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Comparative study of alterated myocardial tissue in infected mice with *Trichinella spiralis* under treatments between doramectin and combined doramectin with dexamethasone

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Abstract

This study was evaluated the alteration of myocardial tissues in mouse model under treatments between doramectin 200µg/kg and doramectin 200µg/kg plus dexamethasone 0.6mg/kg during study period and performed by 120 female ICR mice infected with 450 *Trichinella spiralis* larvae and divided into 5 groups: 2 experimental groups and 3 control groups. Briefly, the experimental groups were infected mice with doramectin and doramectin plus dexamethasone treatments, the control groups were uninfected mice, untreated infected mice, and infected mice with dexamethasone treatments. The evaluation a number of parasites under stereomicroscopic examination and the inflammatory reactions grading in the myocardial tissues based on histological study under microscopic examination. The results showed a number of parasites of infected mice treated with doramectin were significantly less than doramectin plus dexamethasone treated and untreated mice at 1st day post-infection (DPI) (*p-value* < 0.05). Moreover, the inflammatory reactions in myocardial tissues of infected mice treated with doramectin plus dexamethasone is significantly less than doramectin treated mice at 6th and 9th DPI (*p-value* < 0.05). In addition, there is no statistic significant difference of inflammatory reactions compared between infected mice treated doramectin and untreated control throughout the study periods. Furthermore, the inflammatory reactions in doramectin plus dexamethasone and dexamethasone alone is significantly less than untreated mice (*p-value* < 0.05) at 6th, 9th, 12th, 18th DPI and 9th, 12th, 18th DPI, respectively. In conclusion, this study shows an effectiveness of doramectin alone and doramectin plus dexamethasone treatments could reduce parasites only 1st DPI as 95.27% and 58.58 %, respectively. The effectiveness of doramectin plus dexamethasone and dexamethasone could reduce the inflammatory reactions in myocardial tissues within 6th to 18th DPI. Summary, further proper usefulness of this study for applies develop anthelminthic drug and anti-inflammatory drug for improve trichinellosis and prevention serious complications.

Keywords: *Trichinella spiralis*; doramectin; parasite numbers; inflammatory reactions.

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1. Introduction

Trichinellosis is a zoonosis that the majority caused by *Trichinella spiralis* with occurred in the humans and mammalian animals and caused by ingestion of uncooked meat with encysted containing first stage larvae (L1) (Kennedy et al., 2009). Recently, the estimate morbidity rate is approximately 10,000 cases per year (Murrell et al., 2011) while the mortality rate is 0.2% (Gottstein et al., 2009). In Thailand, trichinellosis was first reported in 1962 then the outbreak has been reported each year. The most recent outbreaks have been reported in May 11, 2012 (Mahannop et al., 2012; Depinta et al., 2013). The clinical features of trichinellosis include fever and myositis. Moreover, myocarditis is noted as a serious complication which identified as the leading cause of death in approximately 0.1 % of trichinellosis patients (Tasic et al., 2006). An interaction between host and parasite (immune response) which caused the inflammatory reactions in various tissues and internal organs has been defined as the major factors that determine the disease severity. The first line treatment of trichinellosis is albendazole (20 mg/kg) treatment which revealed more effective in the early stage of infection than the late stage and the reduction was 100 % and 71 % respectively (Siriyasatien et al., 2003). On the other hand, the efficacy of albendazole (250 mg/kg) has been shown to be more than 50% against the encapsulated larva stage of *T. spiralis* (Sheng et al., 2012). The interesting points of this study are focused on an anthelminthic drugs (doramectin) in combination with anti-inflammatory drug (dexamethasone) expected to improvement of effectiveness and efficacy treatment in term of parasites elimination as well as reduction of the inflammatory reactions in various tissues which might prevent the serious complications especially myocarditis that the leading cause of death.

2. Materials and methods

2.1. Sample size calculation

The sample sizes was setting based on general objective of research, statistical analysis of hypothesis test, the optimal of formula, power of test setting and significance level of test setting, according to the formula below*(1)*. The power of the test was set at 90%, significance level 5%, the appropriate standard deviation and mean were obtained from a reviewed literature (Soliman et al., 2011). The sample size was calculated using "Comparison of two mean" formula (Riffenburgh, 1999; Sakpal, 2010). Where: n = total sample sizes per group for each case and control, u = power = 90%, v = significance level = 5%, μ_1 = means of experimental groups from reviewed literature, μ_0 = means of control groups from reviewed literature, σ_1 = standard deviation of experimental groups from review literature, σ_2 = standard deviation of control groups from review literature.

$$n = \frac{(u + v)^2(\sigma_1^2+\sigma_2^2)}{(\mu_1 - \mu_0)^2} \qquad (1)$$

2.2. Preparation of parasite and infecting the mice

Non-free germ female ICR mice aged 8–10 weeks old, and weights 25-35 grams obtained from the National Laboratory Animal Center, Mahidol University (ethic clearance number FTM-ACUC 004/2015, approved by the Faculty of Tropical medicine, Mahidol University). All maintained parasites in mice were acclimated to animal room for at least 1 week prior to infection with *Trichinella spiralis* and care under

conventional conditions at the Faculty of Tropical medicine, Mahidol University. *T.spiralis* larvae supported by Department of Parasitology and Entomology, Faculty of Public Health, Mahidol University and inoculated in ICR mice 8–10 weeks old with 200 *T. spiralis* larvae per mouse and care under conventional conditions for 45 days until performing experiment. After maintenance parasites in mice for 45 days, all infected mice were euthanized via CO_2 inhalation and definitely dead. After that, all infected mice were generalized gross examination and dissection for detecting encysted larvae in striated muscle by crushing technique under standard protocol (Gail et al., 2013). Selected positive encysted larvae muscle for digestion by pepsin solution for recovery and counting *T.spiralis* larvae under stereomicroscopic examination, and then all selected live larvae were washed by PBS and kept into NSS in container for further experiment. Selected 450 live *T.spiralis* larvae were oral feeding via nasogastric-gavage tube No.18 into the experimental mouse and care under conventional conditions.

2.3. Drugs administration

All 120 mice were divided into five groups and administered drugs or placebo after 24 hours post infection on study periods (1st, 6th, 9th, 12th, 18th and 32nd day). Briefly: two experimental groups (EI, EII) and three control groups (CI, CII, CIII). The experimental groups (EI) are composed of 24 infected mice with 450 *T. spiralis* larvae per mouse and administered with a single dose of doramectin 0.2 mg/kg via oral feeding and experimental groups (EII) are composed of 24 infected mice administered with a single dose of doramectin 0.2 mg/kg via oral feeding plus dexamethasone 0.6 mg/kg via intramuscular injection (0.2 mg/kg every 8 hours x 3 doses). Control groups (CI) are composed of 24 uninfected mice administered with a single dose of placebo (sesame oil) via oral feeding; control groups (CII) are composed of 24 infected mice administered with a single dose of placebo (sesame oil) via oral feeding and control groups (CIII) are composed of 24 infected mice administered with dexamethasone 0.6 mg/kg via intramuscular injection (0.2 mg/kg every 8 hours x 3 doses) (Hedrich, 2012).

2.4 *Detection and collection of Trichinella spiralis post-treatment*

All mice in each group were euthanized via CO_2 inhalation at 2nd, 7th, 10th, 13th, 18th and 33rd day post infection (Gail et al., 2013). Dissection and gross examination of dead mice was performed and then selected gastrointestinal tract, heart, lungs, kidneys and muscle were weighed and measured in size, detected of *T. spiralis* larvae under stereomicroscopic examination by crushing technique and digested of each internal organs by artificial digestion method (Siriyasatien et al., 2003; Soliman et al., 2011); then counted the number of recovered *T. spiralis* larvae under stereomicroscope examination. Finally, the selected myocardial tissues were fixed in 10% neutral buffered formalin for histology study with H&E staining.

2.5. Histological hematoxylin and eosin staining (H&E staining) technique

The selected myocardial tissues have been processed H&E staining by automated instrument at Department of Pathology, Chulalongkorn University. Briefly, the serial sectioned were fixed in 10% neutral buffered formalin for 24 hours; dehydrated in ascending grades of alcohols (70% - 95%); cleared in xylene; embedded in paraffin blocks; sectioned at 5 µm in thickness by microtome; and placed the tissue on glass slide and H&E staining was performed.

2.6. The inflammatory reactions grading criteria of myocardial tissues

The modified criteria of inflammatory reactions grading of myocardial tissues were based on reviewed literatures (Leon, 2003; Quijano et al., 2013). The interpretation of inflammatory reactions grading was performed by microscopic examination in consecutive pattern with 400X objective (high power field) in all serial sectioned of myocardial tissues at least 16 fields. The inflammatory reactions grading score was graded as follows: score 0 = normal; 0% involvement of the histological sections, score 1 = mild; < 25% involvement of the histological sections, score 2 = moderate; 25- 50% involvement of the histological sections, and score 3 = severe; > 50-75% involvement of the histological sections.

2.7. Statistical analysis

Total a number of *T. spiralis* larvae in the control groups (CII, CIII) and the experimental groups (EI, EII) were statistical analyzed by independent *t-test*, and were considered significantly at *p-value* < 0.05 and the inflammatory reactions in experimental groups and control groups were compared by Mann-Whitney U-Test. The data were coded, computed and analyzed by using the SPSS statistics.

3. Results

3.1 Evaluation of a number of T. spiralis larvae

A number [mean(standard deviation)] of *T. spiralis* larvae at 1^{st}, 6^{th}, 9^{th}, 12^{th}, 18^{th}, and 32^{nd} DPI in the infected mice untreated as control groups (CII), were: 126.75(51.55), 55(28.67), 67.25(129.22), 29.25(57.84), 724.5(308.28), and 3871.75(3330.22), respectively. In the infected mice treated with dexamethasone 0.6 mg/kg as control group (CIII), were: 143.5(55.77), 96.75(59.22), 9.5(7.33), 1(1.16), 1036.75(648.56), and 3582.25(2052.85), respectively. In the infected mice under treatment with single dose of doramectin 0.2 mg/kg as experimental groups (EI), were: 6(8.17), 112.75(146.44), 12.75(21.85), 3.67(6.35), 432.75(207.02), 2107(282.17), respectively. In the infected mice treated with a single dose of doramectin 0.2 mg/kg plus dexamethasone 0.6 mg/kg as experimental groups (EII), were: 52.5(35.6), 54.25(65.18), 6.5(11.09), 0(0), 409.5(307.72), 3212.50(1207.50), respectively. In the experimental groups (EI) revealed decrease of the percentage of a number of *T. spiralis* larvae on 1^{st}, 6^{th}, 9^{th}, 12^{th}, 18^{th}, and 32^{nd} DPI as 95.27%, 0%, 81.04%, 87.45%, 40.27%, and 45.58%, respectively (Table 1). In the experimental groups (EII) revealed decrease of a percentage of a number of *T. spiralis* larvae in study periods as 58.58%, 1.36%, 90.34%, 100%, 43.48%, and 17.03%, respectively (Table 2). While in the control groups (CIII) revealed decrease of the percentage of a number of *T. spiralis* larvae in study periods as 0%, 0%, 85.87%, 96.57%, 0%, and 7.48% respectively. The statistical analysis by independent *t-test* showed that a number of recovered parasites of infected mice after treated with doramectin (EI) were significantly less than doramectin plus dexamethasone treated (EII) and untreated mice (CII) at 1^{st} DPI (*p-value* < 0.05) (Table 1,3).

3.2 Evaluation of the inflammatory reactions of myocardial tissues

The inflammatory reactions of the myocardial tissues were identified in all infected mice during 6 to 32 DPI as follows; The infected mice treated with doramectin (EI) showed mild, moderate and severe inflammatory reactions as 50%, 33.33-75% and 50-100%, respectively and the infected mice treated with doramectin plus dexamethasone (EII) showed mild and moderate inflammatory reactions as 50-100% and 50-100%, respectively. The untreated infected mice (CII) showed mild, moderate and severe inflammatory

reactions as 75%, 25-75%, and 25-100%, respectively and the myocardial tissues of uninfected mice (CI) showed unremarkable. In conclusion, statistical analysis of the inflammatory reactions in the myocardial tissues of the experimental groups (EI) compared with the control groups (CII) showed no statistic significant difference (*p-value* > 0.05) during 1st to 32nd DPI. However, the inflammatory reactions in infected mice treated with doramectin plus dexamethasone (EII) and infected mice treated with dexamethasone alone (CIII) were significantly less than untreated infected mice (*p-value* <0.05) at 6th, 9th, 12th, 18th DPI and 9th, 12th, 18th DPI, respectively. The experimental groups (EI) compared with the experimental groups (EII) showed the inflammatory reactions in infected mice treated with doramectin plus dexamethasone (EII) were significantly less than infected mice treated with doramectin (EI) at 6th and 9th DPI (*p-value* < 0.05) (Table 4).

Table 1. Statistical analysis compare mean number of *T.spiralis* (SD) between infected mice treated doramectin and untreated infected mice during the study periods at 1st, 6th, 9th, 12th, 18th and 32nd days post infection. (n=4)

Day post infection	Experimental group(EI) x̄(SD)	Control group(CII) x̄(SD	Experimental group(EI) compare to control group(CII) *p-value* (95% CI)	Efficacy %
1	6.00(8.17)	126.75(51.55)	0.004 (56.90, 184.60)*	95.27
6	113.25(146.75)	55.25(29.06)	0.468 (-241.03, 125.03)	0.00
9	14.75(20.84)	67.25(129.22)	0.453 (-107.64, 212.64)	81.04
12	3.67(6.35)	29.25(57.84)	0.490 (-62.72, 113.89)	87.45
18	432.75(207.02)	724.50(308.28)	0.167 (-162.57, 746.07)	40.27
32	2107.00(282.17)	3871.75(3330.22)	0.332 (-2324.23, 5853.73)	45.58

Note; Asterisks (*) denotes; the significant *p-value* < 0.05 and n=3 at 12th DPI as death occurred in the experimental.

Table 2. Statistical analysis compare mean number of *T.spiralis* (SD) between infected mice treated doramectin plus dexamethasone and untreated infected mice during the study periods at 1st, 6th, 9th, 12th, 18th and 32nd days post infection. (n=4)

Day post infection	Experimental group(EII) x̄(SD)	Control group(CII) x̄(SD)	Experimental group(EII) compare to control group(CII) *p-value* (95% CI)	Efficacy %
1	52.50(35.60)	126.75(51.55)	0.055 (-2.39, 150.89)	58.58
6	54.50(65.67)	55.25(29.06)	0.984 (-87.11, 88.61)	1.36
9	6.50(11.09)	67.25(129.22)	0.385 (-97.93, 219.43)	90.34
12	0.00(0.00)	29.25(57.84)	0.351 (-41.51, 100.01)	100.00
18	409.50(307.72)	724.50(308.28)	0.198 (-217.91, 847.91)	43.48
32	3212.50(1207.50)	3871.75(3330.22)	0.723 (-3674.69, 4993.19)	17.03

Table 3. Statistical analysis compare mean number of *T.spiralis* (SD) between infected mice treated doramectin and infected mice treated doramectin plus dexamethasone during the study periods at 1st, 6th, 9th, 12th, 18th and 32nd days post infection. (n=4)

Day post infection	Experimental group(EI) x̄(SD)	Control group(EII) x̄(SD)	Experimental group(EI) compare to control group(EII) *p-value* (95% CI)
1	6(8.17)	52.50(35.60)	0.044(-91.18, -1.82)*
6	113.25(146.75)	54.50(65.67)	0.492(-137.95, 255.45)
9	14.75(20.84)	6.50(11.09)	0.655(-24.23, 35.73)
12	3.67(6.35)	0.00(0.00)	0.286(-4.22, 11.55)
18	432.75(207.02)	409.50(307.72)	0.904(-430.50, 477.00)
32	2107.00(282.17)	3212.50(1207.50)	0.125(-2622.62, 411.62)

Note; Asterisks (*) denotes; the significant *p-value* < 0.05 and n=3 at 12th DPI as death occurred in the experimental.

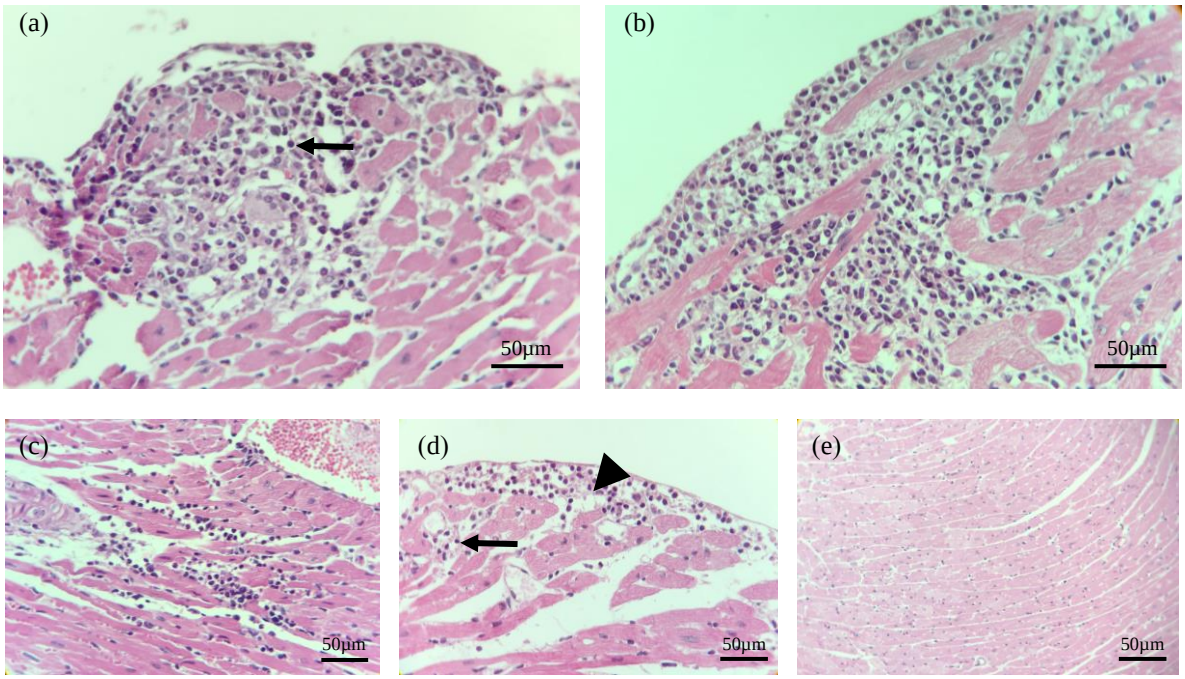

Fig.1. The photomicrographs (400X) of histology in the myocardial tissues with H&E staining at 9th DPI; (a) infected mice with *T.spiralis* without treatment showed acute severe myocarditis, characterized by inflammatory cells infiltrated into the interstitial myocardial tissues (arrow); (b) infected mice with *T.spiralis* treated with doramectin showed acute severe myocarditis; (c) infected mice with *T.spiralis* treated with doramectin plus dexamethasone showed moderate myocarditis; (d) infected mice with *T.spiralis* treated with dexamethasone showed mild (arrow) to moderate (arrowhead) myocarditis and (e) uninfected mice showed unremarkable myocardial tissues.

4. Discussions

This study of the comparison between doramectin and the combination of doramectin and dexamethasone treatments showed effective treatments against a number of parasites are revealed as follows, doramectin treatment alone is greatest reduce parasites burden observed in only 1st DPI (efficacy = 95.27%) this can be explained by the primary mode of mechanisms underlying these effects of doramectin reacting with parasites in the intestinal lumen in which doramectin effects chloride ion channel activity in the nervous system of nematodes and binds to receptors that increase membrane permeability to chloride ions and inhibits the electrical activity of nerve cells in nematodes and also causes paralysis and death of the parasites (Donald, 2008; Omura, 2008; Tambotra et al., 2012) meanwhile, doramectin plus dexamethasone treatment is less effective in reducing parasites burden than doramectin treatment alone only 1st DPI (efficacy = 58.58%) because doramectin is effective against parasites while dexamethasone is mainly an anti-inflammatory actions therefore we can explain this phenomenon based on Genta' s hypotheses. Firstly, dexamethasone acting with worm receptors for host-derived eicosanoids, cytokines or chemokines in their cuticles and responds to this mediators by the synthesis of own reproductive and growth hormones. Secondly, parasites benefit from the suppressed innate and adaptive immune responses of glucocorticoid-exposed host, which fosters parasite reproduction, invasion, and spreading into various internal organs (Genta, 1992) therefore, unless doramectin directly affect killed parasites, but the effective dexamethasone is suppressed immune response to eliminate parasites resulting in the efficacy of doramectin plus dexamethasone treatment is less than doramectin

treatment alone, and also we found that the result of a number of parasites burden in infected mice with dexamethasone treatment compared to untreated infected mice was not different. In terms of infected mice treated with doramectin and dexamethasone against the inflammatory reactions in myocardial tissues in this study revealed as follows, dexamethasone treatment alone greatest suppressed the inflammatory reactions at 9^{th}, 12^{th}, and 18^{th} DPI especially for 12^{th} DPI (*p-value* = 0.011) and doramectin plus dexamethasone treatment could suppress the inflammatory reactions at 6^{th}, 9^{th}, 12^{th}, and 18^{th} DPI especially for 12^{th} DPI (*p-value* = 0.008), this phenomenon can be explained based on life cycle of *Trichinella spiralis* and Genta' s hypotheses. Firstly, myocardial tissue involvement occurs during 6^{th} to 21^{st} DPI (Tasic et al., 2006). Secondly, dexamethasone is mainly an anti-inflammatory action with suppressive immune response in host-parasite interactions resulting in decreasing inflammatory reactions in myocardial tissues. Therefore, the results indicated that effective of doramectin and dexamethasone was not effect on inflammatory reactions in the myocardial tissues in all groups at 1^{st} DPI, meanwhile we found that dexamethasone treatment alone or dexamethasone plus doramectin treatment could be suppressed an inflammatory reactions in the myocardial tissues.

Table 4. The comparison of inflammatory reactions grading of myocardial tissues in experimental groups (EI, EII) and control groups (CII, CIII) during study periods at 1^{st}, 6^{th}, 9^{th}, 12^{th}, 18^{th} and 32^{nd} days post infection.

DPI	Group	n	Number of mice (%) and inflammatory reactions grading of myocardial tissues				CII compare EI	CII compare EII	EI compare EII	CIII compare EI	CIII compare EII	CIII compare CII
			Normal	Mild	Moderate	Severe	*p-value*	*p-value*	*p-value*	*p-value*	*p-value*	*p-value*
1	CI	4	4(100)	0	0	0						
	CII	4	4(100)	0	0	0						
	CIII	4	4(100)	0	0	0	1.000	1.000	1.000	1.000	1.000	1.000
	EI	4	4(100)	0	0	0						
	EII	4	4(100)	0	0	0						
6	CI	4	4(100)	0	0	0						
	CII	4	0	0	3(75)	1(25)						
	CIII	4	0	1(25)	3(75)	0	0.495	0.040*	0.032*	0.096	0.186	0.186
	EI	4	0	2(50)	2(50)	0						
	EII	4	0	3(75)	1(25)	0						
9	CI	4	4(100)	0	0	0						
	CII	4	0	0	1(25)	3(75)						
	CIII	4	0	2(50)	2(50)	0	0.317	0.011*	0.008*	0.013*	0.127	0.032*
	EI	4	0	0	0	4(100)						
	EII	4	0	0	4(100)	0						
12	CI	4	4(100)	0	0	0						
	CII	4	0	0	0	4(100)						
	CIII	4	0	1(25)	3(75)	0	0.248	0.008*	0.074	0.076	0.317	0.011*
	EI	3**	0	0	1(33.33)	2(66.67)						
	EII	4	0	0	4(100)	0						
18	CI	4	4(100)	0	0	0						
	CII	4	0	0	1(25)	3(75)						
	CIII	4	0	2(50)	2(50)	0	0.495	0.032*	0.061	0.061	1.000	0.032*
	EI	4	0	0	2(50)	2(50)						
	EII	4	0	2(50)	2(50)	0						
32	CI	4	4(100)	0	0	0						
	CII	4	0	3(75)	1(25)	0						
	CIII	4	0	4(100)	0	0	0.495	0.317	0.127	0.127	1.000	0.317
	EI	4	0	2(50)	2(50)	0						
	EII	4	0	4(100)	0	0						

Note: Asterisks (*) denotes; The significant *p-value* < 0.05; The data show the number of mice (%) and inflammation reactions grading; n= number of mice per group; DPI= Day Post Infection; Control(CI)= Non-infected mice; Control(CII)= Infected mice untreated; Control(CIII)= Infected mice with dexamethasone; Experiment(EI)= Infected- mice with doramectin; Experiment(EII)= Infected mice with doramectin plus dexamethasone; Asterisks (**)denotes; n=3, a dead mouse during study period at 12^{th} DPI.

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