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Original Article

Toxoplasma gondii seroprevalence and association with equine protozoal myeloencephalitis: A case–control study of Californian horses

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ABSTRACT

While toxoplasmosis is not commonly considered a clinical disease of equines, previous seroprevalence studies have reported differing background rates of *Toxoplasma gondii* infection in horses globally. The objective of this study was to evaluate possible associations between *T. gondii* seroprevalence and clinical signs of equine protozoal myeloencephalitis (EPM) in horses. Using a case–control study design, 720 Californian horses with neurologic signs compatible with EPM were compared to healthy, non-neurologic horses for the presence of *T. gondii* antibodies (using indirect fluorescent antibody tests [IFAT]). *Toxoplasma gondii* seroprevalence among cases and controls was determined at standard serum cut-offs: 40, 80, 160, 320, and 640.

At a *T. gondii* titre cut-off of 320, horses with clinical signs compatible with EPM had 3.55 times the odds of a seropositive test compared to those without clinical signs ($P < 0.01$) when adjusted for covariates. When restricted to the autumn season and at the same titre cut-off, an EPM suspect horse had 6.4 times the odds of testing seropositive to *T. gondii*, compared to non-neurologic horses. The association between high *T. gondii* titres and clinical signs compatible with EPM is potentially reflective of toxoplasmosis in equines. Serologic testing of cerebrospinal fluid and isolation of *T. gondii* in EPM suspect cases should be considered. Future studies investigating the relationship between *T. gondii* and EPM are warranted.

Introduction

Toxoplasma gondii, an obligate intracellular apicomplexan parasite, is an important pathogen of both humans and warm-blooded animals. While toxoplasmosis is not commonly considered a disease of equines, previous seroprevalence assays have been conducted globally over the past decade to determine the potential risk of *T. gondii* in horsemeat destined for human consumption (Tenter et al., 2000; Pomares et al., 2011). These studies have produced differing background rates of *T. gondii* infection in horses, with seroprevalences ranging from >50% in Egypt (Ghazy et al., 2007; Shaapan and Ghazy, 2007) to 6% in the United States (Dubey et al., 2014). While there is no validated immunoassay test for detecting *T. gondii* antibodies in equines, several dilution cut-offs to determine positive tests have been used throughout the literature, ranging from 1:6 for the modified agglutination test (MAT; Paştiu et al., 2015) to 1:64 for the indirect fluorescent antibody test (IFAT; Fonseca de Araujo Valenca et al., 2015).

Riskfactors for clinical infections in equine protozoal myeloencephalitis (EPM), a neurologic disease caused by two other apicomplexan protozoal parasites (*Sarcocystis neurona* and *Neospora hughesi*), are currently unknown. A biological model that can reproducibly transition an asymptomatic infection to clinical disease has not yet been developed (Saville et al., 2000). Experimental studies to induce EPM in horses with severe combined immunodeficiency (SCID) did not progress to infections of the spinal cord with associated signs of EPM, although infection was confirmed by demonstrating the presence of antibody in visceral tissues, (Sellon et al., 2004). However, healthy, immunocompetent horses can develop clinical signs and tissue damage consistent with EPM (as confirmed post-mortem using immunohistochemistry). This seems to suggest that an immune-mediated inflammatory process may be responsible for parasitic migration to the site of clinicaldisease, i.e. the nervous system (Lewis et al., 2014).

In a recent study of Brazilian horses with high antibody titres for *T. gondii* (IFAT titres 64–1024), seropositive horses had elevated

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humoral and cellular immune responses compared to seronegative horses (Do Carmo et al., 2015). Considering a comparative species approach, polyparasitism in wild marine mammals has been shown to modulate the severity of protozoal encephalitis, most notably co-infections by *S. neurona* and *T. gondii,* (Gibson et al., 2011). To help elucidate a possible role for *T. gondii* in the progression of EPM, the present study used a case–control study design to test the hypothesis that horses with neurologic signs compatible with EPM would have higher antibody titres to *T. gondii* compared to horses with no neurologic signs.

Materials and methods

Case selection

Cases were defined as horses with at least one of the following clinical signs compatible with EPM: ataxia, muscle atrophy, head tilt, hypermetria, lameness, weakness, circling, mentation change. Sera were tested at the Immunology Laboratory of the William R. Pritchard Veterinary Medical Teaching Hospital (VMTH), School of Veterinary Medicine, University of California at Davis, for the presence of antibodies against *S. neurona* and *N. hughesi* using Sarcofluor (Conrad Protozoal Laboratory) and Neofluor (Conrad Protozoal Laboratory) IFAT during 2013. Study inclusion criteria included Sarcofluor and Neofluor serum results, availability of serum to be tested for the presence of *T. gondii*; horses must also have been resident in California. Equine health records contained breed, sex, use, and age data, but did not include any information on prior treatment for EPM. There were 5592 diagnostic submission requests for Sarcofluor and/or Neofluor from 1 January, 2013 to 31 December, 2013; this was the study population from which cases were selected based on study inclusion criteria.

Control horse selection

Controls were resident horses of California with no current neurologic signs during 2013. Otherwise, this population was comparable to the cases in terms of residence, availability of data on covariates, and distribution of demographic factors. This was originally a convenience sample of non-neurologic horses for a separate study of leptospirosis. Participating clinics enrolled voluntarily and were asked to sample approximately 110 horses/practice. A 10 mL blood specimen was drawn from the jugular vein, and a questionnaire was completed for each horse to collect data on age, breed, primary use, and sex. Information regarding prior treatment for EPM was not collected. Serum from 5250 horses was collected across 18 states in September and October 2013. This constituted the study population from which the study sample of controls was selected. The presence of antibodies against *S. neurona* and *N. hughesi* was determined as previously described (Sarcofluor and Neofluor IFAT; James et al., 2017).

Toxoplasma gondii *exposure*

To determine exposure status to *T. gondii*,*T. gondii* antibody titres were determined for cases and controls using IFAT, as previously described (Conrad et al., 1993; Miller et al., 2001). The *T. gondii* strain used in testing was a type 2 strain (ME49) available from American Type Cell Culture research foundation (ATCC). Tachyzoites were grown on monkey kidney cells (MA104) in 10% fetal bovine media, incubated at 37 °C. Slide fixation techniques included a 10 min fix with formalin, followed by a phosphate buffered saline wash. However, instead of anti-ferret IgG, anti-horse IgG (Jackson ImmunoResearch Laboratories) conjugated to fluorescein isothiocyanate (FITC) was used as the secondary antibody, diluted 1:100. Due to the lack of validated diagnostic testing for *T. gondii* in equines, each sample was end-titrated to generate quantitative titres of antibodies directed to *T. gondii.* Two investigators were blinded to case or control status during the reading of the IFAT slides and read the slides independently.

Statistical analysis

All statistical analyses and data management were performed using Stata Statistical Software: Release 14 (StataCorp). Demographic frequency tables for the case and control populations were created to compare populations. Demographic factors included breed, sex, age, and use. Breed was divided into Quarter horse, Warmblood, Thoroughbred, Paint horse, Arabian, Draft horse, Pony/miniature, and other. Age was analysed as a 5-year increment categorical variable. Sex was categorised into male (gelding and stallion) and female. Use of animal was as follows: competition animals, resident farm animals, breeding animals, or other uses.

Clinical factors, including *S. neurona* and *N. hughesi* seropositivity, were evaluated for each population. The *S. neurona* seropositive titre cut-off was set at 40; for *N. hughesi* a seropositive titre cut-off was set at 160. Bivariate logistic regression models between the demographic and potential co-infection factors with the clinical outcome were generated to determine possible covariates for a multivariable logistic regression model. Odds ratios (ORs; 95% confidence intervals, 95%CI) with $P < 0.05$ were considered statistically significant and were included in the multivariable logistic regression model in a forward stepping manner. Confounding and first order interaction of predictor variables was assessed for the clinical outcome in preliminary multivariable logistic regression models (Agresti, 2002).

Toxoplasma gondii seroprevalence was determined for case and control populations at multiple titre cut-offs. Due to the lack of a generally accepted IFAT titre cut-off for determining seropositivity in equines, seroprevalence at reciprocal titres of 40, 80, 160, 320, and 640 were determined. Unadjusted effect measures for the association of *T. gondii* exposure at these titre cut-offs and the clinical outcome were created (unadjusted ORs). A multivariable logistic regression model was then created with the statistically significant demographic explanatory variables and the *T. gondii* exposure at the titre cut-offs.

A further refinement of the statistical analysis included matching cases to the same season as controls to increase matching of the two groups. As described, all controls were selected in September and October 2013. In a secondary analysis, only cases with serum submitted to the UC Davis Immunology Laboratory from August to November were included, to investigate the effect of the autumn season. The demographic frequencies, bivariate associations, *T. gondii* exposure prevalences, and final multivariable logistic regression models were generated as described above.

Results

Primary analysis results: All year 2013 cases and controls

Of 5592 horses from the case population, 392 resided in California. Cases were not equally distributed throughout the calendar year and the majority of cases occurred in April–July ($n = 146$); however, this is not statistically different from August to November, in which there were 138 cases ($P > 0.05$). December–March had the fewest cases, with 105 cases total. Of the 5250 horses from the control population, 328 resided in California. Important risk factors from bivariate modeling ($P < 0.05$) were age and breed (Table 1), and were therefore included in the multivariable logistic regression model.

Clinical characteristics for *S. neurona* and *N. hughesi* seropositivity also differed between the case and control populations.