Characterization and the Increase of Chicken Interferon-gamma Production as a Measure of T-cell Responses to *Eimeria tenella* Antigens

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Abstract

Characterization and the increase of chicken interferon-gamma (ChIFN-γ) production were investigated in *Eimeria tenella* (*E. tenella*) infected chicken. Twenty poultry broilers at three weeks old were divided into two groups. The first group was control and those chickens were not infected anything. The second group was infected with 4×10^3 of *E. tenella*. Spleen cells of both the groups were cultured and induced mitogen. This study was to characterize ChIFN-γ protein through extraction technique of supernatant protein of the culture medium of chicken spleen cell that was induced Con-A by SDS-PAGE. The result of characterization showed that molecule weight of ChIFN-γ protein was 17 kDa. Then, Optical Density (OD) of ChIFN-γ protein was detected ELISA reader 450 nm of both *E. tenella* uninfected and infected chickens was 1.29 and 1.95, respectively, whereas, concentration of ChIFN-γ protein of both those groups was 10.312 pg/ml and 15.569 pg/ml, respectively.

Key words: E. *tenella*, chicken interferon-gamma.

Introduction

IFN-γ is a cytokine with a wide range of biological effects including antiviral and macrophage activating capacities and inhibition of the intracellular development of protozoan parasites (Houghum, 1983; Dijkmans and Billiau, 1988; Murray, 1988; Liesenfeld *et al.*, 1996). In mice, elevation in the production of IFN-γ correlates with a genetically-determined disease phenotype; an earlier increase in IFN-γ occurred following primary infection (pi) with *E. cerunifornis* in a resistant mouse strain compared to relatively susceptible mice (Wakelin *et al.*, 1993). Although IFN-γ has been shown to play an important role in host defense against *Eimeria* (Rose *et al.*, 1989, 1991; Lillehoj and Choi, 1998), *Leishmania* (Scott, 1991), *Plasmodium* (Schofield *et al.*, 1987) and *Toxoplasma* (Suzuki *et al.*, 1988), the mechanisms of its action have yet to be clarified.

Cytokines and lymphokines have been shown to influence the course of coccidial infections. Cell culture supernatant from concanavalin A-stimulated lymphocytes inhibited the replication of *Eimeria* parasites in MDBK cell cultures (Lillehoj *et al.*, 1989). The same supernatant, when administered to chickens, reduced oocyst production following both *E. acervulina* and *E. tenella* infections (Lillehoj *et al.*, 1989). Supernatant from concanavalin A-stimulated lymphocytes also inhibited the growth of *E. bovis* and *E. papillata* in bovine monocyte cultures and activated murine macrophages and a bovine monocyte cell line to kill *E. bovis* parasites (Hughes *et al.*, 1987).

IFN-γ production in chickens has been used as a measure of T-cell responses to coccidial antigens (Byrnes *et al.*, 1993; Martin *et al.*, 1994; Prowse and Pallister, 1989). Lymphocytes from *Eimeria*-infected chickens produced a higher level of IFN-γ when induced with concanavalin A than did lymphocytes from uninfected chickens (Martin *et al.*, 1994). Chicken IFN-γ regulates acquired immunity by activating lymphocytes and enhancing expression of MHC class II antigens (Kaspers *et al.*, 1994). Treatment of MDBK, fibroblast, and epithelial cell cultures with recombinant bovine IFN-γ inhibited *E. tenella* and *E. cerunifornis* development (Kogut and Lange, 1989; Rose *et al.*, 1991). Pretreatment of sporozoites with IFN-γ did not affect growth, indicating that IFN-γ alters some aspects of the host cells but not those of the parasites. However, until recombinant chicken IFN-γ become available, the role of IFN-γ in avian coccidiosis remains to be determined.

Recently, the availability of recombinant chicken IFN-γ has led to a better understanding of its physiologic and immunologic roles in chicken coccidiosis (Lillehoj and Choi, 1998; Lowenthal *et al.*, 1997; Song *et al.*, 1997). Chicken recombinant IFN-γ was capable of protecting chick fibroblasts from virus

mediated lysis, induced nitrite secretion from macrophages in vitro, and enhanced MHC class II antigen expression on macrophages (Lowenthal *et al.*, 1997). Administration of exogenous recombinant IFN-γ to chickens significantly hindered intracellular development of *Eimeria* parasites and reduced body weight loss (Lillehoj and Choi, 1998). When chicken fibroblast cells transfected with the IFN-γ gene were infected with *E. tenella* sporozoites, significant reductions in parasite intracellular development occurred although the ability of parasites to bind and to invade host cells was not affected (Lillehoj and Choi, 1998). Briefly, the purpose of this study was to characterize and to know the influence of *E. tenella* infection on the increase of chicken interferon gamma as a measure of T-cell responses to coccidial antigens.

Materials and Methods

Animals: Male CP707 broiler, 3-5 weeks old with around 1000 g body weight (BW) were purchased from Poultry Supplier Co. Surabaya, housed in clean cages and fed with a standard diet without coccidiostat and tap water *ad libitum* in room temperature (24 ± 1°C), under conventional conditions with a 12:12 hr. light: dark cycle. They were kept as outlined in the guide for the care and use of laboratory animals by the Faculty of Veterinary Medicine, Airlangga University.

Parasites: The pathogenic agent used in this study was *E. tenella* obtained from field and routinely maintained in our laboratory by oral passage through CP707 broiler.

Experimental procedures: Twenty CP707 broilers were divided into two groups, each group composed ten chickens. The first group was control group and to those chickens were not infected anything. The second group was infected with *E. tenella* sporulated oocyst. All infective doses of *E. tenella* sporulated oocysts were orally given by 1 ml spuite, as 4×10^3 oocysts/chicken in 1 ml of distilled water. The second infection with the same doses as the first infection was given at the second group to know protective immunity due to the first infection by oocysts production. Fecal pellets were collected from the infected chicken between days 6 until 12 post infection (pi). Spleen cells of both the groups were cultured at 1.7×10^6 cells/ml and placed in 16 well plastic tissue culture test plates, for 48 hr in RPMI-1640 medium containing 2 mM L-glutamine, 10% fetal calf serum, 100 U/ml of penicillin, 100 U/ml of streptomycin and supplemented with 1.2 µg/ml Concanavalin A (Con-A, Sigma, St. Louis, MO), incubated at 37°C and 5% CO_2. Supernatant of tissue culture was predicted to contain ChIFN-γ isolated and characterized by SDS-PAGE and confirmed with ELISA and Dot Blot.

Data Analysis: Oocysts output was analyzed descriptive. The character of ChIFN-γ expressed by molecule weight and described with comparison analysis, concentration and OD of ChIFN-γ were statistically analyzed using student *t*-test and a *p* value below 0.05 was considered significant (Steel and Torrie, 1995).

Results and Discussion

The pattern and total of oocyst output of *E. tenella* infection: The temporal pattern of oocyst output per day confirms those previously reported (Stiff and Bafundo, 1993) with this isolate of *E. tenella*. Oocyst first appeared on the seven days pi, then reached peak on the 10 days pi before numbers declined rapidly and the fewest oocysts were detected on 12 days pi. Basically, the pattern of daily oocyst output was clearly seen in the first infection of infected chickens groups, but in the second infection, oocyst output per day as well as totally were significantly very lower and very few than the first infection (Figs. 1 and 2).

The total numbers of oocysts output of the 1st *E. tenella* infected chickens in this study was [23.5 ± 5.3] x 10^6/chicken and the 2nd *E. tenella* infected chickens [1.1 ± 0.2] x 10^6/chicken, the period of patency was [12.4 ± 0.7] days (Fig. 2). Total of oocysts ouput in the 2nd *E. tenella* infected chickens were significantly decreased ($p<0.01$) about 95 % compared with the 1st *E. tenella* infected chickens (Fig. 2). Clinical signs (such as anemia, anorexia) of the 2nd *E. tenella* infected chickens were slighter than the 1st *E. tenella* infected chickens. Consistency and colour of feces appeared normal in the 2nd *E. tenella* infected chickens compared the 1st *E. tenella* infected chickens. Pathological changes and lesion score of cecum in the 2nd *E. tenella* infected chickens more slight compared than the 1st *E. tenella* infected chickens (Unpublished data). Endogenous development of *E. tenella* (schizogony and gametogony) in the 2nd *E. tenella* infected chickens was suppressed and/or incompleted undergone. Several generations of schizont appeared degenerated consequently unbreak schizont, damaged cecal mucosa epithelial cell was not occurred and automatically there were no bleeding in cecum. Many abnormal endogenous developments of parasites such as gametogony result in disturbancing syngany of microgamete and macrogamete. Thus, oocyst formation was not perfectly continued. In contrast, endogenous development of parasites in the 1st *E. tenella* infected chickens occurred well and no inhibition. Infection with one species of *Eimeria* induces protective immunity in the host that is long lasting and exquisitely specific to that particular parasite (Yun *et al.*, 2000). While a large number of

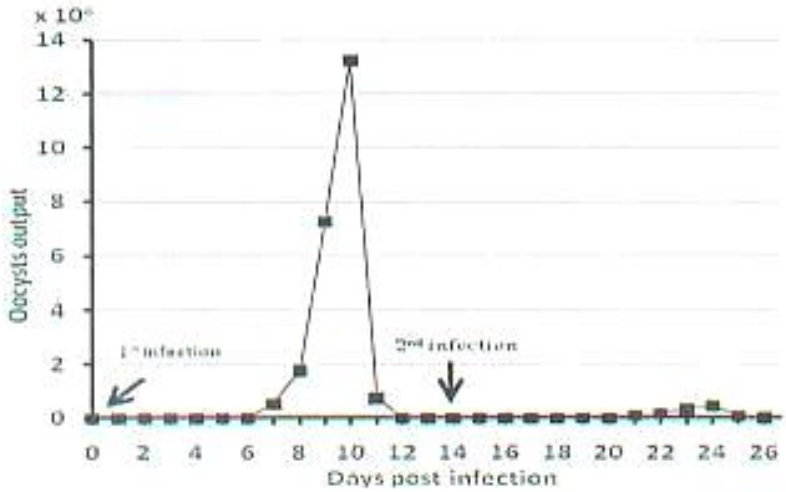

Figure 1. The pattern of oocyst output per day of 1st and challenge infection of *E. tenella* infected chickens. The initially oocyst output on the 7th day, then to peak level the 10th day and for limit around 12 days post infection. Each value of oocyst output per day represents mean of 10 chickens.

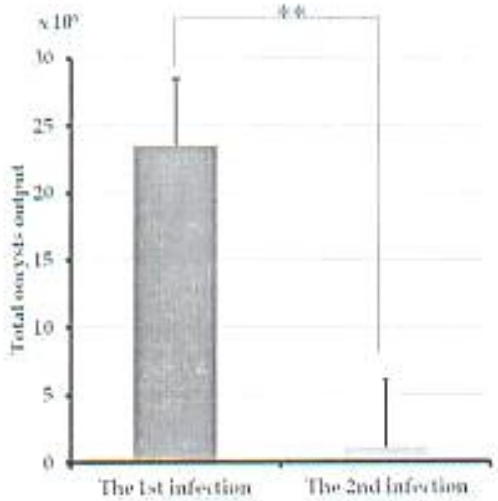

Figure 2. The differences among 1st and challenge infection on total of oocysts output from *E. tenella* infected chickens. From left to right, each column represents 1st and challenge infection status. Each value represents mean ± SD of 10 chickens. ** $p < 0.01$.

inoculating oocysts is generally required to generate an immune response against *Eimeria*, some exceptions have been noted, e.g. *E. maxima* is highly immunogenic and requires only a small number of oocysts to induce almost complete immunity. The early endogenous stages of the parasite life cycle are considered to be more immunogenic than the later sexual stages (Yun *et al.*, 2000) although Wallach *et al.* (1990 and 1995) showed that immunization with recombinant gamete associated antigen induced partial protection against challenge infection. Studies using oocysts irradiated to prevent intracellular development, but not invasion, demonstrated partial protection against challenge infection, thereby suggesting that sporozoites may also be immunogenic (Jenkins *et al.*, 1991).

Spleen cell culture of uninfected and infected chickens in induction of Con-A for isolation of ChIFN-γ protein. Spleens of *E. tenella* infected chickens and *E. tenella* uninfected chickens were cultured in 16 well plastic tissue culture test plates, for 48 hr in RPMI-1640 medium containing 2 mM L-glutamine, 10% fetal calf serum, 100 U/ml of penicillin, 100 U/ml of streptomycin and supplemented with 1.2 µg/ml Concanavalin A (Con-A, Sigma, St. Louis, MO), incubated at 37°C and 5% CO_2.

The daily check up on the growth of spleen cell cultured appeared the good growth and proliferation of both spleen cells infected and uninfected chickens (Fig. 5). Additional mitogen (Con-A) be able to induce release and production of IFN-γ by lymphocytes. Lymphocytes from *Eimeria*-infected chickens produced a higher level of IFN-γ when induced with Con-A than did lymphocytes from uninfected chickens (Martin *et al.*, 1994).

The examination of concentration of Ch-IFN-γ that expressed in supernatant of spleen cell cultured with Con-A induction of *E. tenella* infected chickens compared *E. tenella* uninfected chickens using indirect ELISA showed significantly differences ($p<0.05$)

(Fig. 4) which concentration of Ch-IFN-γ *E. tenella* infected chickens increased 46% higher than *E. tenella* uninfected chickens that was 15.569 pg/ml and 10.312 pg/ml, respectively. Pattern of optical density (OD) of both *E. tenella* infected chickens and *E. tenella* uninfected chickens was also same as pattern of concentration that was 1.95 and 1.29, respectively (Fig. 5).

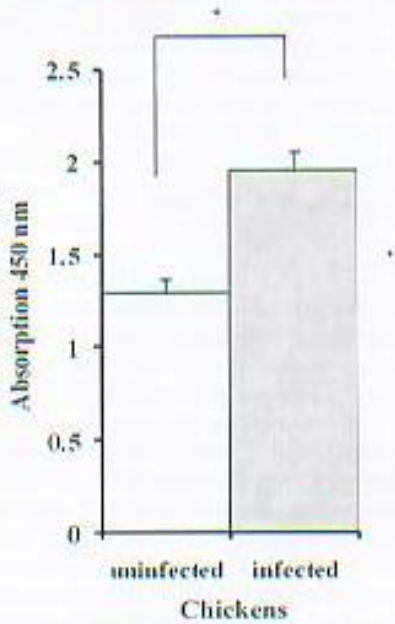

Figure 3. The comparison of optical density (OD) of ChIFN-γ protein between *E. tenella* infected and uninfected chickens. *, $p < 0.05$.

Cytokines (i.e. IFN-γ) are proteins that are naturally produced by the body's immune system immediately following infection or vaccination, resulting in protection from disease. Cytokine activities during avian coccidiosis are major roles (Choi *et al.*, 1999; Lillehoj and Choi, 1998). Chicken IFN-γ regulates acquired immunity on *Eimeria* infection by activating lymphocytes and enhancing expression of MHC class II antigens (Kaspers *et al.*, 1994; Lillehoj, 1989). IFN-γ production in mice (Rose *et al.*, 1991) and chickens (Martin *et al.*, 1994; Yun *et al.*, 2000) has been used as a measure of T cell responses to coccidial antigens. Study by Yun *et al.*, (2000) showed that production of IFN-γ was high in intestine tissue of coccidia development.

IFN-γ mRNA expression is significantly increase in infected chickens compared uninfected chickens. Correlation of immunity on disease with local IFN-γ production early indicates important roles of IFN-γ in protective immunity. Level of IFN-γ increased in SC compared TK chickens and this cytokine appeared in intestine particularly in circulation (Yun *et al.*, 2000).

Characterization of Ch-IFN-γ Protein by SDS-PAGE. In supernatant of chicken spleen cell cultured was identified Ch-IFN-γ protein by SDS-PAGE and confirmed using Dot Blot. Identification Ch-IFN-γ protein of *E. tenella* infected as well as uninfected chickens was shown several bands in variety several bands in conformity with marker in certain molecule weight standard (Fig. 7). Based on measurement of molecule weight, all supernatants showed the same band of molecule weight of 17 kDa.

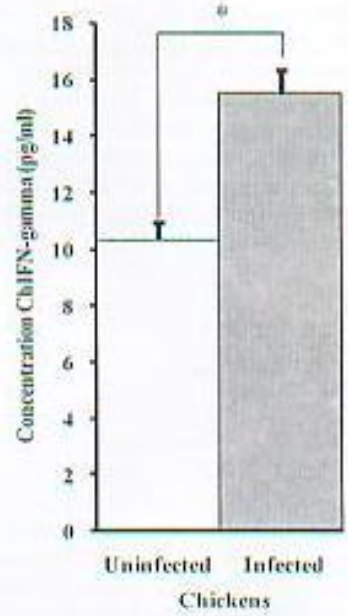

Figure 4. The comparison of concentration of ChIFN-γ protein between *E. tenella* infected and uninfected chickens. *, $p < 0.05$.

The result analysis of SDS-PAGE to be sure more specific of Ch-IFN-γ protein confronted with *immunoblotting* (*Dot Blot*) (Fig. 8). Characterization of Dot Blot showed protein was specific for monoclonal antibody anti Ch-IFN-γ, except panel A (antigen, mouse IFN-γ). Panel B and C expressed Ch-IFN-γ protein that known by monoclonal antibody anti Ch-IFN-γ. In *E. tenella* infected chickens was shown clear spot which intensity was stronger than *E. tenella* uninfected chickens. Briefly, concentration of Ch-IFN-γ protein of *E. tenella* infected chickens higher than *E. tenella* uninfected chickens, however both those groups had strong antigenicity which proved the result of examination both showed positive reaction.

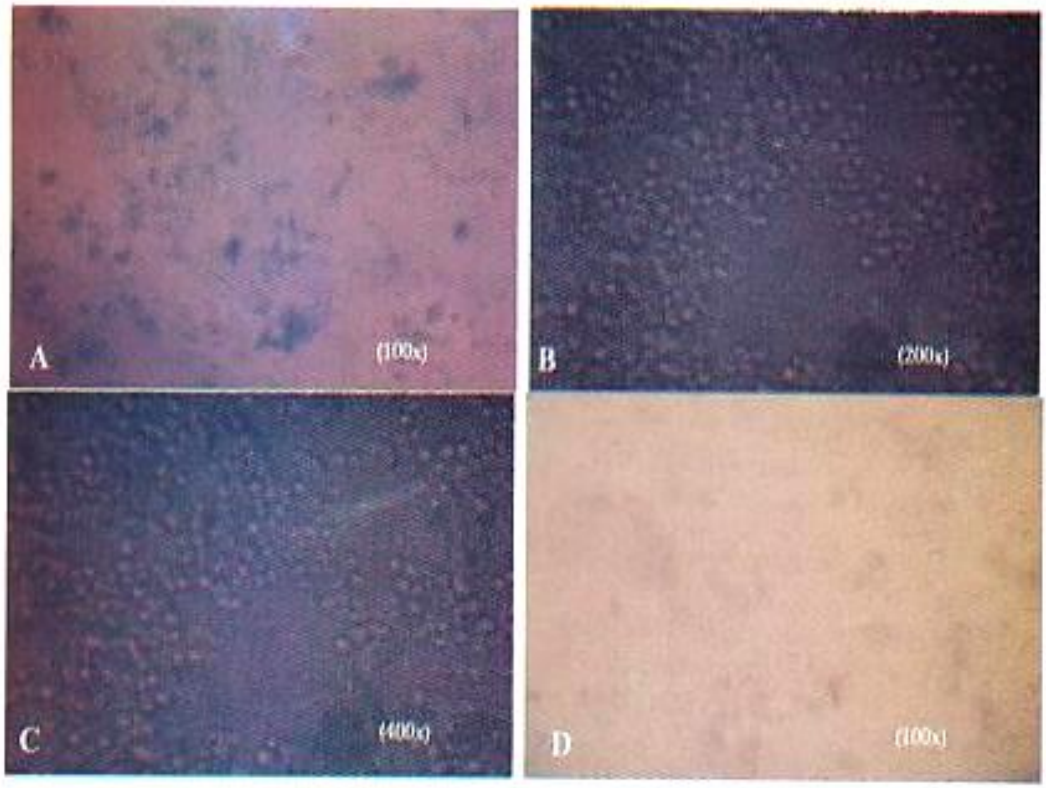

Figure 5. Spleen cell cultured and cell number in initial culture ~ 1.7 x 10⁶/ml (panel A, B, C), 48 hours after undergoing development (panel D).

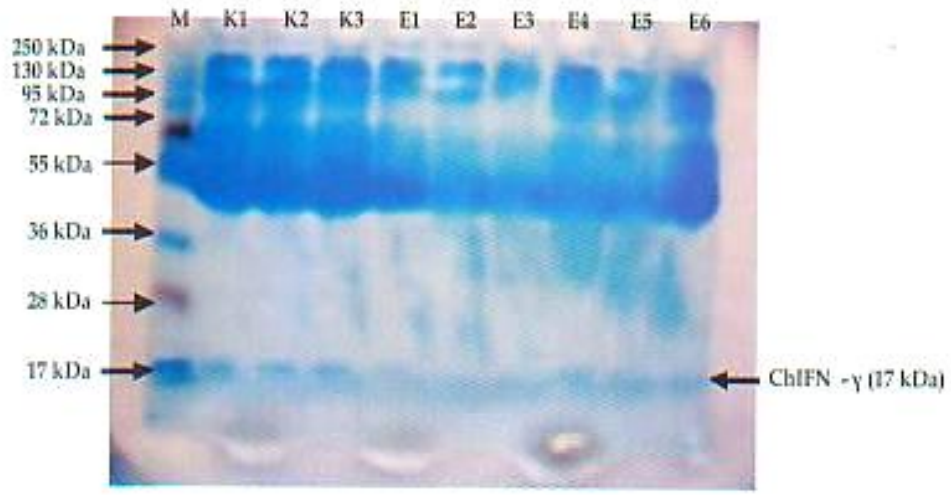

Figure 6. Molecule weight of ChIFN-γ protein. M: marker, K1-3: *E. tenella* uninfected chickens, E1-6: *E. tenella* uninfected chickens (Song, *et al.*, 2007, molecule weight of ChIFN-γ protein recombinant is around 17-18 kDa).

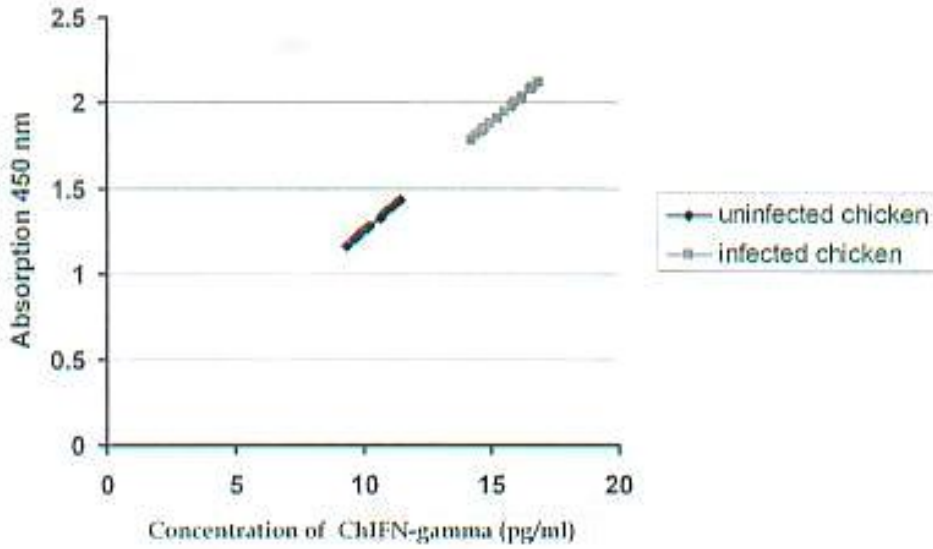

Figure 7. Comparison optical density and concentration ChIFN-γ between *E. tenella* infected and uninfected chickens.

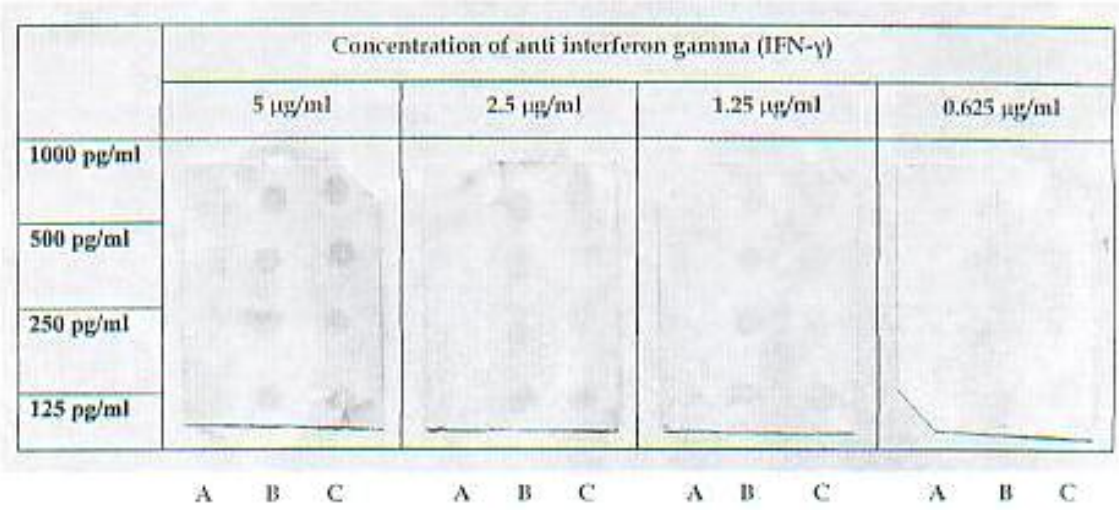

Figure 8. Immunoblotting for characterization of ChIFN-γ protein by *Dot Blot*. A. mouse interferon-gamma; B. *E. tenella* infected chickens interferon-gamma; C. *E. tenella* uninfected chickens interferon-gamma.

Conclusions

Measurement of molecule weight of chicken interferon gamma protein of *E. tenella* uninfected and infected chickens showed the same band of molecule weight of 17 kDa. Lymphocytes from *Eimeria*-infected chickens produced a higher level of IFN-γ when induced with Con-A than lymphocytes from uninfected chickens. Moreover, the concentration of Ch-IFN-γ that expressed in supernatant of spleen cell cultured with Con-A induction of *E. tenella* infected chickens higher compared *E. tenella* uninfected chickens.

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