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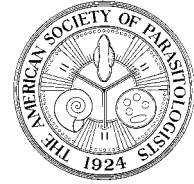
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SARCOCYSTIS NEURONA-INDUCED MYELOENCEPHALITIS RELAPSE FOLLOWING ANTICOCCIDIAL TREATMENT

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KEY WORDS ABSTRACT

Diclazuril
Sarcocystis neurona
Equine Protozoal Myeloencephalitis
Relapse
Horse
Opossum
Didelphis virginiana

Sarcocystis neurona is a ubiquitous parasite in the eastern United States, which is the principal causative agent in the neurologic disorder equine protozoal myeloencephalitis (EPM). While much is known about this protozoa's life cycle in its natural host, the opossum (*Didelphis virginiana*), little is known of how it acts in the aberrant equine host, which displays a high incidence of exposure with a relatively low rate of morbidity. For this study, we employed the popular interferon gamma knockout mouse model to determine the potential for recrudescence of *S. neurona* infection after treatment with the anticoccidial drug diclazuril. Mice were infected with *S. neurona* merozoites, and 7-days post-infection (DPI) they were treated with diclazuril for 30 or 60 days or not treated at all. All infected non-treated mice developed neurologic signs consistent with *S. neurona* infection within 30 DPI. All diclazuril-treated infected mice remained clinically normal while on treatment but developed neurologic signs within 60 days of treatment cessation. Histological examination of cerebella from all infected mice demonstrated characteristic lesions of *S. neurona* infection, regardless of treatment status. Cerebellar samples collected from infected treated mice, displaying neurologic signs, produced viable *S. neurona* in culture. However, cerebellar samples collected from infected and neurologically normal mice at the end of a 30-day treatment period did not produce viable *S. neurona* in culture. Analysis of the humoral immune response in infected mice showed that during treatment IgM antibody production decreased, suggesting the organism was sequestered from immune surveillance. The cessation of treatment and subsequent development of neurologic disease resulted in increased IgM antibody production, suggesting recognition by the immune system at that time. Based on the study results the authors propose that diclazuril was able to inhibit the replication and migration of *S. neurona* but not fully eliminate the parasite, suggesting recrudescence of infection after treatment is possible.

Equine protozoal myeloencephalitis (EPM) is the most prevalent protozoal disease of horses in North America with the predominant cause being ingestion of *Sarcocystis neurona* sporocysts deposited by opossums (*Didelphis virginiana*) (Dubey and Lindsay, 1999; Dubey et al., 1999; Dubey, 2001). The resulting neurological disorder affects approximately 14 of every 10,000 horses older than 6 mo of age in the United States (NAHMS, 2001). The reason for high exposure rates but relatively low morbidity remains unclear as does the life cycle of the parasite in the aberrant horse host. Previous studies indicate a potential aberrant immune response to *S. neurona* in susceptible horses (Spencer et al., 2004; Scott et al., 2005; Yang et al., 2006; Lewis et al., 2014), indicating EPM susceptibility may persist for the life of the animal and is not a onetime occurrence. Clinical

treatment consists of the use of anti-protozoal drugs such as ponazuril, diclazuril, and sulfa drugs as well as corticosteroids to combat associated inflammation (Reed et al., 2016). Based on the Saville et al. (2000) study, which included all stages of disease as well as all different treatments, the average equine patient is expected to improve only one neurological grade. Approximately 10–25% of EPM affected horses that respond positively to appropriate treatment are at risk for developing recurrent or novel neurological signs after discontinuing therapy. Whether the horses responding to treatment or are relapsing from their initial infection (i.e., persistent infection) or from re-exposure to the environmental stages of *S. neurona* is not fully understood (MacKay et al., 2006). Challenges of using the horse to study EPM are numerous and include the complexity of experimental

infection, the terminal end-point nature of many studies, and the difficulty in controlling environmental factors (Saville et al., 2001; Witonsky et al., 2008; Lewis et al., 2014). Alternatively, immune compromised, C57bl/6J or BALB/c interferon gamma gene knockout (*Ifn γ ^{-/-}*) mice offer a regularly utilized model for addressing certain aspects of *S. neurona* pathology (Dubey and Lindsay, 1998; Dubey et al., 2001; Witonsky et al., 2003). The goal of the current study was to determine the potential for persistent infection by *S. neurona* in mice after receiving diclazuril treatment previously shown to inhibit disease in this model (Dubey et al., 2001).

MATERIALS AND METHODS

Sarcocystis neurona culture and preparation of inoculum

Merozoites of the sixth passage of the Sn-15OP isolate were grown in African green monkey (*Cercopithecus aethiops*) kidney cells (CV-1[ATTC CCL-70]) American Type Culture Collection, Manassas, Virginia, separated from host cells, collected and enumerated for inoculations as previously described (Lindsay et al., 2013). The Sn-15OP isolate was isolated from the brain of an *Ifn γ ^{-/-}* mouse fed sporocysts obtained from the feces of a naturally infected opossum from Virginia (Dubey et al., 2000). This isolate was originally a gift from Dr. J. P. Dubey, United States Department of Agriculture, Beltsville, Maryland, and has been maintained cryopreserved in liquid nitrogen.

Mice

Male and female B6.129S7-*Ifng^{tm1Ts}/J* (B6.*Ifn γ ^{-/-}*) mice, age 8–10 wk old, were obtained from Jackson Laboratory (JR2287, Bar Harbor, Maine). Mice were maintained at Virginia Tech in accordance with an Institutional Animal Care and Use Committee (IACUC) breeding protocol and experimental studies were conducted under other IACUC-approved protocols. There were 3 experimental mouse groups that were infected with *S. neurona* merozoites: infected untreated (n = 13), 30-day treatment (n = 15), and 60-day treatment (n = 12). Mice in the treatment groups were fed rodent diet compounded with diclazuril for the duration of 30 or 60 days beginning 7 days post-infection (DPI). At the end of the 30- or 60-day treatment period, mice were changed to the control diet for the 60-day observation period. Another group of mice did not receive treatment and remained uninfected (naïve, n = 9). Another group of mice was fed diclazuril chow for 60 days and not infected (medicated diet, n = 5). The final group of infected, untreated mice received control diet.

For infection, mice were injected subcutaneously with 2×10^6 *S. neurona* merozoites. Blood was collected from the submandibular vein from live mice for sera antibody analysis at 7 DPI and on the final day of 30-day treatment. Post-euthanasia blood was collected via cardiac puncture for sera antibody analysis. Mice were euthanized using CO₂ asphyxiation when clinical neurological signs relating to lack of balance and coordination were observed.

Diclazuril treatment

Diclazuril (Sigma-Aldrich, St. Louis, Missouri and LKT Laboratories, St. Paul, Minnesota) was compounded into 5LG4 rat and mouse chow (Test Diet, St. Louis, Missouri) at a concentration of 50 ppm. Medicated chow provided 10 mg of

diclazuril/kg of body weight to each mouse, a dose previously shown to prevent the development of *S. neurona*-associated disease in *Ifn γ ^{-/-}* mice (Dubey et al., 2001). The rat and mouse chow (5LG4), without diclazuril, served as the control diet.

Cerebellum preparation and histology

At the time of euthanasia, cerebella were collected, and those intended for the use of histology were fixed in 10% buffered formalin solution. The cerebellum was then embedded in paraffin and processed for hematoxylin and eosin (H&E) staining. Triplicate sections were cut from paraffin blocks and used for immunohistochemical staining. One sample section was assessed for the total number of perivascular cuffs, gliosis foci, and number of organisms for each cerebellum collected and given a score of 1 (mild) to 6 (severe). The total number of perivascular cuffs and gliosis foci increased with score where a score of 1 = 0–10 cuffs or foci and 6 = 55–70 cuffs or foci. The total number of organisms was scored in a similar manner, score 1 = 0–20 organisms and 6 = number organisms >100. Sections of cerebellum were stained with polyclonal rabbit anti-*S. neurona* anti-sera as described by Gerhold et al. (2005) with the exception that sections were digested with peroxo-block (Novex, Life Technologies, Carlsbad, California) prior to incubation with anti-sera at 1:500 dilution.

Sera immunoglobulins

Blood was collected from infected untreated (n = 10), naïve (n = 9), and 30-day (n = 10) and 60-day (n = 10) treatment groups at the time of euthanasia, for sera IgM, total IgG, and *S. neurona* immunoglobulin analysis with enzyme-linked immunosorbent assay (ELISA). Serum was collected from whole blood following centrifugation and stored at –20 C until utilized for ELISA. Immunoglobulin ELISA tests were performed as described previously (Christianson et al., 1997) using IgG and IgM isotypes (Southern Biotech, Birmingham, Alabama). Plates were read on an Infinite M200 Pro plate reader with Magellan 7.0 software (Tecan, Mannedorf, Switzerland).

For *S. neurona*-specific ELISA, a *S. neurona* merozoite protein lysate was created as a plate coating agent for antibody capture. For the lysate, frozen Sn-15OP merozoites were thawed and centrifuged at 2,500 g for 5 min at 4 C, merozoites were washed twice in ice-cold 1× PBS and re-suspended to a concentration of 1×10^7 /ml in 1× PBS, and 10 ml of radioimmunoprecipitation assay buffer (Thermo Fisher Scientific, Waltham, Massachusetts) was added per 1 ml of merozoite suspension. The merozoite suspension was incubated on ice for 15 min with intermittent manual shaking and then centrifuged at 1,400 g for 15 min at 4 C. After centrifugation, the supernatant was collected, and 100 μ l was used to coat the ELISA plate wells (Thermo Fisher, Ref. No. 9018) incubating overnight at 4 C. The protocol then proceeded as described in Christianson et al. (1997). The detection antibody was goat anti-mouse-Ig kappa-alkaline phosphatase (Southern Biotech) and optical density (OD) values were reported for data analysis.

Cerebellum culture and observations

At the time of euthanasia cerebella intended for the use of culture were collected from infected 30-day treated mice at the

Table I. Mouse group, treatment, sample collection and neurologic sign development. Abbreviations: DPI, days post-infection; DPT, days post-treatment.

Group	Infected (day 0)*	Treatment diet (7 DPI)†	Sample collection: serum and cerebellum	Neurologic sign development
Negative control (n = 12)	No	No	Yes	No
Positive control (n = 13)	Yes	No	Yes	Yes within 30 DPI
30-day treatment (n = 15)‡	Yes	Yes	Yes	Yes within 60 DPT
60-day treatment (n = 12)	Yes	Yes	Yes	Yes within 60 DPT

* Mice infected with 2×10^6 merozoites subcutaneous injection.

† Diet changed to control after treatment duration (30 or 60 days) control mice received control diet.

‡ Serum samples collected on day 0, 7 DPI, and the final day of 30-day treatment.

following time points: on the final day of treatment (n = 3), 12 days post-cessation of treatment (n = 3), and at the time of neurologic sign development post-treatment (n = 2). Additionally, cerebella were collected from infected untreated mice (n = 2). Immediately after collection, cerebella were placed in ice-cold 1× PBS and then homogenized in 1 ml Hanks balanced salt solution (HBSS) using a sterile Teflon-coated tissue grinder and 12 ml grinding tube. The homogenized brain-HBSS solution was inoculated onto 2 (25 cm²) tissue culture flasks containing monolayers of CV-1 cells. The homogenate was incubated with CV-1 cells for 2 hr at 37 C. After 2 hr, the inoculum was removed, the cell monolayers were washed with HBSS, and 5 ml of maintenance medium was added for long-term culture. Cultures were examined twice weekly and the medium replaced twice weekly for a month and then once weekly thereafter until *S. neurona* was detected or for 2 mo when the study was ended, and cultures were considered negative.

Statistical analysis

Neurologic disease incidence was determined using a log-rank Mantel-Cox test with a significant level set at $P < 0.05$ and Bonferroni correction to account for multiple comparisons with a significance threshold at $P < 0.017$. Significance for immunoglobulin changes during treatment was determined using ANOVA accounting for repeated measures and multiple comparisons with Tukey's *t*-test. To assess the immunoglobulin changes that occurred between the naïve mouse group and throughout treatment ANOVA was used with Dunnett's test to account for multiple comparisons. All other statistical analysis was calculated using the Kruskal-Wallis test and Dunn's test for post hoc comparisons. The significant level was set at $P < 0.05$. All analyses were performed using GraphPad Prism software version 6 for Mac (GraphPad Software, La Jolla, California).

RESULTS

Sarcocystis neurona-induced myeloencephalitis

All infected untreated mice developed neurologic signs within 23 days of starting the non-medicated diet. *Sarcocystis neurona*-infected diclazuril-treated mice did not develop clinical signs while on the medicated diet. Both the infected 30- and 60-day treated mice remained clinically normal on the non-medicated diet for significantly longer than the infected untreated mice. Neurologic disease did occur within 60 days of ceasing diclazuril treatment in all infected treated mice (Table I). There was no statistically

significant difference in the incidence of neurologic disease between the 30-day treatment and 60-day treatment groups after switching to the control diet (Fig. 1).

Histopathological analysis

Histology results confirm *S. neurona*-induced encephalitis. When assessed at the time of neurologic sign onset, the cerebella of infected untreated mice possessed significantly more gliosis foci by number (Fig. 2A) and perivascular cuffs (Fig. 2B) compared to infected 30- and 60-day treated mice. *Sarcocystis neurona* organisms were found in significantly greater numbers in cerebellum sections from infected untreated mice compared to mice in the 60-day treatment group at the time of necropsy. There were no significant differences in number of gliosis foci (Fig. 2A), perivascular cuffing (Fig. 2B), or *S. neurona* organisms (Fig. 2C)

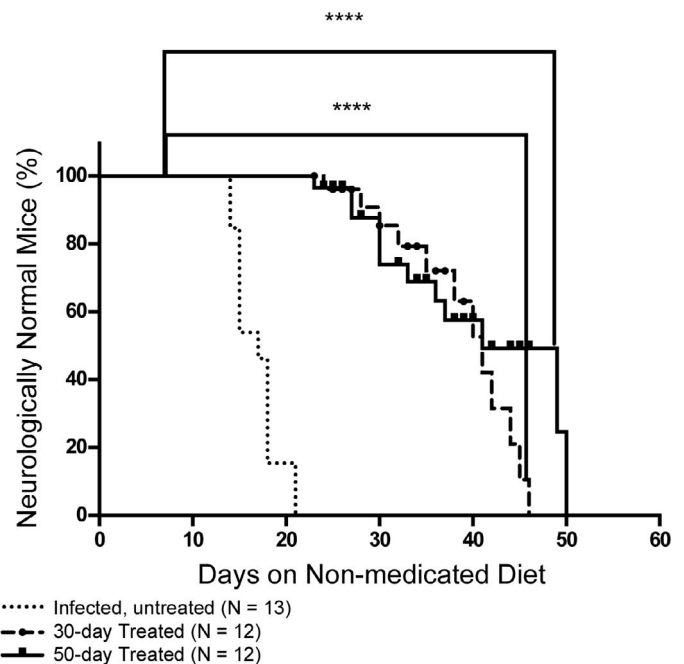


Figure 1. The percent neurologically normal mice vs. the number of days mice have been consuming non-medicated diet. Time point day 0 represents the day mice were placed on the control diet. The infected untreated mice started consumption of non-medicated diet 7 days post-infection, and 30- and 60-day treated mice started at the end of the treatment periods. Closed circles represent the number of neurologically normal mice at that time point within each group. **** $P \leq 0.0001$.

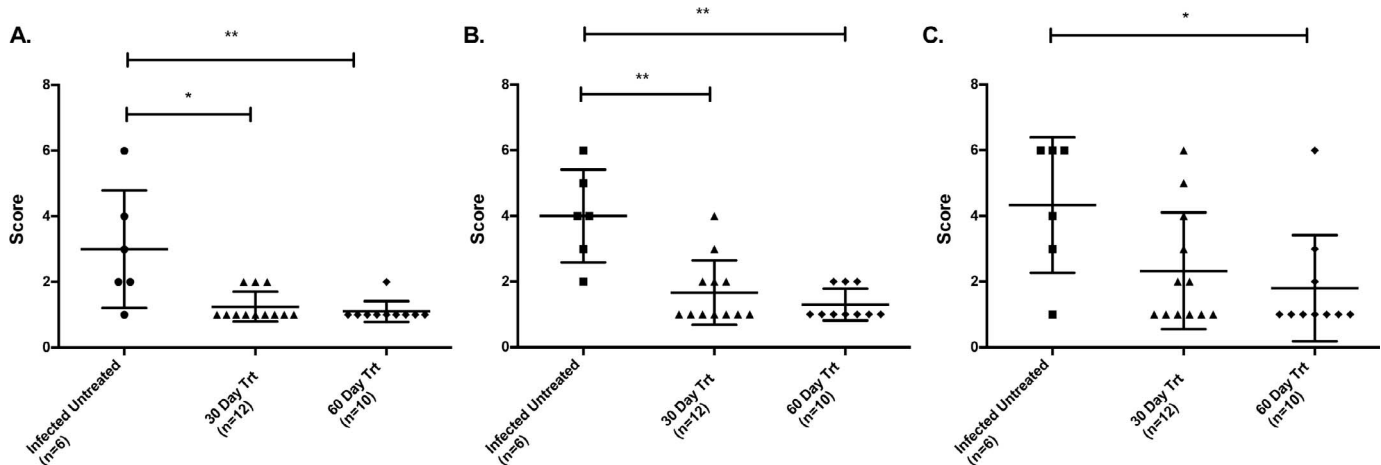


Figure 2. Scores of histopathologic changes associated with infection and treatment (trt) in cerebella collected from mice displaying neurologic signs. (A) Perivascular cuffing foci in cerebellum hematoxylin and eosin (H&E) stained sections. (B) Gliosis foci in cerebellum H&E stained sections. (C) *Sarcocystis neurona* organisms in H&E and immunohistochemistry (IHC) stained cerebellum sections. Male B6.*Ifng*^{-/-} mice, age 8–10 wk at start of experiment. * $P \leq 0.05$, ** $P \leq 0.005$

between the 30- and 60-day treatment groups. These results indicate that diclazuril treatment resulted in less severe cerebellar lesion pathology at the time of neurologic sign development. Representative H&E and immunohistochemistry (IHC) cerebellum images are depicted to display pathological changes among all infected study groups (Fig. 3A–F).

Sera ELISA

Mice that developed *S. neurona*-induced myeloencephalitis, regardless of prior treatment, produced significantly greater IgM (Fig. 4A), and total IgG (Fig. 4B) sera concentrations in comparison to the naïve mouse group. *Sarcocystis neurona* merozoite-specific antibody optical density (OD) values were significantly greater in infected 30- and 60-day treated mice compared to naïve mice. However, these levels did not reach significance when comparing infected untreated mice and naïve controls (Fig. 4C). There was not a significant difference in serum IgM or total IgG concentration between infected untreated mice and the 30- and 60-day treatment groups. Therefore, at the time of neurologic disease development, treatment did not impact IgM or total IgG immunoglobulin concentrations (Fig. 4A, B). However, treating infected mice for 60 days with diclazuril resulted in a significantly higher *S. neurona* antibody OD value compared with infected untreated mice. There was a non-significant increase in the 30-day treatment group compared to infected untreated mice (Fig. 4C). The diclazuril medicated diet itself had no significant effect on IgM or total IgG production (Fig. 4A, B).

Although treatment did not impact sera IgM and IgG levels at the time of neurologic disease development, it did result in a strong decrease in sera IgM levels before neurologic disease incidence. IgM levels trended upwards at 7 DPI compared with naïve mice and then fell by the end of treatment (Trt End) with a significant elevation appearing at the time of neurologic sign development (Fig. 5A). Total IgG levels increased at the end of treatment and terminal time points with no significant difference between the Trt End and terminal time point (Fig. 5B).

Sarcocystis neurona-specific antibody OD values become significantly elevated at the terminal time point (Fig. 5C).

Cerebellum culture and observations

Of 3 cerebellum samples collected on the final day of a 30-day treatment period and of 3 collected 12 days post-treatment, no viable *S. neurona* was recovered after culture. However, of 2 cerebellum samples harvested from mice demonstrating neurologic signs within 60 days after a 30-day treatment period, one produced viable *S. neurona* that replicated in culture (Table II). Our results in these immunodeficient mice show that viable parasites can remain in the mice even after appropriate therapy with diclazuril.

DISCUSSION

Using the immunocompromised *Ifng*^{-/-} mouse, the current study demonstrates that continuous diclazuril treatment for 30 or 60 days prevents the development of *S. neurona*-induced protozoal myeloencephalitis and associated neurologic signs. However, within 60 days after cessation of diclazuril treatment mice develop neurologic signs. This suggests that treatment prevents replication and/or migration of *S. neurona*, but cessation of treatment leads to resumption of pathogenic activity. Previous in vitro cell culture studies demonstrated that diclazuril limited the development of *S. neurona* merozoites, but parasite replication was observed after the removal of diclazuril from cell culture media. This also suggests that diclazuril has only an inhibitory effect on parasite activity (Lindsay and Dubey, 2000). In the current study, cerebellum samples collected on the final day of a 30-day diclazuril treatment period yielded no viable *S. neurona*. However, a cerebellum sample collected from a neurologic mouse post-treatment yielded viable *S. neurona* capable of replicating in culture. The results of our histology data reveal positive *S. neurona* antibody staining in the cerebellum regardless of treatment duration. The decrease in the number of gliosis foci and perivascular cuffs in the treated mice compared to untreated mice suggests that treatment decreases the severity of *S. neurona*-

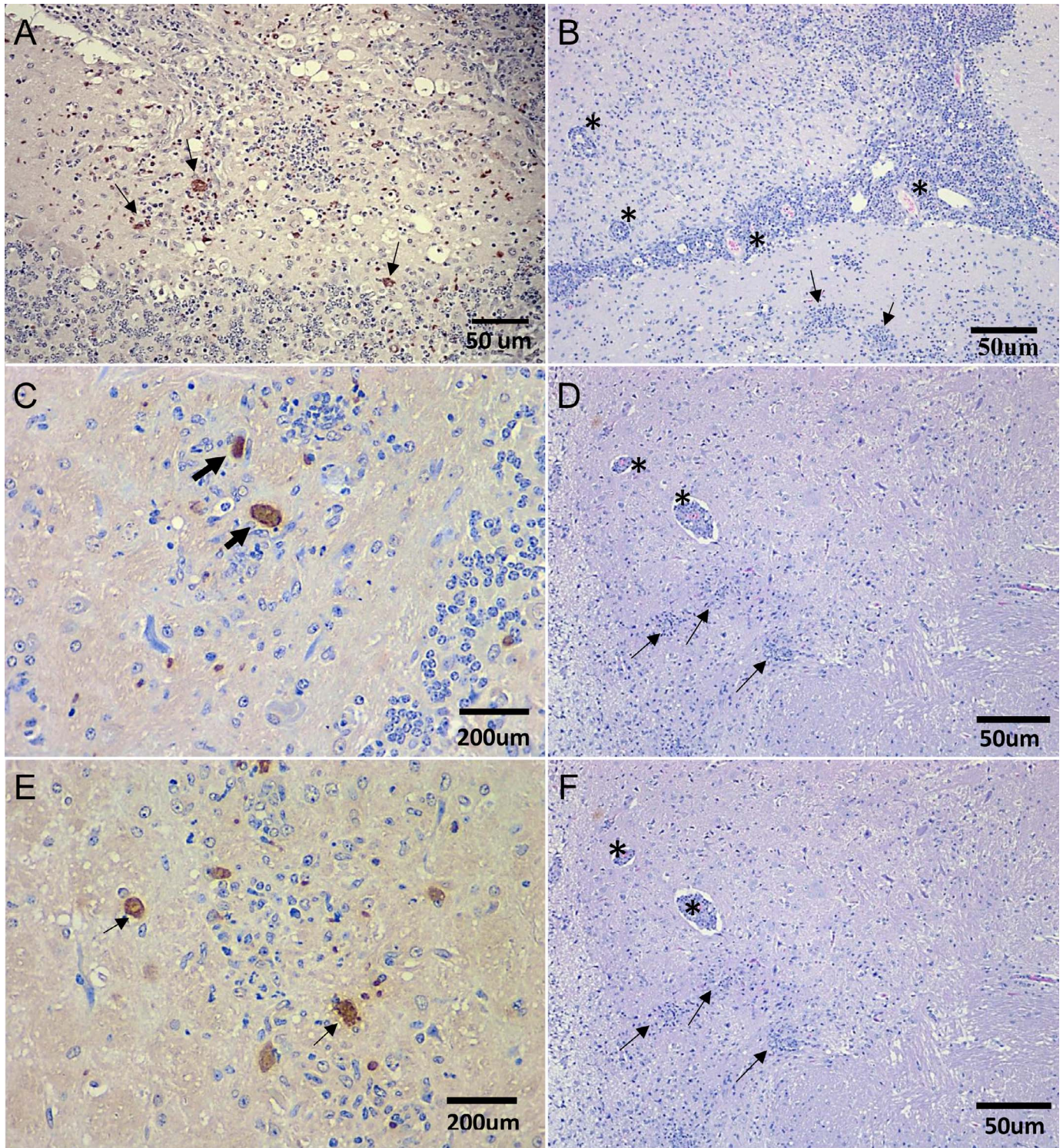


Figure 3. Cerebellar immunohistochemistry (IHC) and hematoxylin and eosin (H&E) images. All images are of cerebellar samples collected when neurologic signs were displayed by infected untreated mice (**A, B**) 30-day treated mice (**C, D**) and 60-day treated mice (**E, F**). (**A**) IHC image where the asterisk (*) indicates eosinophilic inflammation and arrows indicate *Sarcocystis neurona* asexual stages. (**B**) H&E image where asterisks (*) indicate eosinophilic inflammation and arrows gliosis + eosinophilic inflammation. (**C**) IHC image arrows and dark brown staining indicate *S. neurona* asexual life stages. (**D**) H&E stained asterisks (*) indicate perivascular cuffing and arrows indicate gliosis. (**E**) IHC image arrows and dark brown staining are *S. neurona* asexual life stages. (**F**) H&E staining asterisks (*) indicate gliosis and eosinophilic inflammation and arrows *S. neurona* asexual life stages. Color version available online.

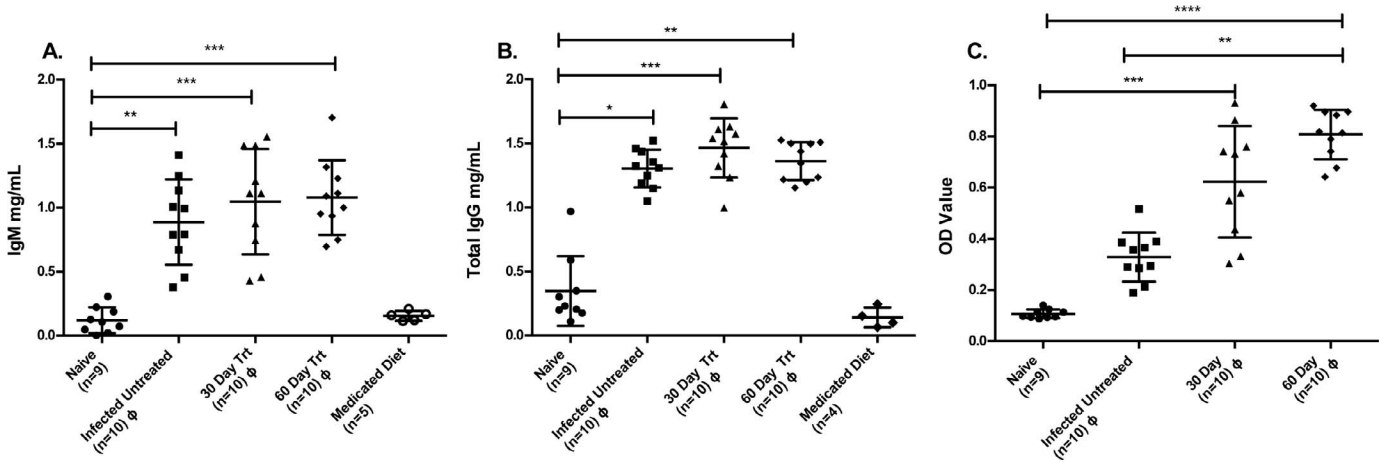


Figure 4. Sera immunoglobulin data. (A) Sera IgM concentrations. (B) Sera total IgG concentrations. (C) Optical density values of *Sarcocystis neurona* merozoite-specific antibodies. * $P \leq 0.05$, ** $P \leq 0.005$, *** $P \leq 0.0005$, **** $P \leq 0.0001$. Φ Sera sample collected from mice displaying neurologic signs.

associated lesions. It should be acknowledged that Dubey et al. (2001), administered diclazuril compounded rodent diet for 39 days, to *Ifn γ ^{-/-}* mice 7 days after infection with 1,000 *S. neurona* sporocysts from the isolate Sn-15OP and sacrificed mice 8 days after treatment. They found that 8 days after the cessation of treatment, cerebella subpassaged to naïve *Ifn γ ^{-/-}* mice were not pathogenic nor did cerebellum histology results show positive *S. neurona* antibody staining (Dubey et al., 2001). In the current study, cerebellum samples collected 12 days after 30 days of treatment from infected mice did not yield *S. neurona* in culture, suggesting that the brains were not yet parasitized at this point after treatment. We hypothesize that if treated infected mice in Dubey’s study were left alive for 60 days post-treatment, clinical disease may have developed, as in our study. Collectively, these results indicate *S. neurona* can resume its activity after cessation of diclazuril in vivo barring an adequate immunological response from the host.

While the immunologic response to *S. neurona* in *Ifn γ ^{-/-}* mice is not directly translatable to equine patients, quantifying the humoral response gives clues to immune awareness of parasitic infection. Extraneural *S. neurona* replication occurs 7 DPI (Dubey

et al., 2000), which allows for antigen recognition by naïve B-lymphocytes resulting in the modest elevation of sera IgM concentrations 7 DPI compared to the naïve mouse group. At the end of 30 days of diclazuril treatment, sera IgM concentrations declined from the beginning of treatment (7 DPI) similarly to those observed in the naïve mouse group, suggesting that few new naïve B-lymphocytes were encountering parasites during treatment. The resumption of active *S. neurona* infection and parasite replication and migration is probable after treatment was discontinued as indicated by the significant increase in sera IgM concentrations at the time of neurologic disease development. The significant increase in total IgG sera concentration throughout the course of treatment can be attributed to maturation of the initial immune response, with IgM B-lymphocytes switching isotypes to IgG through the process of class switch recombination. The significant increase in *S. neurona* antibody OD values at the time of neurologic sign also supports maturation of an immune response to *S. neurona* and relates the humoral immune response to *S. neurona* infection. Pusterla et al. (2015) demonstrated that foals residing in a high-risk EPM area treated with a low dose of diclazuril (0.5 mg/kg) had significantly less sera conversion of

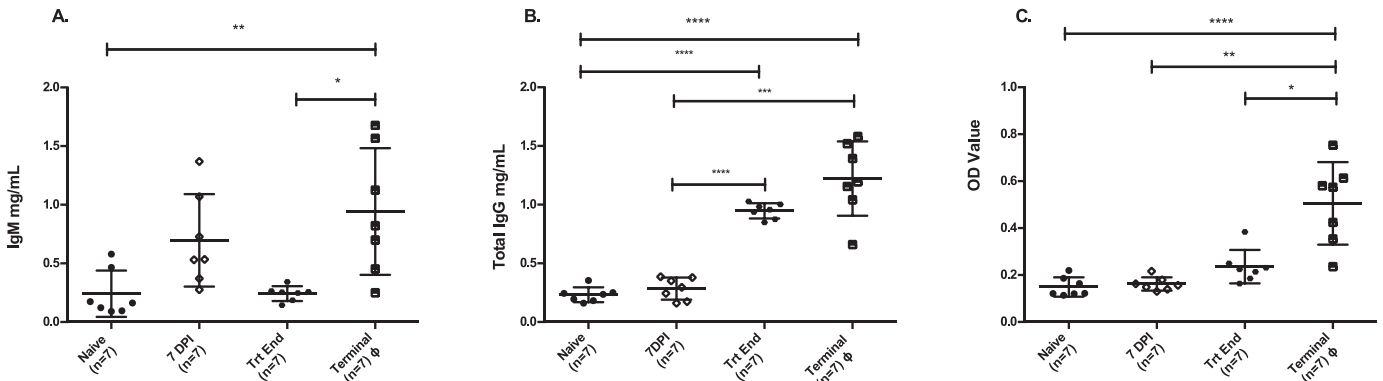


Figure 5. Sera immunoglobulin data during treatment and time points during treatment compared to naïve mice. Abbreviations: 7 DPI = 7 days post-infection; Trt End = at the end of 30 days of diclazuril treatment. (A) Sera IgM concentrations. (B) Sera total IgG concentrations. (C) Optical density values of *Sarcocystis neurona* merozoite specific antibodies. * $P \leq 0.05$, ** $P \leq 0.005$, *** $P \leq 0.0005$, **** $P \leq 0.0001$. Φ Sera sample collected at time of symptom development.

Table II. Cerebellum culture time point and organism retrieval. Abbreviation: trt, treatment.

	Cerebellum culture time point			
	End of 30-day trt	12 days post-30-day trt	Infected 30-day treated*	Infected untreated*
Live organism retrieval	0/3	0/3	1/2	2/2

* Cerebella collected from mice displaying neurologic signs.

antibodies against *S. neurona* compared to untreated foals following the decline of maternal antibodies. Furr et al. (2006) also demonstrated the prophylactic nature of the coccidiostat Ponazuril in decreasing the incidence and severity of experimentally induced EPM, assessed by sera and CSF antibodies and clinical neurologic signs. Murphy et al. (2006) experimentally infected horses with *S. neurona* and demonstrated significant increases in *S. neurona*-IgM concentrations in serum and CSF samples collected from experimentally infected horses. The horses in the mentioned study developed clinical neurologic disease as a result of the experimental *S. neurona* infection. While our study was performed in the immunocompromised mouse model and cannot be directly correlated to disease in the horse, the previously mentioned equine model studies by Furr et al. (2006), Murphy et al. (2006), and Pusterla et al. (2015) also support the efficacy of humoral immune response analysis. The sera immunoglobulin results from the current study also highlight the potential for evaluating *S. neurona*-specific IgM antibodies clinically in equine patients, before the start of anti-protozoal treatment, at the time of treatment discontinuation and periodically after to monitor for the development of recurrent infection.

Recurrent disease in horses may be a result of persistent infection due to treatment failure as opposed to re-exposure to parasite. Other EPM treatment efficacy studies demonstrate potential treatment failure that could lead to disease relapse. Furr et al. (2001) demonstrated that within 118 days after appropriate ponazuril treatment only 62% of horses in the study improved neurologically 1 grade, and 10% of treated horses had cerebral spinal fluid negative for *S. neurona* antibodies by 90 days post-treatment. In the same study, 8% of horses that displayed improvement after treatment demonstrated recurrent disease by 118 days post-treatment. Horses in the study were not housed in a manner to determine if recurrent disease could be attributed to re-exposure to *S. neurona* or persistent infection due to treatment failure, but it was hypothesized to be a result of treatment failure (Furr et al., 2001). Additionally, horses treated with sulfadiazine/pyrimethamine have a disease relapse rate of 25% (Fenger, 1998). To the best of the authors' knowledge, no studies have been done in horses that evaluate the disease relapse rate related to diclazuril treatment. In many cases, the discussed anti-protozoal drug coupled with an appropriate immune response in an otherwise normal horse is a successful treatment strategy for clinically affected patients, but for those patients that present with recurrent signs, other treatment strategies should be considered. The results of this study imply that diclazuril can inhibit *S. neurona* activity but not eliminate the parasite, providing evidence that recurrent disease could be a result of persistent infection and treatment failure rather than simple reinfection.

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