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Toxoplasma gondii-positive human sera recognise intracellular tachyzoites and bradyzoites with diverse patterns of immunoreactivity

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ABSTRACT

Antibody detection assays have long been the first line test to confirm infection with the zoonotic parasite *Toxoplasma gondii*. However, challenges exist with serological diagnosis, especially distinguishing between acute, latent and reactivation disease states. The sensitivity and specificity of serological tests might be improved by testing for antibodies against parasite antigens other than those typically found on the parasite surface during the acute stage. To this end, we analysed the reactivity profile of human sera, identified as positive for anti-*Toxoplasma gondii* IgG in traditional assays, by indirect immunofluorescence reactivity to acute stage intracellular tachyzoites and in vitro-induced latent stage bradyzoites. The majority of anti-*Toxoplasma gondii* IgG positive sera recognised both intracellularly replicating tachyzoites and in vitro-induced bradyzoites with varying patterns of immune-reactivity. Furthermore, anti-bradyzoite antibodies were not detected in sera that were IgM-positive/IgG-negative. These results demonstrate that anti-*Toxoplasma gondii*-positive sera may contain antibodies to a variety of antigens in addition to those traditionally used in serological tests, and suggest the need for further investigations into the utility of anti-bradyzoite-specific antibodies to aid in diagnosis of *Toxoplasma gondii* infection.

1. Introduction

Toxoplasmosis is a parasitic infection caused by the protozoan *Toxoplasma gondii*. Infection may be acquired through ingestion of infective oocysts shed in feline stool or tissue cysts in raw or undercooked meat of chronically infected animals. Infection may also be transmitted vertically by organ transplantation or in utero. During its life cycle, the parasite transitions between stages with differing replicative, metabolic, antigenic and transmissive properties (Dubey, 1998; Bohne et al., 1999; Weiss and Kim, 2000; Gross et al., 2004; Skariah et al., 2010). In the gastrointestinal tract of the definitive host (any feline), the parasite undergoes sexual replication with differentiation into macro- and microgametocytes, and the development of sporozoites in oocysts, which are shed in the faeces and are stable in the environment. After oocysts are ingested by an intermediate host (non-feline warm-blooded animal), sporozoites are released and the parasite develops into two forms: tachyzoite and bradyzoite. The tachyzoite is the rapidly replicating, pathogenic stage associated with manifestations of acute disease. Symptoms of acute infection are typically mild in the immune-competent host. However, immune-suppressed individuals are at risk of severe disease and fatal outcomes (Halonen and Weiss, 2013; Jones et al., 2014). Chronic infection occurs due to parasite stage conversion from tachyzoite to bradyzoite, a slowly replicative cell type which survives for long periods in the intermediate host. Bradyzoite conversion is induced by multiple factors including the host immune response, host cell type and metabolism, and the parasite strain (Fux et al., 2007; Ferreira-da-Silva et al., 2008; Weilhammer et al., 2012; Swierzy and Lüder, 2015). While humans can be infected with any of the three major stages (sporozoites, tachyzoites, bradyzoites), serological assays cleared by the United States Food and Drug Administration (FDA) target tachyzoite stage antigens (Boothroyd, 2009) (Supplementary Table S1).

Diagnostic methods for toxoplasmosis include isolation and cultivation of the parasite in an animal model or tissue culture, detection of a serological response to infection, histology and, more recently, molecular detection of parasite-specific nucleic acid (Jorgensen and Pfaller, 2015). Drawbacks to these methods include invasive procedures required to sample potentially infected tissue, the cost of maintaining animals or tissue cultures, and a lack of FDA approved tests for molecular detection. Thus, serological detection of parasite-specific antibodies is typically performed to test for exposure to the parasite, and a negative serological result is

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thought to exclude the possibility of infection (Montoya, 2002; Press et al., 2005).

A challenge related to serological diagnosis is the difficulty in distinguishing between acute and latent or chronic infection. This is important in guiding the treatment of pregnant women due to the risk of congenital transmission during acute infection. While IgM is typically associated with acute infection, it may not be detected in some acutely-infected individuals and may persist for long periods in others (Fricker-Hidalgo et al., 2013; Beal et al., 2014). Another drawback for IgM testing is the potential for false-positive results (Wilson et al., 1997; Garry et al., 2005). The FDA issued a public health advisory regarding *Toxoplasma* IgM commercial kits in 1997, which included recommendations for follow-up testing at a laboratory with specialised experience in *Toxoplasma* serological testing (Burlington, 1997). Avidity testing is currently recommended to aid in the timing of infection for IgG/IgM-positive pregnant individuals (Jorgensen and Pfaller, 2015). High avidity IgG, typically found in past infections, is useful for ruling out recent infection. However, low avidity IgG, which should be found only in acute or recent infection, has been found to persist long-term in some individuals; this confounds the clinical picture if a single sample is tested (Findal et al., 2015).

One of the major parasite antigens recognised by the human immune system is the surface protein SAG1/p30 (Kasper et al., 1983; Santoro et al., 1985). Accordingly, commercially available serology assays test for antibodies (IgG, IgM) to major surface antigens of the tachyzoite (Supplementary Table S1). Since *T. gondii* is an obligate intracellular pathogen, many antigens are produced during replication within the host cell and are exposed to the immune system upon host cell lysis. Whether antibodies against other antigens play a role in the humoral response against *Toxoplasma*, especially putative ones from intracellularly replicating tachyzoites and latent bradyzoites, is underexplored. To investigate this possibility, we tested human sera positive for anti-*T. gondii* antibodies by an immune-fluorescence assay for immune-reactivity to intracellularly replicating tachyzoites and in vitro switched bradyzoites. Our results demonstrate that anti-*T. gondii*-positive sera may contain antibodies to a variety of antigens in addition to those traditionally used in serological tests. The detection of antibodies to parasite structures found in host cells infected with tachyzoites and bradyzoites opens new avenues to investigation of the humoral response to *Toxoplasma* infection.

2. Materials and methods

2.1. Serum samples

Samples used in this study were remnants of human sera that had been tested at the Indiana University (IU) Health Pathology laboratory (IUHPL), United States for IgG to *T. gondii* by enzyme-linked fluorescent assay (ELFA) ($n = 89$ study samples of 818 clinical samples tested in 2014) (Vidas, bioMérieux, Durham, NC, United States and for IgM by an IFA ($n = 18$ study samples of 341 clinical samples tested) (Hemagen Diagnostics, Columbia, MD, United States) by routine laboratory protocols and stored at −20 °C. Two study specimens were positive for both IgG and IgM. Sample selection criteria were IgG and/or IgM positivity, availability of stored specimen and sufficient residual volume (≥0.25 ml). Clinical laboratory test results were recorded with patient age and gender, and samples were de-identified for further investigations. This study was granted exempt status upon protocol review by the Indiana University School of Medicine Institutional Review Board (IRB).

2.2. Host cell and parasite maintenance and reagents

Human foreskin fibroblasts (HFF, purchased from American Type Culture Collection (ATCC, Manassas, VA, United States) were grown in DMEM supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, and 100 units of penicillin/100 μg of streptomycin per ml, in a humidified incubator at 37 °C and 5% CO_2. Green monkey kidney cells (Vero) were grown under the same conditions. Parasite strains were maintained by passage through HFFs in normal culture medium and grown in Vero cells for all studies using human sera. All experiments were performed with *T. gondii* strain PRUΔC32 (Singh et al., 2002). This strain carries GFP under the control of the bradyzoite stage-specific *ldh2* promoter.

2.3. Immunofluorescence assays and western blots

For IFA, 1×10^4 PRUΔC32 tachyzoites were inoculated into Vero cells on coverslips for IFA. After 35 h of incubation, coverslips were fixed with 4% paraformaldehyde, and an IFA was performed using a routine laboratory protocol (Arrizabalaga et al., 2004). The primary antibody was the human serum (1:500 for IgG sero-positive specimens, 1:20 for IgM sero-positive specimens) and the secondary antibody was Alexa-fluor 594 conjugated goat anti-human IgG or anti-human IgM (1:2000 for both classes) (ThermoFisher Scientific, Waltham, MA, United States). DAPI (1:1000) was used to stain nuclear material. In vitro bradyzoites were induced with alkaline stress (RPMI pH 8.2) incubation for 4–5 days in an ambient-air incubator (Weiss et al., 1995) and IFA performed as for tachyzoites. Slides were examined in a zig-zag pattern on a Nikon microscope at 600× total magnification. For each serum sample tested, photomicrographs were taken from three fields of view with parasitic structures observable by phase contrast microscopy. Sera were classified as positive for anti-*Toxoplasma* antibody if specific signal was observed for parasite-associated structures as identified by phase contrast. Staining patterns were compared with previously published patterns for different parasite antigens: cyst wall (Tobin et al., 2010), matrix (Zhang et al., 1999), bradyzoite surface (Saeij et al., 2008), parasitophorous vacuole (PV) membrane (PVM) (Karsten et al., 1998), surface antigen 1 (SAG1), PV-internal structures/intravacuolar network (IVN) (Rome et al., 2008). For in vitro bradyzoites, only GFP-positive structures were imaged. The 4 μm scale bar was determined from the narrowest (non-dividing) tachyzoite or bradyzoite cell width (reported as approximately 2 μm (Dubey et al., 1998)) in each image and doubling the measurement.

Co-localisation experiments were performed with human serum and mouse anti-SAG1 (Genway Inc., San Diego, CA, United States), mouse anti-GRA7 (dense granule antigen 7, (Coppens et al., 2006)) for intracellular tachyzoites, and mouse anti-BAG1 (bradyzoite antigen 1, Weiss et al., 1992; McAllister et al., 1996) antibodies and *Dolichos biflorus* agglutinin (lectin that recognises cyst wall, Vectorlabs Inc., Burlingame, CA, United States).

For western blots, extracellular parasites were filtered, washed in PBS, and pelleted by centrifugation. The parasite cell pellet was lysed in SDS sample buffer with a final concentration of 5×10^7 tachyzoites/ml. Vero cells were trypsinised, centrifuged, washed with PBS, and lysed in SDS sample buffer at a final concentration of 5×10^6 cells/ml. Ten μl of each lysate were used for western blot per routine laboratory protocol (Garrison and Arrizabalaga, 2009); primary antibody was IgG sero-positive human serum or anti-SAG1-positive or -negative mouse serum (1:5000) and the secondary antibody was horseradish peroxidase (HRP)-conjugated goat anti-human IgG or Goat anti-mouse IgG (1:20,000) (Thermo Fisher Scientific). Chemiluminescent substrate (SuperSignal West Femto Substrate, Thermo Fisher Scientific) was applied to the