant chickens.

Acknowledgments

This work was supported by the Priority Academic Program Development of the Jiangsu Higher Education Institutions (PAPD), the National Sci-Tech Support Plan (2014BAD13B02), the Natural Science Great Research Projects of Jiangsu Province Universities (11KJA230001), and the Union Innovation Project of Production, Education and Scientific Research of Jiangsu Province (BY2013063-06).

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