

IMMUNOLOGICAL STUDY OF *Toxocara canis* IN DOGS AND MICE AT BASRAH CITY

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Abstract

Sixty-five sera samples were collected from stray dogs in Basrah which included 31 dogs infected with *T. canis*, 21 dogs infected with cestodes, while 13 dogs were infected with other nematodes than *T. canis*. The antibody concentration for eggs crude antigen (ECA) and adult crude antigen (ACA), adult purified antigen (APA) was evaluated using IHAT test for monitoring toxocariasis in dogs. The sensitivity of the above antigens was 64.5, 86.2 and 93.5%, respectively, while the specificity was 84.4, 93.8 and 100 %, respectively. Furthermore, the predictive values were 80.0, 92.6 and 100 %, respectively, while, the likelihood ratio was 4.5, 14.3 and ∞ for the above antigens.

No cross reaction was detected between the above three antigens and the infection with cestodes.

A total of 48 sera samples from mice experimentally infected with 2nd stage larvae of *T. canis* with two doses (250 and 500 larvae) at 1, 2, 3 weeks and 2, 4, 6 days post-infection. IHAT was used with larval excretory/ secretory and tegumental larval antigens. The total sensitivity was 91.66% and 83.33% for E/S, while it was 58.33 and 50% for TES, respectively. The specificity was 100% for E/S and TES at both doses, respectively. The predictive value for E/S was 91.66% in dose (500) larvae and 95.83% at second dose (250, larvae). It was found that in case of TES antigen, the predictive value was 75% and 79.16% at both doses (500 and 250 larvae, respectively).

There was no cross-reaction between the above larval antigens and the infection with cestodes and nematodes

Introduction

Toxocara canis is originally a parasitic nematode of canine, bitches and their puppies (Kuroda *et al.*, 2001). However, *T. canis* eggs can also hatch in a large number of non-canid species, including human beings (Kayes *et al.*, 1985). Larvae in non-canid species hatch and distribute themselves throughout the viscera; most often these larvae become encapsulated in a granulomatous response (Kayes and Oaks, 1978) or accumulated in the brain, where they elicit little or no histological reaction (Dunsmore *et al.*, 1983).

Infection with *T. canis* to the newborn puppies results from the tracheal migration of larvae which have arrived from their mothers during transmammary after birth (Overgaauw, 1997b).

It was hypothesized that the immunosuppressive effect of pregnancy and lactation may permit tissue larvae or larvae from a newly acquired infection to initiate tracheal migration in the adult bitch and maturation in the intestine (Lloyd, 1993). It could be accounted by ingestion of vomit or faeces from their puppies (Sprenst, 1961).

Moyo (2002) isolated larvae of *T. canis* and *T. vitulorum* from experimentally infected mice and reported that larvae increased in size but still have a somatic type of migration.

Dvoroznakova *et al.* (2002) reported that mice immunized with somatic antigen of *T. canis* larvae increased specific antibody response. Furthermore, E/S antigen was more immunogenic and more efficient way to protect against larval toxocariasis in paratenic hosts.

Sugane and Oshima (1984a) reported that infection with the parasites induces a variety of immunological alterations in the hosts, including an increase level of serum immunoglobulin IgE, polyclonal B cell activation and depression of T-cell function with increased level of eosinophilia. Furthermore, Welch *et al.* (1983) identified *T. canis* antibody from definitive and paratenic hosts by

serological assays including skin test, indirect haemagglutination, flocculation and immunofluorescence.

The changes in the number and ratio of CD4⁺ and CD8⁺ T lymphocytes in the cell immune reponse are typical phenomena in larval toxocariasis (Kayes, 1997). Moreover, Reiterova *et al.* (2004) reported increases at CD4⁺ / CD8⁺ ratio in mice infected with *T. canis* mothers and their offspring for the first time on the fifth day after the birth.

The aim of this study was to investigate the sensitivity, specificity and predictive value for five antigens prepared in chapter three and used for the diagnosis of *Toxocara* infection in definitive host (dogs) and experimentally infected mice as paratenic hosts.

Materials and Methods

-Blood Samples

1. from Dogs

A total of 97 blood samples were taken by puncturing heart from dissected dogs by using disposable syringe 5 ml. Blood samples were allowed to clot at room temperature, and then centrifuged at 3000-5000 rpm for 15 minutes (Hettich EBA111/Germany). Sera were obtained, placed into glass vials and stored at -20C⁰ until used later.

2. from Mice

Fourty-eight blood samples from mice infected experimentally with *T. canis* larvae (250 and 500) after 1,2 and 3 weeks and 2,4 and 6 days post-infection and control group were taken by puncturing heart with disposable syringe (1 ml.). Blood was allowed to clot for one hour at room temperature, and then centrifuged at 1000-1500 rpm for 15 minutes. Sera were placed at a glass vials and stored at -20C⁰ until used later.

- Indirect Haemagglutination Test (IHAT)

The procedure of Herbert (1967) was used which can be briefly stated as follows:

1. Chemicals and Equipments

1- Phosphate Buffer Saline (PBS) pH: 7.2, 0.15M

Prepared by:

Sodium Chloride NaCl	8 gm
Potassium Chloride KCl	0.2 gm
Disodium hydrogen phosphate	1.15 gm
Potassium dihydrogen phosphate	0.2 gm
Dissolved in 1 liter of distilled water.	

2- Alsever's solution prepared according to Mishell and Shiigi (1980):

Dextrose	20.5 gm
Sodium citrate dehydrate	8.0 gm
Sodium chloride	4.2 gm
Dissolved in 1 liter of distilled water.	

The above ingredients were dissolved in distilled water, autoclaved at 121C⁰ and 1.5 Kg/Cm³ for 15 min. The pH was adjusted to 6.1.

3- Sheep red blood cells (SRBCs) were collected from Basrah abattoir.

4- Adult crude and purified antigens, egg crude antigen, E/S antigen and TES antigens as described in chapter three.

5- Heamagglutination titer plates.

2. Preparation of SRBCs

After four days, 50 ml of SRBCs with alsever's solution was taken in test tube and centrifuged at 1500 rpm for 10 min. The supernatant was discarded and RBCs were washed with PBS and centrifuged three times at 1500 rpm. The volume was then adjusted the PBS to 20% cell suspension, 50 ml of suspension was diluted for 1:8 PBS and used as an untanned control.

The rest volume of 20% RBCs suspension was mixed with equal freshly prepared 0.00025% tannic acids in PBS and used as tanned cell control. The suspension was incubated in water bath (Guwina-Huvman GmbH/Germany) at 37C⁰ for 15 min. Then, centrifuged at 1500 rpm for 30 min. The SRBCs

were washed with PBS and centrifuged at 1750 rpm for 5 min. The RBCs were resuspended in PBS to make 5% PBCs.

One ml of 5% PBCs mixed with equal volume of PBS and used as tanned cell control. The antigens (crude and purified adult, crude egg, E/S and TES larval antigens) were diluted for 1:10 with PBS. Equal volume of 5% tanned SRBCs suspension and diluted antigen were mixed and incubated at 37C⁰ for 30 min. and then centrifuged at 1750 rpm for 5 min. The coated RBCs were washed with PBS and centrifuged at 1750 rpm for 5 min. (three times). The final 2.5% concentration was used as sensitized SRBCs.

- **The Run Test of Sera**

Two hundred and fifty microliter of PBS was put in each well of haemagglutination titer plate except the 1st well (left row) where another 250 µl were added. Fifty µl of test serum was added to 1st well (left row) and mixed well with the micropipette. 250 µl were transferred from 1st well to 2nd well and from 2nd to 3rd until the last well where 250 µl were discarded. 250 µl of antigen coated RBCs were added to each well. The wells were set up as control containing 250 µl PBS and 250 µl PBS of untanned SRBCs, 250 µl of tanned SRBCs, 250 µl and 250 µl of sensitized SRBCs.

-Cross Reaction with other Parasites

The cross reactivity of adult and larvae of *T. canis* antigens in dogs and mice with antibodies of other parasites, monospecific sera of cestodes and nematode parasites in dogs and mice were tested with IHAT.

Results

1. The Sensitivity and Specificity of the Antigens in the Dogs

The sensitivity of the test with three antigens (adult crude, adult purified and eggs crude antigens) using IHAT was evaluated depending on the results in table (4.1). IHAT titer ≥ 160 was considered positive of adult crude antigen which confirmed the diagnosis of toxocariasis in dogs. While, the titer ≥ 40 was considered positive for purified adult of *T. canis* worms and titer ≥ 160 was positive for eggs crude antigen (table 1).

Serum samples collected from dogs were classified according to the titer ($=160$ and $=40$) because no one of control groups reach these titer and with the presence or absence of *T. canis* in the intestine, as the golden or validating test.

The sensitivity of the test was calculated and it was found 64.5, 86.2 and 93.5 % using eggs crude, adult crude and adult purified antigens in dogs, respectively. The specificity was 84.4, 93.8 and 100% with the above three antigens, respectively. The predictive values were 80.0, 92.6 and 100% respectively. The likelihood ratio was 4.5, 14.3 and ∞ (Table 2, Fig. 1).

Cross-reactivity between IHAT and three antigens were recorded using sera from dogs infected with *Echinococcus granulosus*, *Dipylidium caninum*, *Taenia sp.*, *Toxascaris leonina* and *Ancylostoma caninum* (Table 3). There was no cross-reaction between all cestodes species and the three antigens under study. Also, no cross-reaction between adult purified antigen and infection with nematodes. A low cross-reaction was found between adult crude and eggs antigens and infection with both nematode species (Table 3).

Table (1): The number of positive (titer ≥ 160 , ≥ 40) and negative (titer ≤ 160 , ≤ 40) IHAT in dogs with or without *T. canis* worms in the intestine using adult crude, purified and eggs crude antigens, respectively.

Antigen	+ worm	- worm	<i>Total</i>
	+ ve	- ve	
Adult crude (ACA)	25	2	27
Adult purified (APA)	6	30	36
Egg crude (ECA)	29	0	29
	2	32	34
Total	20	5	25
	11	27	38
	31	32	63

Table (2): The sensitivity, specificity, predictive value and likelihood ratio of IHAT with the three antigens.

Antigen	Sensitivity %	Specificity %	Predictive value %	*Likelihood Ratio +
ECA	64.5	84.4	80.0	4.5
ACA	86.2	93.8	92.6	14.3
APA	93.5	100	100	∞

*LR+ = Sensitivity/ 100- Specificity

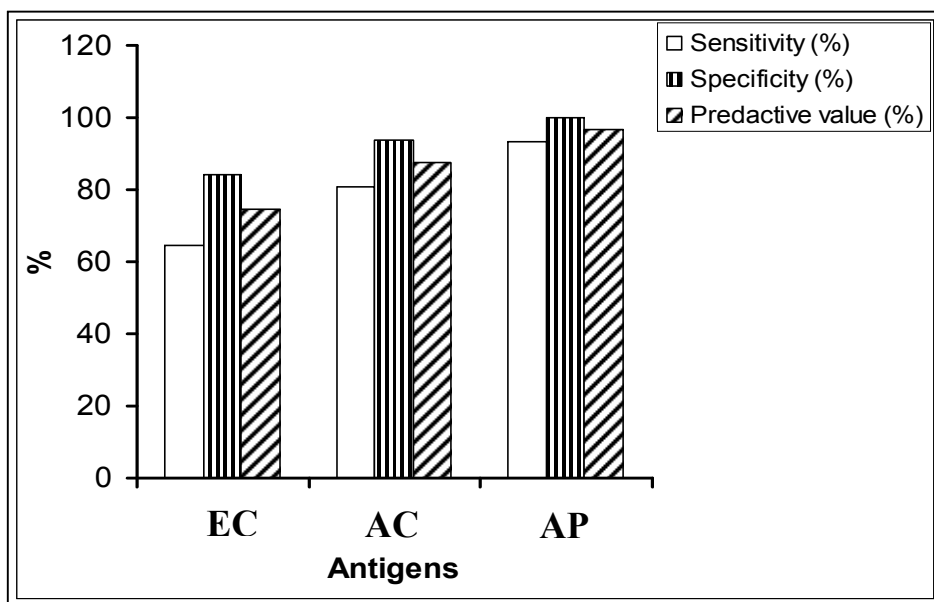


Figure (1): The sensitivity, specificity and predictive value of Adult Crude (ACA), Adult Purified (APA) and Eggs Crude Antigens (ECA) by using IHAT in dogs.

Table (3): Evaluation of cross-reactivity of the three antigens with different sera using IHAT.

Parasites	No. of test	ACA		APA		ECA	
		+ ve	- ve	+ ve	- ve	+ ve	- ve
<i>E. granulosus</i>	10	0	10	0	10	0	10
<i>D. caninum</i>	2	0	2	0	2	0	2
<i>Taenia sp.</i>	9	0	9	0	9	0	9
<i>T. leonina</i>	8	2	6	0	8	1	7
<i>A. caninum</i>	5	1	4	0	5	1	4
Total	34	3	31	0	34	2	32

2. The Sensitivity and Specificity in Mice

Mice were experimentally infected with two doses (250 and 500) embryonated eggs of *T. canis* and within three different periods each (1, 2, 3 weeks and 2, 4, 6 days post-infection respectively). The IHAT titer ≥ 40 was considered positive which confirmed the diagnosis of toxocariasis

(larvae) in paratenic host (mice) using E/S antigen, while, titer ≥ 160 was considered positive with TES antigen (Table 4)

From these data, the mean sensitivity of the tests was found to be 91.66% and 83.33% for both doses (250 and 500 respectively) with E/S antigen, while the sensitivity of TES antigen was 58.33% and 50% for both doses (Table 5, Figs. 2, 3).

The specificity of the tests was 100% with both doses and antigens (E/S, TES) (table 5).

Generally, the high sensitivity and specificity were found in long periods (weeks) as compared with short period (days) in E/S and TES larval antigens as compared with specificity which was fixed for both doses and antigens. There was no cross reaction between infection with cestodes, nematodes and mice infected with *T. canis* larvae.

Table (4): The number of positive (titer ≥ 40 and ≥ 160) and negative (titer ≤ 40 and ≤ 160) IHAT in mice infected with 500 and 250 embryonated eggs of *T. canis* with two antigens (E/S, TES).

Antigen	Dose	Period	Serum antibody	+ ve	- ve
E/S	500	2 days	Titer ≥ 40	3	1
		4 days		3	0
		6 days		4	0
TES		2 days	Titer ≥ 160	1	3
		4 days		2	2
		6 days		3	1
E/S	250	1 week	Titer ≥ 40	3	1
		2 weeks		4	0
		3 weeks		4	0
TES		1 week	Titer ≥ 160	1	3
		2 weeks		2	2
		3 weeks		4	0

Table (5): The sensitivity, specificity and predictive value of IHAT in mice infected with 500 and 250 embryonated eggs of *T. canis* with E/S and TES antigens.

Antigen		Sensitivity %						Specificity %						Predictive value
		Days			Weeks			Days			weeks			
E/S	Dose	2	4	6	1	2	3	2	4	6	1	2	3	91.66
	500		75	75	100				100	100	100			
250					75	100	100				100	100	100	95.83
Total		83.33			91.66			100			100			
TES	Dose	25	50	75				100	100	100				75
	500													
250					25	50	100				100	100	100	79.61
Total		50			58.33			100			100			

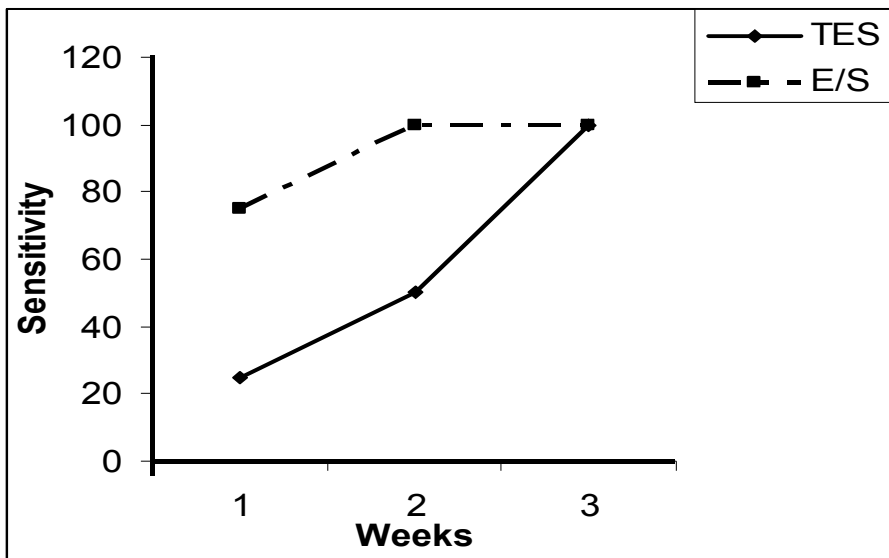


Figure (2): The sensitivity of two antigens E/S, TES of mice infected with (500) embryonated eggs of *T. canis*.

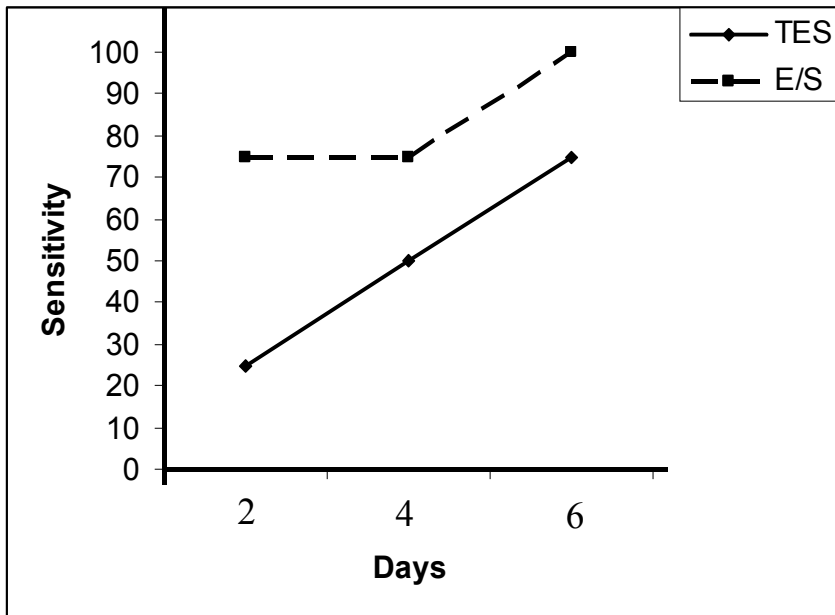


Figure (3): The sensitivity of two antigens E/S, TES of mice infected with (250) embryonated eggs of *T. canis*.

Discussion

The definitive diagnosis of intestinal parasites is usually determined by finding eggs or segments of the parasites in faeces of the host. It was difficult to diagnose *T. canis* in paratenic hosts including human because *Toxocara* larvae do not develop to adult stage in other hosts than the dogs. In dogs a microscopic examination of the faeces was beneficial for diagnosis at late infection with *T. canis* (Inoue and Tsuji, 1989).

In the present study, an immunological test (IHAT) was used to diagnose the infection with *T. canis* worms in dogs as definitive host and in mice experimentally infected with 2nd stage larvae of *T. canis* as paratenic host. Three antigens (adult crude, purified and egg crude antigens) were used with dogs, while, E/S and TES larval antigens were used with mice.

In dogs, IHAT was found to be highly sensitive in adult purified antigens as compared with the other two antigens.

This may be due to the high concentration of the immunized materials in the adult purified antigen rather than adult or egg crude antigens. The same results were obtained in case of specificity. Dragneva and Rupova (1991) showed by using passive haemagglutination test (PHAT) and ELISA that the increase in the degree of the purification of adult antigen of *T. canis* worms led to a high sensitivity and specificity. Moreover, Overgaauw (1997a) pointed out the specifications of the floatation test for finding *Toxocara* eggs in dogs 51% for specificity and 100% for sensitivity, while the predictive value of a positive test was 100% and 81% for a negative value.

In the current study could not recognized cross-reaction between cestodes and three antigens. This may be due to the differences in the structure and basic materials of cestodes. There was low cross reaction between *T. canis* (adult and eggs crude) antigens and other nematodes. This indicated that *T. canis* shared *T. leonina* and *A. caninum* with basic materials. Kulkarni *et al.* (1990) showed that *Ascaris suum* antigen shared *T. canis* worms extracted by titer ≥ 80 using ELISA in dogs experimentally infected with *A. suum* and *T. canis*. Similar result was obtained by Cuellar *et al.* (1992). No cross-reaction between *A. suum* E/S, adult worms extract antigens and *T. leonina* adult worm extract antigens with *T. canis* adult worm extract in Balb/c mice infected experimentally, also there was no cross-reaction between oncosphere antigens of *Taenia hydatigena*, *T. pisiformis* and *T. ovis* in dogs experimentally infected with *T. canis* (Jenkins and Rickard, 1986)

A high sensitivity (total) was shown in E/S antigens as compared with TES antigen of 2nd stage larvae of *T. canis* at dose 250. This difference may indicate that the E/S materials were more specific and immunogenic (nature of enzymes and proteins) than tegumental materials. Long post-infection period may stimulate high immune response in the host as compared with short period. Furthermore, there was no cross-reaction of both antigens with cestodes and nematodes. The high sensitivity and specificity of E/S antigens compared with

TES could be due to the excretion of enzymes and other materials from larvae which have a high immunogenicity than the tegument of the larvae of *T. canis*. This created a group of mucine-like glycoprotein implicated in parasitic immune evasion. Moreover, Wnukowska and Dzbencki (2001) pointed out that the cell mediated immunity was depressed in mice 3 weeks post-infection with *T. canis* larvae and the blastogenic responses increased, reaching a level significantly higher at 8th weeks post-infection. Also, specific toxocaral IgG and IgM antibodies were first detected at 4th weeks post-infection.

A glycoprotein with mollecular weight of 31000 dalton antigens was isolated from infective stage larvae of *T. canis* with photolytic properties (Mc Gillivery *et al.*, 1990). Furthermore, immunized rabbits with *T. canis* larvae glycoproteins made a significant increase in serum IgE (El-Ganayni, 1990). Cuellar *et al.* (1990) reported that the specific antibodies against excretory-secretory antigen of *T. canis* larvae were detected with peaks in rabbits at 10 and 12 weeks post-infection depending on the dose and the period of post-infection. Low dose of *T. canis* larvae induced high degree of eosenophils levels. Gupta *et al.* (1992) found that excretion/secretions of the larvae indicated the specific humoral and cell mediated immune response in mice infected with second stage larvae of *T. canis*.

Yamashita *et al.* (1993) pointed out that the splenic cells of infected mice with embryonated *T. canis* larvae failed to respond to T-cell mitogen. Also, they found that B-cell activity and the production of interlukin-1 were enhanced in the spleen of the infected mice. On the other hand, Iglesias *et al.* (1996) showed that ELISA indicated high antigenic cross-reactivity between *Anisakis simplex* and other ascaridoid nematodes. Courtade *et al.* (1995) used two immunodiagnostic methods of ELISA (BP and LMD) and Western blot for diagnosis of toxocariasis in mice and they proved that Western blot had a higher sensitivity and specificity as compared with ELISA.

Sommerfelt *et al.* (2001) reported that pigs experimentally infected with 1000 and 2000 infected eggs of

T. canis shared an increased eosinophil counts 2-7 weeks post infection, furthermore, the immunological values were increased from 7th day post-infection and remained till 56th day post-infection.

ELISA was more sensitive than IFAT test as an immunodiagnostic method for toxocariasis in dogs (Scheuer, 1987). On the other hand, Fan *et al.* (1998) showed that IgG and IgM antibodies in mice infected with E/S antigens of *T. canis* larvae and homogenized larval extracts antigens were detected 1-2 weeks post-infection. Moreover, they proved that the E/S and homogenized larval extracts antigens were excellent for diagnosing murine toxocariasis by using IgG-ELISA in mice. In contrast, Fan *et al.* (2003) proved that serum IgG antibody titers in mice infected with *T. canis* larvae was higher as compared to IgG3 antibody titers (indicator for the Th1 type response) which was not effected.

Inoue and Tsuji (1989) reported that in rats experimentally infected with *T. canis* larvae the antibody of *T. canis* were detected one week post-infection, but it would be decrease 9-18 weeks post-infection till one year.

The immune response depended upon the host species, route of inoculation and the number of larvae used for infection. Antibody was found as early 4-7 days after oral infection. But more often the antibody response was detectable three weeks post-infection and peaked sometime during the second month of infection (Glickman and Summers, 1983).

Welch *et al.* (1983) showed that pure antigen contained fewer but more specific proteins than crude antigen. Pure antigen showed a higher specificity and sensitivity than crude antigens of *T. canis* adult worms and larvae in many serological tests.

Migrating larvae in dog induced high levels of liver enzymes (AST, ALT) and the total IgG levels in serum were double during 20th day post-infection (Stejkskal and Johansson, 1983).

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