CHAPTER 4

EVALUATION OF INSECTICIDE INDUCED DEVELOPMENTAL IMMUNOTOXICITY IN RIR CHICKS

INTRODUCTION

The immune system is a remarkably versatile defensive system that has evolved to protect the animals from various pathogens and tumour cells. It detects a wide variety of agents, from viruses to parasitic worms, and distinguishes them from the organism's own healthy cells and tissues in order to function properly.

The bird's immune system consists of mainly the lymph vessels and lymphoid tissue. The primary lymphoid tissue includes the thymus and bursa of Fabricius while the secondary tissue includes spleen, bone marrow, mural lymph nodules and lymph nodes. There is also a circulatory system of vessels and capillaries that transport lymph fluid through the bird's body and communicate with the blood supply. The avian immune system shows the specific and non specific type of immune responses. The specific immune response consists of humoral and cell mediated type of immune response, mediated mainly by the B and T lymphocytes specifically. The specific immune responses need a processed antigen and their response is to create a specific antibody for that particular antigen. The non specific immune response responds to all antigens. The macrophages, heterophils and thrombocytes are the main cells associated with the non specific immune responses.

The bird's immune system begins developing before hatch and is complete by sexual maturity. They are the first vertebrates in which a clear dichotomy of the lymphoid system has been established: 1) Thymus-derived (T) lymphocytes, the effector cells in cell-mediated immunity and 2) Bursa-derived (B) lymphocytes, the precursor cells of the antibody-synthesizing plasma cell. By embryonic day 9 or 10, the haemopoietic stem cells from the embryonic yolk sac and liver migrate into the thymus and under the influence of a thymus hormone proliferate and differentiate into thymus lymphocytes (**Owen and Raff, 1970**). Birds unlike mammals do not have a library of genetic information for the B cells to use during the production of antibodies. During the first six weeks of age, the B lymphocytes

undergo the gene conversion (education of B-lymphocytes) in the bursa, in order to provide the diversity needed to protect against the great variety of potential pathogens (**Wakenell, 1999**).

The physical blockade (skin, cilia, mucous) is the body's first line of defence. Then the next level is taken up by the non-specific immune responses from the macrophages, heterophils, thrombocytes and natural killer cells which converge on the invader to kill it or slow down until the components of the specific immune system join the battle.

The most sophisticated feature of the immune system expressed in vertebrates is recognition of foreign molecules by distinct types of immunocompetent cells. There is compelling evidence that antigen entering the body stimulates a conventional type of systemic immune response. A fully responsive immune system is essential for survival from infections and toxic agents, as well as from antigenic patterns abnormal to the genotype which arises by somatic mutation. A proliferation and differentiation of lymphoid cells is initiated in response to foreign macromolecules and finally two different types of immunological reaction may occur: (1) Synthesis and release of immunoglobulin (Ig) molecules and/or (2) development of specifically reactive lymphocytes, the effector cells of cell-mediated immunity. Humoral antibodies as well as sensitized cells can recirculate, passing from the blood into the peripheral lymphoid organs and other tissues capable of reacting specifically with antigens. As the result of this reaction, foreign macromolecules may be inactivated (toxins) or killed (bacteria, viruses), and finally phagocytosed, by cells of the reticulo-endothelial system (**Kindt *et al.*, 2006**).

The immune system is both target and mediator of environment-induced injury (**Garg *et al.*, 2004**). It shows vulnerability to any chemical, including pesticides, which can cause structural and functional alterations to the system. The immunotoxic effects of xenobiotics include: histopathologic effects in immune tissues and organs; cellular pathology; altered maturation of immunocompetent cells; changes in B and T cell subpopulations; and functional alterations of immunocompetent cells (**Blakley *et al.*, 1999**). A range of studies have been conducted by several investigators to report the immunotoxic effects of the pesticides in the adult animals (**Moon *et al.*, 1986; Holladay *et al.*, 1996; Blakley *et al.*, 1999; Aly and El-Gendy, 2000; Girón-Pérez *et al.*, 2006; Suke *et al.*, 2006**). The humoral and cell mediated immune responses were depressed in murines treated with various pesticides like fenitrothion, fenthion, diazinon (**Moon *et al.*, 1986**), dimethoate (**Aly and El-**

Gendy, 2000), phosphamidon (**Suke *et al.*, 2006**), and chlorpyrifos (**Blakley *et al.*, 1999**). The phagocytic function was reported to be diminished in fish by the chlorpyrifos treatment (**Holladay *et al.*, 1996; Girón-Pérez *et al.*, 2006**). However, developmental immunotoxic studies on avian models are scanty and needs more attention.

The ontogeny of the vertebrate immune system involves highly regulated sequences of cell development and differentiation which are meticulously timed and co-ordinated. These processes begin very early in the foetal life and continue through the early post natal development. Even minor changes to differentiating cells during this period can have permanent implications (**Dean and Thurmond, 1987**). The developing organisms are at a greater risk because chemicals may alter their organ systems with effects that are more persistent and/or more severe than those observed in adults, or they may alter these systems at lower doses than in adult animals (**Ladics *et al.*, 2005**). Considering specific organ systems, the nervous and immune systems have been identified as possibly exhibiting a unique susceptibility during development that may not be apparent if toxicological data are only acquired in adult animals (**Claudio *et al.*, 2000; Dietert *et al.*, 2003, Hussain *et al.*, 2005**). Moreover, the immune function which is programmed during fetal and neonatal life is under the close coordination with the neural input (**Navarro *et al.*, 2001**). Consequently, the development of immunocompetence is vulnerable to changes evoked by any xenobiotic that acts on the nervous system.

The test chemicals in the present study *viz.*, combination insecticide, as well as the Spinosad, are both neurotoxicants. Therefore, to understand the status of immunocompetence in the chicks which were earlier treated with either the combination insecticide or the Spinosad, it was felt crucial to evaluate the immuntoxicological inflictions.

OBJECTIVE

The analysis of the clinical evaluations in chapter 3 showed that there was a significant alteration in the total leukocyte count, differential leukocyte count and also the cholinesterases in both the combination insecticides as well as the Spinosad treated groups (thought the effect was more significant with the combination insecticide). The substantial decrease in the white cell counts can be attributed to an immunosuppression or a pancytopenic effect on the haemopoietic system. Also, developmental exposure to cholinergic stimulants evokes long lasting deficits on immune system. Therefore, the present

study was designed to evaluate the non specific immune response in the chicks (7 day old) after an initial *in ovo* insecticide treatment. The methodology adopted for the evaluation included:

(i) Lymphoid organ weight

(ii) Bacterial clearance assay

(iii) NBT salt reduction test

MATERIALS AND METHODS

The control and pesticide treated hatchling groups were housed in separate pens. Layer starter mash and water were provided *ad libitum*. After one week the immunotoxicity was evaluated through the NBT salt reduction test and bacterial clearance assay and weights of thymus, spleen, bursa of Fabricius. A detailed description of the following methods is mentioned earlier in the section materials and methods.

Lymphoid Organ Weight

Day old hatchlings were sacrificed and the thymus, spleen and bursa of Fabricius were excised, carefully blotted and weighed on a Sartorius balance (BS-223S). The relative weights of these organs were then calculated.

NBT Salt Reduction Test

NBT test was done as per **Singh *et al.* (1990)** on the impression smears of the spleen.

Bacterial Clearance Assay

The assay was performed as per **Peterson *et al.* (1999)** using *Escherichia coli* colonies to challenge the chick immune function.

RESULTS

The insecticide treatment groups i.e. 0.01 and 0.05µg/egg of Ci did not show any significant variation in the body weight or relative weights of thymus, spleen or bursa of Fabricius, respective to the vehicle control group (**Table 4.1**). However, the absolute body weight and also the relative weight of thymus and spleen were found to be significantly low ($p \leq 0.05$) in 0.1µg/egg combination insecticide treated chicks, while the relative weight of bursa showed no variation compared to control. The other treatment groups i.e. 0.15, 0.75 and 1.50mg/egg

of Spinosad did not show any significant variation in the body weight or relative weights of thymus, spleen or bursa of Fabricius, respective to the vehicle control groups (**Table 4.1**).

In chicks treated with 0.01μg/egg of Ci, the rate of bacterial clearance from the blood circulation was comparable to the controls. In chicks treated with 0.05μg/egg combination insecticide, bacterial clearance from the blood circulation was significantly delayed at 60min ($p \leq 0.05$) and 80min ($p \leq 0.01$). A viable bacterial load which was significantly higher than the controls was retrieved from the blood samples of chicks with 0.1μg/egg of Ci treatment collected at 20, 40, 60 and 80min after inoculation and the greatest disparity was observed at 80min ($p \leq 0.001$) (**Table 4.2; Figure 4.1**). The viable bacterial colonies retrieved (after 110min of inoculation) from the spleen and liver of 0.01μg/egg of Ci treated chicks were comparable to those of the vehicle control. The spleen and liver in 0.05 and 0.1μg/egg of combination insecticide treated chicks showed lowered bacterial clearance, with the higher dose being more significantly ($p \leq 0.01$) lagging (**Table 4.3; Figure 4.2**). The 0.15 and 0.75mg/egg of Sp tested doses did not induce any significant changes in phagocytic functions in either the blood, spleen or liver of the chicks, as evident from the bacterial clearance which was comparable to the vehicle control. Spinosad treated chicks revealed a lag in bacterial clearance ability only at a dose of 0.75mg/egg; however, the clearance rate was delayed only towards the end of the observation period i.e at 60 and 80min (**Table 4.4; Figure 4.3**). The bacterial colony counts from the spleen and liver of 0.15, 0.75 and 1.50mg/egg of Sp treated chicks were comparable to the control (**Table 4.5; Figure 4.4**).

The 0.01 μg/egg of Ci treatment groups did not show any considerable variation in the number of active phagocytic cells in the spleen as shown by the NBT test. Nevertheless, the NBT test revealed a decreased number of active phagocytic cells in the spleen at 0.05 ($p \leq 0.05$) and 0.1μg/egg ($p \leq 0.01$) doses of combination insecticide treatment. None of the tested Spinosad doses induced changes in the phagocytic activity when compared to the vehicle control (**Table 4.6**).

DISCUSSION

The immune response in birds is highly regulated and breakdown in regulation often results in immunodepression (**Sharma, 1991**). The developing organisms are more vulnerable to a vast majority of known immunotoxicants than do the adults (**Dietert, 2005**). Therefore, diagnosis and prevention of pesticide intoxication in terms of developmental immunotoxicty is of considerable importance.

The weights of thymus, bursa of Fabricius and spleen can be used to assess the relative immune status in poultry birds (**Rivas and Fabricant, 1988**). In the current study, with 0.01 and 0.05 μg/egg of Ci treatment, neither the body weight nor the relative weight of lymphoid organs showed any significant changes. However, the absolute body weight and also the relative weight of thymus and spleen were significantly low at 0.1μg/egg of combination insecticide treated chicks, while the relative weight of bursa showed no variation compared to control. These lowered weights might be associated to a direct necrotic effect of the insecticide on the lymphoid tissue and leading to lowered numbers of T cells and macrophages, and thereby causing a deficit in antigen recognition and phagocytosis. However, in none of the Spinosad treated groups changes relating absolute body weight or relative lymphoid organ weight were observed.

The defence mechanisms in a vertebrate evoke an appropriate immune response whenever they identify a foreign antigenic invasion. These responses of the immune system are quite sophisticated and function on an intricate balance. The first step of host defence against bacterial invasion is phagocytosis and degradation of phagocytic cells. The mononuclear phagocytic system takes up the function of phagocytosis in the form monocytes in blood and macrophages in the tissues like spleen, liver and lymph nodes. The macrophages are unique in that they are crucial players in both innate and adaptive immune responses (**Qureshi, 2003**). The heterophils which offer an innate immune response are also important phagocytic cells against microbial pathogens (**Stedman *et al.*, 2001**).

Considering the above discussed facts, the present investigation was designed to study and compare the immune responses evoked in two groups of *in ovo* insecticide intoxicated chicks by employing an *E.coli* challenge into their blood streams. It is expected that the immune system would evoke an immune response against the injected antigen and try clearing them off from the circulation. The bacteria in the circulation which would also reach the filtering systems like the liver and spleen. The spleen is an integral part of the immune system. The phagocytes in the spleen engulf and digest not only the old and damaged RBC, but also the invading microorganisms and debris as well. In the liver also, the Kupffer cells are strategically stationed to phagocytose the invading microorganisms. Therefore, after an initial encounter with the *E.coli* into the blood circulation, the rate of bacterial clearance was determined in the blood, liver and spleen at fixed intervals of time. The results of the experiment revealed significantly high recovery of viable bacterial colonies from the blood streams of Ci treated (0.05 and 0.1μg/egg) group of chicks. Further, the liver and spleen

homogenates too showed higher numbers of uncleared bacterial colonies. This indicates a compromised bacterial clearance, which might be a consequence of diminished phagocytic and lytic potential of the monocytes and/or heterophils in the blood and macrophages in the liver and spleen. On the other hand, the decline in the immune response could also be due to diminished numbers of phagocytic cells owing to the cytotoxic nature of the insecticide.

Further, NBT-salt reduction test was performed to assess the functional ability of splenic macrophages of insecticide-intoxicated RIR chicks. The NBT is a non specific test for phagocyte membrane stimulation. The NBT dye in an oxidized state is pale yellow in colour, but when reduced turns blue. When the phagocytes are active and release the oxidase, the NBT gets reduced to NBTH, which can be microscopically observed as blue intracellular inclusions (Formazan deposits). The number of active phagocytic cells can thus be enumerated. In the current study, the percentage of active macrophages in the intoxicated chicks was significantly lower in combination insecticide treated chicks than that of control. This impaired phagocytosis might also be due to the adversely modulated immunogenic potency of phagocytes during the development of the immune system.

The immune function is programmed during the foetal and neonatal life and is under close coordination by neural input and therefore, agents or drugs that interfere with the development of the nervous system elicit corresponding immunologic deficits (**Navarro *et al.*, 2001**). Through different mechanisms the organophosphates (**Rice and Barone, 2000; Landrigan, 2001; Qiao *et al.*, 2004**) and also the pyrethroids (**Shafer *et al.*, 2005; Farag *et al.*, 2007**) are known to induce developmental neurotoxicity. Exposure of rats to chlorpyrifos during a developmental period in which this organophosphate pesticide is known to produce lasting changes in neural function, elicits corresponding, long term deficits in immune competence (**Navarro *et al.*, 2001**). It is therefore likely that the immune suppression in the combination insecticide dosed chicks could be due to disturbances in neural development. The results of evaluations in chapter three have shown that the combination insecticide treatment lead to a decline in the activity of cholinesterases in the day old chicks as well as in the 8 day old embryos . Therefore, the present study is in agreement with the earlier reports that the immune development is under the control of neural input (**Felten and Felten, 1994; Madden *et al.*, 1995, Navarro *et al.*, 2001**); and that the disturbances in the neural coordination during the embryonic development might lead to disorders in the immune development. Further, chlorpyrifos and cypermethrin as a combination have shown an enhanced insecticidal effect (**Wielgomas and Krechniak, 2007**) and therefore an enhanced

neurotoxic effect. And this could be the reason for the combination insecticide being immunotoxic at levels as low as 0.1 and 0.05μg.

With the Spinosad treatment of 1.5mg/egg, there was a decrease in bacterial clearance as evident by relatively high number of bacterial colonies retrieved from blood samples collected after 60 and 80 min post bacterial inoculation. However, the liver and spleen showed no significant variations. The other lower doses nevertheless, showed no significant variation in bacterial clearance when samples from the blood, liver and spleen were tested. The NBT test showed no significant difference in the number of active macrophages in the spleen for all the three tested doses of Spinosad. Further, the study is in agreement with the earlier scientific literature that the immune development is in close coordination with the neural input, since Spinosad seemed to be showing only mild effects on immune system as well as nervous system. The clinical estimations in chapter 3 showed that Spinosad induced a decline in the acetylcholinesterase activity (though only at higher doses of the present study) in both the 8 day embryo as well as day old chicks. However, the neurotoxicty is not as severe as in the case of the combination insecticide. Further, Spinosad was reported to be non-neurotoxic to rats in acute, sub chronic or chronic toxicity studies and had shown no developmental effects (**EPA, 1997**). Due to its low toxicity and perceived low impact on the environment, EPA registered Spinosad as a reduced-risk material (**Jachetta, 2001**). The present results are also in agreement with the earlier reports that the Spinosad is relatively milder in inflicting the neurotoxic effects and also the possibility that it would be a developmental immunotoxicant is also meagre.

SUMMARY

Comparison of rate of bacterial clearance in the circulating blood, liver and spleen; and the splenic phagocytic activity in both the insecticide treated groups, a clear conclusion can be drawn that the combination insecticide is far more potentiated to induce immunotoxicity in developing chick at quite lower doses; while Spinosad was relatively mild in terms of inducing developmental immunotoxicity in chicks.

TABLE 4.1 Body weight and relative weights of lymphoid organs of controls and insecticide treated chicks (day old)

Treatment	Weight			
	Body weight (gm)	Relative weight of Thymus	Relative weight of Spleen	Relative weight of Bursa
VC1	62.84±0.83[@]	0.314±0.009	0.067±0.004	0.207±0.012
0.01Ci	65.01±1.67	0.321±0.018	0.065±0.004	0.201±0.016
0.05Ci	59.11±1.12	0.339±0.026	0.068±0.002	0.223±0.013
0.10Ci	57.96±1.54↓*	0.233±0.025↓*	0.055±0.002↓*	0.201±0.014
VC2	63.74±1.14	0.332±0.014	0.069±0.003	0.219±0.015
0.15Sp	64.60±1.16	0.343±0.019	0.066±0.003	0.203±0.012
0.75Sp	62.68±0.92	0.314±0.029	0.063±0.002	0.205±0.014
1.50Sp	63.24±1.17	0.346±0.017	0.065±0.002	0.204±0.014

[@] Values expressed as mean ± SEM; n=6

VC1: Corn oil; VC2: methyl cellulose

Ci: Combination insecticide (µg/egg); Sp: Spinosad (mg/egg)

↓ Significant decrease; *p ≤0.05

TABLE 4.2 Bacterial load in blood of vehicle control (VC1) and combination insecticide (Ci) treated chicks

Time (minutes)	Bacterial Load in Blood (CFU/ml blood)			
	VC1	0.01µg/egg	0.05µg/egg	0.1µg/egg
10	127820.02 ± 7857.75[@]	129020.00 ± 8638.46	133286.14 ± 3143.21	136201.42 ± 9272.34
20	89883.40 ± 7698.50	103602.12 ± 3897.69	104767.00 ± 3951.48	117060.05 ± 9315.04↑*
40	65410.31 ± 5581.54	81096.11 ± 6633.37	82263.51 ± 3656.06	88236.81 ± 5651.10↑*
60	42512.22 ± 6531.18	60331.09 ± 5072.90	65706.07 ± 2282.22↑*	73914.12 ± 6500.05↑**
80	30011.20 ± 1657.28	35885.09 ± 4669.84	55550.21 ± 4230.09↑**	64666.32 ± 6293.82↑***

[@] Values expressed as mean ± SEM; n=6

VC1: Corn oil vehicle control

CFU: Colony forming units

↑ Significant increase; *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$

TABLE 4.3 Bacterial clearance assay in spleen and liver of Ci treated chicks after 110min of bacterial inoculation

Tissue	Bacterial Load in Tissue (CFU/0.1g tissue)			
	VC1	0.01µg/egg	0.05µg/egg	0.1µg/egg
Spleen	70840.11 ± 3189.018[@]	77885.84 ± 3518.917	83021.50 ± 1575.577↑*	85656.16 ± 3089.449↑**
Liver	51612.33 ± 2368.516	53800.67 ± 2793.578	61779.01 ± 1742.266↑*	64145.83 ± 1979.715↑**

[@] Values expressed as mean ± SEM; n=6

VC1: Corn oil

CFU: Colony forming units

↑ Significant increase; *$p \leq 0.05$; **$p \leq 0.01$

TABLE 4.4 Bacterial load in blood of VC2 and Spinosad (Sp) treated chicks

Time (minutes)	Bacterial Load in Blood (CFU/ml of blood)			
	VC2	0.15mg/egg	0.75mg/egg	1.5mg/egg
10	126112.02 ± 9659.01[@]	130112.50 ± 9857.75	125210.51 ± 9988.11	131783.18 ± 6636.15
20	89765.29 ± 9698.49	90765.71 ± 5698.49	91832.40 ± 6155.12	92412.21 ± 9593.26
40	62235.08 ± 5558.48	60219.10 ± 5558.48	67941.31 ± 8638.62	71812.32 ± 8156.94
60	39112.41 ± 4698.49	41179.24 ± 6988.26	48517.32 ± 4897.17	60995.56 ± 6561.67↑*
80	28980.61 ± 2158.48	30268.16 ± 3896.20	39265.51 ± 3390.11	43134.09 ± 3521.83↑*

[@]Values expressed as mean ± SEM; n=6

VC2: methyl cellulose

CFU: Colony forming units; ↑: Significant increase; *$p \leq 0.05$

TABLE 4.5 Bacterial clearance assay in liver and spleen of vehicle control (VC2) and Spinosad treated chicks

Tissue	Bacterial Load in Tissue (CFU/0.1g tissue)			
	VC2	0.15mg/egg	0.75mg/egg	1.5mg/egg
Spleen	74173.34 ± 2311.62[@]	69385.84 ± 3020.31	68462.51 ± 3742.71	74384.66 ± 4485.41
Liver	53127.33 ± 3425.33	51634.14 ± 3390.56	59279.00 ± 1581.25	61312.50 ± 2892.63

[@] Values expressed as mean ± SEM

VC2: Methyl cellulose

CFU: Colony forming units

TABLE 4.6 NBT salt reduction test

Treatment	Attribute	
	No. NBT positive cells	**%NBT positive cells**
VC1	99.16±3.37[@]	49.58
0.01Ci	93.83±3.84	46.91
0.05Ci	84.00±3.91↓*	42.00
0.10Ci	76.00±4.76↓**	38.00
VC2	101.6±3.08	50.83
0.15Sp	100.6±2.55	50.33
0.75Sp	95.33±3.73	47.66
1.50Sp	98.66±2.99	49.33

[@] Values are expressed as mean ± SEM, n = 5

VC1: Corn oil vehicle control

Ci : Combination insecticide (µg/egg)

VC2: Methyl cellulose vehicle control

Sp : Spinosad (mg/egg)

NBT: Nitroblue tetrazolium

↓ Significant decrease; *$p \leq 0.05$; **$p \leq 0.01$

FIGURE 4.1 Bacterial load in blood of control and combination insecticide treated chicks

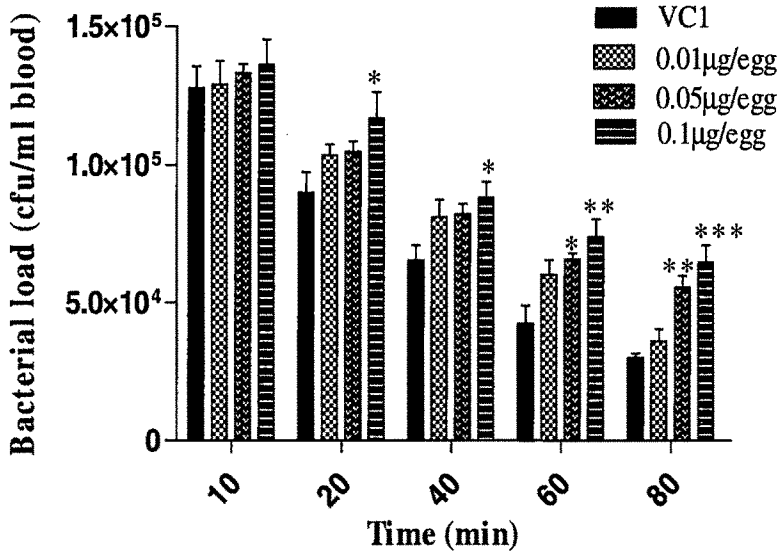

FIGURE 4.2 Bacterial load in spleen and liver of control and combination insecticide treated chicks

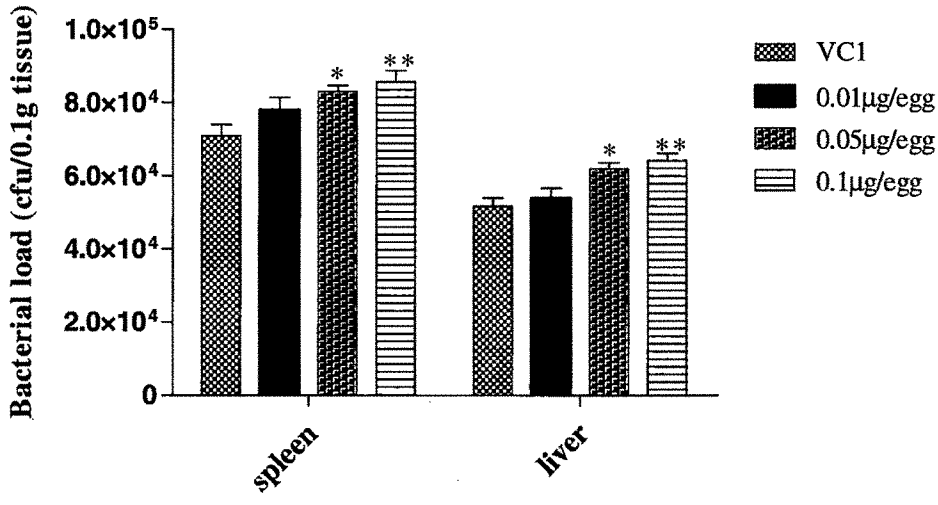

FIGURE 4.3: Bacterial load in blood of control and Spinosad treated chicks

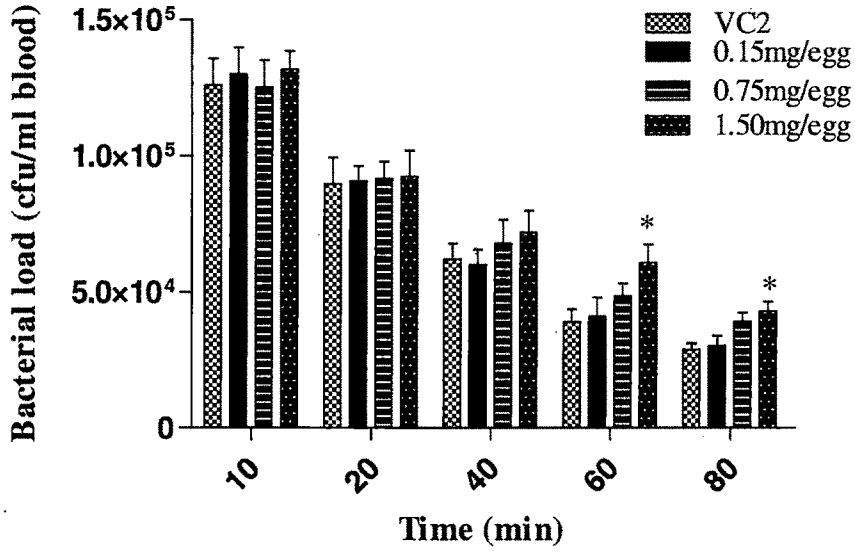

FIGURE 4.4: Bacterial load in spleen and liver of control and Spinosad treated chicks