HHS Public Access

Author manuscript

J Infect Dis. Author manuscript; available in PMC 2017 December 07.

Published in final edited form as:

J Infect Dis. 2010 October 15; 202(8): 1226–1233. doi:10.1086/656397.

Identification of an Atypical Strain of *Toxoplasma gondii* as the Cause of a Waterborne Outbreak of Toxoplasmosis in Santa Isabel do Ivai, Brazil

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Abstract

Multilocus DNA sequencing has identified a nonarchetypal strain of *Toxoplasma gondii* as the causal agent of a waterborne outbreak in Brazil in 2001. The strain, isolated from a water supply epidemiologically linked to the outbreak, was virulent to mice, and it has previously been identified as BrI. Using a serologic assay that detects strain-specific antibodies, we found that 13 (65%) of 20 individuals who were immunoglobulin (Ig) M positive during the outbreak possessed the same serotype as mice infected with the purported epidemic strain. The remaining 7 individuals, plus additional IgM-negative, IgG-positive individuals, possessed 1 of 4 novel serotypes, the most common of which matched the serotype of mice infected with strains isolated from chickens foraging near the outbreak site. The latter strains likely reflect the genetic diversity of *T. gondii* circulating in highly endemic regions of Brazil. The serotyping assay proved a useful tool for identification of specific individuals infected with the outbreak agent.

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The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the US Department of Health and Human Services, the National Institutes of Health, or the Centers for Disease Control and Prevention.

Potential conflicts of interest: none reported.

Between October and December 2001, an outbreak of human toxoplasmosis occurred in the town of Santa Isabel do Ivai, Brazil [1]. The outbreak was epidemiologically linked to a cistern that served as a municipal water supply, and it was characterized by a high prevalence of symptomatic, systemic disease [1] and associated eye involvement (C.S., C.M., J.L.J., R.B., and G.N.H., unpublished data). IgM antibody evidence of infection was found in 426 people.

It has been hypothesized that the genotype of the parasite may contribute to variations in the prevalence and severity of *Toxoplasma gondii* infections in different populations [2, 3]. Preliminary study of the Santa Isabel outbreak identified a “type I” lineage allele at the SAG2 genotyping locus for parasites isolated from the local water supply [1]; however, most strains in Brazil are nonarchetypal and genetically distinct from the clonal type I, II, and III strains common to North America and Europe [4]. Consequently, the genotype analysis performed using the single genetic marker most likely failed to capture the true genetic “type” of the agent thought to be associated with the epidemic. The single marker was also insufficient to resolve whether 3 isolates linked to the epidemic point-source possessed the same genotype. Furthermore, no test was performed on symptomatic people infected during the outbreak to establish whether these studied strains were the true source of infection in humans.

Polymerase chain reaction (PCR) analyses have been used successfully in the past to identify the genotypes of strains infecting people, but this technique requires isolation of parasite DNA from host body fluids or tissues. The morbidity associated with specimen collection usually precludes such determinations. In the absence of parasite DNA from individuals infected during the Santa Isabel outbreak, serotyping was performed to test whether isolates epidemiologically linked to the outbreak were the causal agents. The serologic assay identifies strain-specific antibodies against polymorphic epitopes in the *T. gondii* dense granule proteins GRA6 and GRA7, without requiring isolation of parasite DNA [5]. Specifically, we compared serologic profiles obtained from individuals infected during the outbreak with serologic profiles from mice experimentally infected with epidemic strains isolated from the contaminated water supply, to confirm that isolates epidemiologically linked to the outbreak were responsible for the infections in humans [1].

METHODS

Genotyping analysis

A *T. gondii* isolate was recovered from a cat living near the cistern implicated in the outbreak [1, 6, 7]; this isolate (TgCatBr85) previously was mistakenly designated by Dubey et al. [7] as TgCatBr38. Two isolates were obtained from pigs fed filters through which water from the cistern had been passed. Isolates were also obtained from 11 free-ranging chickens foraging in the vicinity of the cistern shortly after the outbreak. Strains isolated from chickens are believed to reflect the diversity of genotypes naturally circulating in an area where the disease is endemic [6].

Multilocus PCR DNA sequencing was performed on genomic DNA from each isolate at the B1, GRA6, and GRA7 loci.Primer sequences and PCR reaction conditions for B1, GRA6,

and GRA7 have been described elsewhere [8–10]. PCR products were incubated for 15 min with ExoSAP-IT (USB) before DNA sequencing. DNA sequencing was performed by the RML Genomics Unit.

Mouse infection studies

A total of 5 *T. gondii* isolates recovered from chickens foraging near the outbreak site were tested against the epidemic strain by infecting mice; serum samples obtained from infected mice at 17 days after infection were tested using the serotyping assay to determine serologic profiles for each mouse.

Serologic analysis

In July 2003, serum samples (labeled B21-B40) were collected from 20 individuals in Santa Isabel who had anti–*T. gondii* immunoglobulin (Ig) M antibodies and symptomatic toxoplasmosis during the outbreak [1]. Specimens were anonymized before serologic testing and were provided without corresponding clinical data about disease manifestations, in compliance with regulations associated with the public health investigation.

Also in 2003, potential control samples (samples B1–B20) were collected from 20 additional individuals who were seronegative for anti–*T. gondii* antibodies during the outbreak. Two samples (B5 and B10) possessed anti–*T. gondii* IgG antibodies when they were retested using a commercial immunofluorescence assay (IFA; General Biometrics) and 2 in-house assays, utilizing either recombinant SAG1 (rSAG1) or *Toxoplasma*-solubilized tachyzoite antigen preparation to detect anti–*T. gondii* IgG antibodies. These 2 samples presumably represent individuals infected with *T. gondii* during the 18 months after the outbreak. Thus, there were 22 seropositive (20 outbreak-associated samples and 2 other types) and 18 seronegative samples.

This study was conducted in accordance with the tenets of the Declaration of Helsinki. Because specimens were collected from volunteers during a public health emergency, the study was not subject to institutional review board approval by the Federal University of São Paulo. Testing of anonymized serum samples was considered to be exempt from institutional review board approval at the University of California, Los Angeles; the US Centers for Disease Control and Prevention; and the National Institutes of Health.

Peptide-based enzyme-linked immunosorbent assay

The GRA6 and GRA7 strain-specific polymorphic peptides were coupled to keyhole limpet hemocyanin (KLH; Biosource), as described elsewhere [5]. Peptide names were abbreviated accordingly, with “6” denoting peptides from GRA6; “7” indicating peptides from GRA7; “I/III” or “II” denoting the peptide epitope allele; and “d” indicating a truncated version of the diagnostic peptide. Coupled peptides were diluted to 2 micrograms per milliliter in 0.1 mol/L carbonate buffer, pH 8.5. The assay was performed as described elsewhere [5] and was summarized in Table 1, which appears only in the electronic version of the *Journal*. Enzyme-linked immunosorbent assay (ELISA) data were presented as an optical density (OD) index by dividing the OD value obtained at 405 nm for each of the 5 serotyping peptides (6-I/III, d6-I/III, 6-II, d6-II, and 7-II) by the mean of OD readings for 2 control

peptides with results expressed as arbitrary units. Serum samples obtained from unrelated individuals infected with *T. gondii* for whom the genotype of the infecting parasite was known were included in each experiment as positive controls.

Determination of threshold values

Threshold values (above which normalized assay values were considered to denote positive results) were determined by averaging the normalized OD ratio from 16 of the 18 immunofluorescence assay–negative samples and adding 2 standard deviations (SDs). The other 2 samples (B14 and B15) showed strongly false-positive reactions against single diagnostic peptides: B14 with peptide 6-I/III and B15 with peptide d6-I/III. Neither sample had a positive result, according to our 3 screening assays (immunofluorescence assay, rSAG1, and solubilized tachyzoite antigen) for anti–*T. gondii* IgG antibodies. False reactivity occurred in <2% of samples for each of the 5 diagnostic peptides, as determined for a cohort of 115 seronegative individuals throughout North and South America (E.R.J. and M.E.G., unpublished data).

RESULTS

Strain characterization

In Brazil, the prevalence of *T. gondii* infection is high, and the population genetic structure for this parasite is highly diverse. To determine whether the waterborne outbreak was caused by a single, epidemic clone, parasite DNA was obtained from 3 isolates (designated outbreak 1, 2, and TgCatBr85) linked to the outbreak source. To interrogate the diversity of strains endemic to Santa Isabel, parasite DNA isolated from 11 chickens (TgCkBr93–TgCkBr103) foraging nearby the outbreak was tested. DNA sequences obtained at the highly polymorphic B1, GRA6, and GRA7 genotyping loci were aligned against archetypal type I, II, and III strain alleles (Figure 1). At B1, 2 alleles were identified that were equally distributed among the 14 specimens analyzed. An archetypal type I and a unique allele were detected, but the latter was closely related to a type I allele and consequently was given the designation U_I (for type I–like). At GRA7, 3 alleles were identified. Two were archetypal (I or III), and the other was unique, differing from the type III allele at 2 polymorphic sites (nucleotides 168 and 436); it was designated U_{III}. At GRA6, 4 alleles were present. Three novel alleles were detected that diverged substantially from archetypal alleles; as such, they were designated u-1, u-2, and u-3, respectively. Intriguingly, a canonical type II allele was identified at GRA6 in 3 isolates, suggesting that type II strains have introgressed with indigenous strains circulating in this region of Brazil.

The 3 isolates linked to the outbreak (outbreak 1, outbreak 2, and TgCatBr85) were genotypically identical according to multilocus DNA sequencing; all possessed the same alleles across 3 highly polymorphic typing loci, indicating a common, clonal origin for these epidemic strains (Table 2). Previous PCR–restriction fragment–length polymorphism (RFLP) genetic analyses performed on chicken isolates from Santa Isabel [11] identified 4 isolates (TgCkBr98, TgCkBr101, TgCkBr102, and TgCkBr103) that possessed the same alleles as the outbreak strains at B1, GRA6, and GRA7, and they were identical genotypically (ie, they shared the same inheritance pattern of alleles) across an additional 9

loci. This finding supports the conclusion that the outbreak genotype was nonarchetypal, clonal, and the dominant type recovered among animals foraging near the reservoir. This Brazilian clonal type has previously been designated BrI [11]. Furthermore, for the remaining 7 chicken isolates, 6 additional atypical genotypes were identified, confirming that a vast array of genetically heterogeneous strains indigenous to this endemic niche was circulating at the time of the outbreak (Table 2).

Experimental infection and serotyping analysis

To determine whether the purported outbreak isolate was responsible for the waterborne epidemic, Swiss-Webster mice were experimentally infected with the outbreak clone, and then the resulting mouse serotype was compared with the serotypes for people infected during the outbreak. The serologic assay identifies strain-specific IgG antibodies and previously has been successfully applied against both mice and human samples [5, 12]. As controls, mice were infected with archetypal type I and II strains; a chicken isolate (TgCkBr98) that possessed the outbreak clone BrI genotype; and 4 other chicken isolates that each possessed novel, nonarchetypal genotypes indigenous to the Santa Isabel area.

All parasite strains recovered from the Santa Isabel region were virulent; mice typically died within 20 days of inoculation with as few as 50 tachyzoites (data not shown). Serum collected at day 17 from mice infected with the outbreak clone (outbreak 1), as well as with the chicken isolate that possessed the identical multilocus sequence type (TgCkBr98), reacted only with the 6-I/III epitope. IgG reactivity was not dependent on the allele present at GRA6 (u-1) but, rather, on the epitope sequence. The outbreak clone possessed the GRA6 epitope identical to that expressed by type I strains, and both yielded a type I/III serotype in the assay (Table 3). The GRA6 epitope in strain TgCkBr97 had one polymorphism (an arginine-to-histidine substitution); however, serum antibodies to infection still reacted specifically with the 6-I/III peptide. For the other non-archetypal strains TgCkBr94 and TgCkBr100 (which possessed type II alleles at GRA6), antibodies in the infection serum reacted specifically with the 6-II and 6 d-II peptides. These 2 chicken isolates did not react with the 7-II epitope, presumably because these 2 strains did not possess a type II allele sequence at GRA7 (shown by DNA sequencing). Intriguingly, strain TgCkBr95, which possessed a novel GRA6 epitope sequence LHPGSVNVFDY (containing neither the type I/III “ER” nor the type II “E” diagnostic polymorphisms), produced antibodies that reacted to both the type I/III and type II diagnostic peptides. The most parsimonious explanation for this result is that the GRA6 epitope is immunodominant and that antibodies induced by TgCkBr95 infection reacted against the nonpolymorphic residues shared in the 6-I/III and 6-II epitopes. Hence, the epitope expressed by the infecting strain ultimately determined the reactivity against the diagnostic peptides, not the genotype of the infecting strain.

To test our hypothesis, serum samples from the 20 IgM-positive individuals believed to be infected during the epidemic, the two other individuals believed to have subsequent endemic disease, and the 16 seronegative control individuals were tested using the strain-typing assay, to compare serologic profiles obtained from outbreak-infected individuals with those from mice infected experimentally with (1) the epidemic clone isolated from the contaminated water supply, or (2) indigenous strains circulating in Santa Isabel. Hence, 22 *T. gondii*–

positive samples were tested against the polymorphic peptides, and the serotype(s) of the strain(s) associated with the outbreak in humans was determined. A type II–positive control sample established that the assay was reproducible and was working within the detection and cutoff limits, as shown in Table 4 [5]. This outbreak was also particularly remarkable for the high antibody titers achieved, compared with previously tested samples from North America and Europe [5, 12]. Assay values of ≥7.0 at 6-I/III were achieved by 11 samples, which is unprecedented.

Table 4 shows serotyping assay results for individuals believed to be infected in the outbreak; all values at or above the cutoff values are denoted by references to footnote *c* in the table. The serologic profile of mice infected with the outbreak clone was identical to the dominant serotype of individuals infected during the epidemic. Strong reactivity only at the 6-I/III peptide was possessed by 13 (65%) of 20 people. No type II serologic profiles were identified. Analysis of the remaining seropositive samples revealed a unique collection of serologic signatures (Figure 2). In 3 (17%; B21, B23, and B33) of 20 samples, the infecting strain type could not be predicted on the basis of assay results, because only weak or no reactivity against the serotyping peptides was detected. Four additional samples (B22, B30, B31, and B36) showed no allele specificity, with high reactivity to both type-specific GRA6 serotyping peptides but no reactivity to the GRA7-derived 7-II peptide. Because mixed infections are highly unusual, this latter serotype suggests infection with a nonarchetypal strain equivalent to that found in mice infected with strain TgCkBr95, which was indigenous to the Santa Isabel environs. One sample (B26) showed consistently high reactivity across the entire panel of serotyping peptides, which may also suggest infection with a nonarchetypal strain. One of the postoutbreak seropositive samples (B10) strongly reacted to both 6-I/III and 7-II, indicating yet another unique serologic signature indigenous to the area. In all, 5 serotyping patterns emerged for infected individuals. For 6 (27%) of the total 22 samples, the different combinations of reactivity with the type II and type I/III diagnostic peptides suggested either (1) the possibility of mixed infections, or (2) infections with atypical strains that possessed cross-reacting alleles different from the 3 archetypal genotypes, consistent with the diversity of endemic strains identified to be circulating in animals in Santa Isabel at the time of the outbreak.

DISCUSSION

This study was undertaken to determine whether *T. gondii* strains recovered from the water supply implicated as the source of the Santa Isabel epidemic were identical genetically and were responsible for the human disease outbreak. Because no parasites were recovered from infected individuals, no direct determination of the responsible strain type(s) could be performed. We therefore used a serologic strain-typing assay to determine “serotypes” for a group of such individuals. There was a dominant serotype in the majority (65%) of individuals, which was indistinguishable from the serotype found in serum from mice that had been infected with isolates from the implicated water supply. Furthermore, the outbreak strains were genotyped at multiple polymorphic loci and found to be clonotypic and nonarchetypal. Hence, our data support prior evidence that the municipal cistern was the point source of a clonal outbreak and that the causal agent possessed a novel genotype.

It is believed that the cistern was contaminated by feral cats [1]. The water was chlorinated before consumption but not filtered. Drinking unfiltered water has been identified as a risk factor for *T. gondii* infection in Brazil [13, 14], but the reason for the severity of this outbreak remains enigmatic. Current evidence suggests that strain type may influence the severity of toxoplasmosis in immunocompetent hosts [2, 15, 16]. Type I or recombinant strains that possess different combinations of type I and type III alleles (like those found in Brazil) have been associated with severe ocular toxoplasmosis in people [16]. Although it was not our purpose to identify the clinical manifestations of infection with the outbreak strain, it would be useful to perform a follow-up study that investigates the relationship between disease severity and strain type.

Previous studies reported the outbreak strain to be type I by PCR-RFLP analysis performed using a single genetic marker [1, 7]; however, strains in Brazil do not fit the clonal pattern prevalent in Europe and North America, and extensive testing at multiple genetic loci, followed by DNA sequencing, is necessary to resolve a true genotype. Multilocus PCR-RFLP analyses have shown that type I strains are rare in Brazil. Among 149 chicken isolates examined by this technique, only one type I strain was identified, in a chicken from southern Brazil [11]. No type I isolates have been recovered from cats and chickens in the environs of Santa Isabel; instead, there is a rich diversity of nonarchetypal strains [11, 17, 18].

Our data show that the nonarchetypal outbreak clone was also the dominant genotype infecting local chickens (4 [36%] of 11 isolates) at the time of the outbreak (Table 2). More recent multilocus PCR-RFLP analyses have shown a decreasing prevalence of the outbreak clone. In 2003, it was isolated from only 5 (18%) of 28 cats; in 2006, it was isolated from only 1 (7%) of 15 chickens [7, 17]. In both studies, new genotypes had replaced the outbreak clone as the dominant genotype. In this situation, single-locus determinations can fail to provide an accurate description of the population genetic structure. A greater level of genetic resolution is required to derive the informative associations pertaining to the evolution, expansion, and dynamics of potential epidemic clones that cause disease in high transmission zones, such as Brazil, where there is an abundance of genetically diverse, nonarchetypal strains.

Nonarchetypal strains found in Brazil share alleles with type I, II, and III strains, despite the fact that these archetypal lineages are rare in Brazil [11, 19]. This fact prompted us to test whether a serotyping assay, which captures antibodies restricted to key polymorphic amino acids present in diagnostic GRA6 and GRA7 epitopes, might be able to identify accurately those individuals infected with the outbreak strain. DNA sequencing of the outbreak isolates identified a divergent GRA6 allele; however, the 12-amino-acid sequence containing the GRA6 diagnostic epitope was identical to that of type I and III strains, which was sufficient to produce a type I or III strain serotype when mice were infected with the outbreak strain. This serotype was also identified in the majority of people infected during the outbreak, clearly demonstrating that it is the epitope, not the underlying allele or genetic type, that determines the specificity of the serotyping assay. Taken together, the data provide compelling evidence that the isolates recovered from the water supply were the agents responsible for causing the outbreak.

We also identified 4 additional, nonarchetypal serologic profiles among the other 7 human samples associated with the outbreak (Figure 2). We interpret these unique serologic "signature" profiles as being diagnostic in their own right, the result of infection with genetically distinct *T. gondii* genotypes, a variety of which were identified in chickens and cats from Santa Isabel. Atypical strains possessing different epitopes at GRA6 and GRA7 generally induce antibodies that either do not react or cross-react with nonpolymorphic amino acids present in the diagnostic epitopes [20]. These signatures suggest that the 7 individuals may have been infected before the outbreak (with systemic symptoms caused by some other, unrelated disease process) or at the time of the outbreak, but from a different source.

Another possible, but unlikely, explanation for the unusual serologic results is sequential, mixed infections with 2 genetically distinct strains (assay results would reflect the presence of antibodies against both strains). Such mixed infections have been reported in cases of human toxoplasmosis [21, 22], but they remain rare. The mouse infection experiment utilizing TgCkBr95 supports a cross-reaction against nonpolymorphic amino acids as the likely cause for the nonarchetypal serotypes. It is also possible that the cistern was contaminated with more than one *T. gondii* strain. Without isolation and genetic characterization of the strains giving rise to specific serotypes from different individuals, further clarification is impossible. Peptides restricted to diagnostic polymorphisms contained in epitopes from indigenous strains will be promising reagents for future studies attempting to track the epidemiological dynamics of nonarchetypal strains that emerge in areas like Brazil.

In summary, our results support epidemiologic evidence that a contaminated cistern was the source of the Santa Isabel epidemic, and that a nonarchetypal parasite, designated BrI, was the causal agent. The identification of a dominant serologic profile in the context of 4 additional serologic profiles indicates that a heterogeneous group of unique or divergent *T. gondii* genotypes was circulating in this endemic niche. The 4 additional serotypes reflect infection, unrelated to the outbreak, with other indigenous strains circulating in animals at the time of the outbreak. Our findings highlight the potential difficulty of studying an outbreak superimposed on a disease that is otherwise endemic. The strain-typing assay identified those specific individuals infected with the outbreak agent, and it may be a useful epidemiologic tool for future outbreaks that occur in areas having a large genetic diversity of parasites.

Acknowledgments

Financial support: This work was supported by the Intramural Research Program of the National Institutes of Health and the National Institute of Allergy and Infectious Diseases (grant AI001018 to M.E.G.), the US Department of Agriculture (Agricultural Research Service, Current Research Information System [CRIS] Project 1265-32000-076 to J.P.D.), the US Centers for Disease Control and Prevention (contract to J.D.V., G.N.H., and J.L.J.), Fonds National Suisse pour la Recherche Scientifique (PBLA33102339 to J.D.V.), Research to Prevent Blindness (RPB; support to G.N.H.), Conselho Nacional de Desenvolvimento Científíco e Tecnológico (CNPq I-A Investigator Grant to C.M., C.S., and R.B.), the Skirball Foundation, (support to G.N.H.), and the Jack H. Skirball Endowed Professorship (to G.N.H.). G.N.H. is the recipient of an RPB Physician-Scientist Award. M.E.G. is a scholar of the Michael Smith Foundation for Health Research and the Canadian Institute for Advanced Research Program for Integrated Microbial Biodiversity.

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A

B1	366	413	504	
Consensus	C/T	C	C/G	Allele
RH	T	.	G	I
76K	.	.	.	II
CEP	.	.	.	III
TgCkBr 93	T	C/T	G	U_I
TgCkBr 94	T	C/T	G	U_I
TgCkBr 95	T	C/T	G	U_I
TgCkBr 96	T	C/T	G	U_I
TgCkBr 97	T	C/T	G	U_I
TgCkBr 98	T	.	G	I
TgCkBr 99	T	C/T	G	U_I
TgCkBr 100	T	C/T	G	U_I
TgCkBr 101	T	.	G	I
TgCkBr 102	T	.	G	I
TgCkBr 103	T	.	G	I
Outbreak1	T	.	G	I
Outbreak2	T	.	G	I
Outbreak3	T	.	G	I

B

GRA7	101	168	394	421	436	450	556	559	577	587	594	604	622	631	659	673	679	683	737	744	763	769	
Consensus	T	A	C	A	C	A	A	C	C	C	C	A	A	T	C	A	C	C	T	A	G	G	Allele
RH	C	.	.	.	.	G	C	G	G	G	.	.	G	.	.	.	.	.	.	.	.	.	I
76K	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	A	.	II
CEP	.	G	G	C	A	.	.	.	.	.	T	.	A	T	C	A	A	C	C	.	A		III
TgCkBr 95	.	.	G	C	.	.	.	.	.	.	T	.	A	T	C	A	A	C	C	.	A		U_{III}
TgCkBr 96	.	G	G	C	A	.	.	.	.	.	T	.	A	T	C	A	A	C	C	.	A		III
TgCkBr 97	.	.	G	C	.	.	.	.	.	.	T	.	A	T	C	A	A	C	C	.	A		U_{III}
TgCkBr 98	C	.	.	.	.	G	C	G	G	G	.	.	G	.	.	.	.	.	.	.	.	.	I
TgCkBr 99	.	.	G	C	.	.	.	.	.	.	T	.	A	T	C	A	A	C	C	.	A		U_{III}
TgCkBr 100	.	.	G	C	.	.	.	.	.	.	T	.	A	T	C	A	A	C	C	.	A		U_{III}
TgCkBr 101	C	.	.	.	.	G	C	G	G	G	.	.	G	.	.	.	.	.	.	.	.	.	I
TgCkBr 102	C	.	.	.	.	G	C	G	G	G	.	.	G	.	.	.	.	.	.	.	.	.	I
TgCkBr 103	C	.	.	.	.	G	C	G	G	G	.	.	G	.	.	.	.	.	.	.	.	.	I
Outbreak1	C	.	.	.	.	G	C	G	G	G	.	.	G	.	.	.	.	.	.	.	.	.	I
Outbreak2	C	.	.	.	.	G	C	G	G	G	.	.	G	.	.	.	.	.	.	.	.	.	I
Outbreak3	C	.	.	.	.	G	C	G	G	G	.	.	G	.	.	.	.	.	.	.	.	.	I

C

GRA6	41	71	106	162	170	171	304	336	403	412	561	576	614	635	636	637	638	639	640	641	642	643	644	645	646	647	648	649	658	659	660	661	671	677	690	692	693	702	711	
Consensus	C	T	C	G	A	A	T	T	C	A	A	A	G	T	A	C	G	G	A	G	G	C	A	G	A	G	G	T	A	G	G	C	A	C	A	C	G	T	A	Allele
RH	.	G	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	I
76K	T	.	.	.	G	.	C	.	.	.	G	.	.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C	-	-	.	.	G	A	.	A	T		II
CEP	.	.	T	A	.	.	.	.	.	.	C	.	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	.	-	-	.	G	.	.	.	.	.		III
TgCkBr 93	T	.	.	.	G	.	C	.	.	.	G	.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C	-	-	.	.	G	A	.	A	T		II
TgCkBr 94	T	.	.	.	G	.	C	.	.	.	G	.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C	-	-	.	.	G	A	.	A	T		II
TgCkBr 95	.	.	.	A	.	.	.	.	.	.	C	.	.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	.	.	.	.	G	G	A	.	.	.		u-2
TgCkBr 96	.	.	.	A	.	.	.	.	.	.	C	.	.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	.	.	.	.	G	G	A	.	.	.		u-2
TgCkBr 97	.	.	.	.	.	.	.	T	G	C	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	A	.	.		u-3
TgCkBr 98	.	.	.	C	.	.	.	.	.	C	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	G	.	.	.	.	.		u-1
TgCkBr 99	.	.	.	C	.	.	.	.	.	C	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	G	.	.	.	.	.		u-1
TgCkBr 100	T	.	.	.	G	.	C	.	.	.	G	.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C	-	-	.	.	G	A	.	A	T		II
TgCkBr 101	.	.	.	C	.	.	.	.	.	C	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	G	.	.	.	.	.		u-1
TgCkBr 102	.	.	.	C	.	.	.	.	.	C	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	G	.	.	.	.	.		u-1
TgCkBr 103	.	.	.	C	.	.	.	.	.	C	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	G	.	.	.	.	.		u-1
Outbreak1	.	.	.	C	.	.	.	.	.	C	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	G	.	.	.	.	.		u-1
Outbreak2	.	.	.	C	.	.	.	.	.	C	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	G	.	.	.	.	.		u-1
Outbreak3	.	.	.	C	.	.	.	.	.	C	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	G	.	.	.	.	.		u-1

Figure 1.

Polymorphic sites at the B1 (*A*), GRA7 (*B*), and GRA6 (*C*) loci among archetypal lineages were compared with sequences obtained from the 3 outbreak isolates and 11 strains recovered from chickens grazing in the vicinity of the outbreak in Parana State, Brazil. Consensus sequences were determined by the nucleotide common in at least 2 of the 3 archetypal lineages. Periods (.) denote agreement with the consensus sequence. Dashes (−) denote insertions and deletions (INDELS) in the nucleotide sequence. Nucleotide positions were determined based on the published GenBank sequences for *Toxoplasma gondii* at the B1 gene (AF179871), GRA7 gene (Y13863.1), and GRA6 gene (AF239283). The first 3 rows contain sequences for archetypal strains I (RH), II (76K), and III (CEP), obtained from laboratory stock; TgCkBr (rows 4–12) designates isolates from chickens in Brazil; outbreak strains (last 3 rows) were obtained from filtered water from a cistern that was epidemiologically linked to the outbreak (strains 1 and 2) and a cat living near the cistern (strain 3). U_I and U_{III} denote novel alleles that are closely related to types I and III, respectively; u-1, u-2, and u-3 denote novel alleles that are related to, but substantially divergent from, types I, II, and III, respectively.

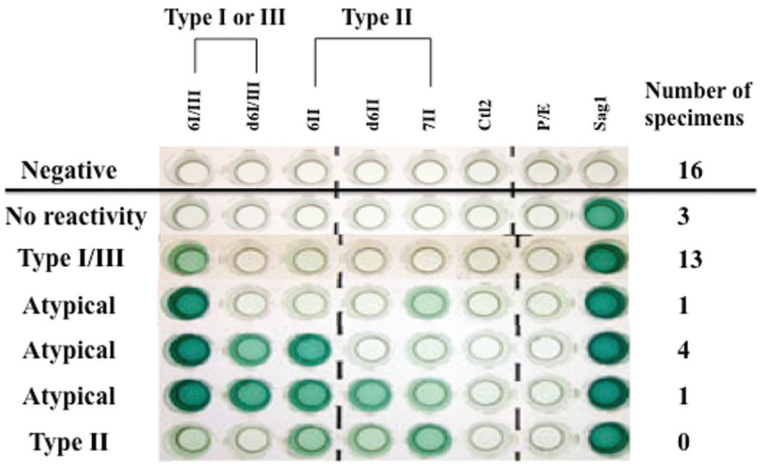

Figure 2.

An enzyme-linked immunosorbent assay plate depicting the 5 distinct serotype patterns identified. The majority of samples exhibited a reactivity pattern consistent with infection by a non–type II strain (labeled “type I/III”), which supported epidemiological evidence. Three additional patterns (labeled “atypical”) were suggestive of infection by nonarchetypal strains, as evidenced by high reactivity to more than 1 allele. In 3 cases, no peptide reactivity was detected, although infection with *Toxoplasma gondii* was confirmed by SAG1 reactivity (labeled “no reactivity”). Reaction with a type II strain–infected control serum *(bottom row)* confirmed the specificity of the assay.

Table 1

Cut-off Values for Five Genotype-specific Peptides Used in an IgG Serotyping Assay and for rSAG1 Based on Sera from Uninfected Individuals.

ID[a]	Genotype-specific Peptides[b]					rSAG1[b]
	6 I/III	6 d-I/III	6 II	6 d-II	7 II	
B 01	1.42	1.48	1.63	1.27	1.36	1.01
B 02	1.69	1.19	1.32	1.31	1.79	1.03
B 03	1.42	1.29	1.33	1.05	1.01	0.87
B 04	2.07	1.85	2.28	1.48	1.25	0.93
B 06	1.94	2.14	1.52	1.60	1.23	1.02
B 07	2.13	2.11	1.86	1.51	1.35	0.96
B 08	1.46	1.78	1.73	1.50	1.21	1.01
B 09	1.46	1.90	1.64	1.42	1.02	1.13
B 11	0.90	1.00	1.00	0.90	0.90	1.14
B 12	1.69	2.46	2.44	2.06	1.13	0.83
B 13	1.88	1.94	1.42	1.36	1.21	1.07
B 16	1.79	2.12	1.91	2.07	1.35	0.96
B 17	1.21	1.31	1.35	1.33	0.80	1.09
B 18	1.53	2.14	2.00	2.08	1.31	1.02
B 19	1.30	2.81	2.08	2.17	0.93	1.08
B 20	1.56	1.65	1.31	1.34	1.24	0.95
Mean[c]	1.64	1.88	1.72	1.57	1.21	1.01
SD[c]	0.28	0.45	0.36	0.35	0.23	0.09
Cut-off values[d]	2.2	2.8	2.4	2.3	1.7	1.2

SD=standard deviation.

[a] Anonymized serum specimen number. Serum was from individuals who were living in Santa Isabel do Ivai at the time of the outbreak under investigation. All were confirmed to be seronegative for *T. gondii* IgG antibodies using a commercial assay.

[b] For serum samples, values represent optical density (OD; obtained at A405 nm) values for the diagnostic antigens (five serotyping peptides or rSAG1) divided by the mean of the OD values for two control peptides (randomized sequence of the GRA6 peptide; mix of peptides derived from human and *Leishmania* sp. EF1α proteins); these normalized values represent background reactivity among the uninfected individuals, expressed as arbitrary units. Values from one representative experiment are shown.

[c] The mean value of the normalized data from four independent experiments.

[d] Defined as the mean value of the normalized data for all four independent experiments +2 SD. Cut-off values were rounded to the nearest decimal place. Results above these cut-off values were considered positive for the serotyping assay and for determining rSAG1-specific reactivity in this study.

Table 2

Genotypes of *Toxoplasma gondii* Isolates Associated with an Outbreak of Toxoplasmosis in Santa Isabel do Ivai, Brazil

	DNA sequence			PCR-RFLP[a]										
Isolate	**B1**	**GRA6**	**GRA7**	**SAG1**	**SAG2**	**SAG3**	**BTUB**	**GRA6**	**c22–8**	**c29–2**	**L358**	**PK1**	**Apico**	**Clonal type**
Outbreak 1[b]	I	u-1	I	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	Outbreak
Outbreak 2[b]	I	u-1	I	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	Outbreak
TgCatBr85[c]	I	u-1	I	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	Outbreak
TgCkBr98	I	u-1	I	I	I	III	I	II	u-1	I	I	I	I	Outbreak
TgCkBr101	I	u-1	I	I	I	III	I	II	u-1	I	I	I	I	Outbreak
TgCkBr102	I	u-1	I	I	I	III	I	II	u-1	I	I	I	I	Outbreak
TgCkBr103	I	u-1	I	I	I	III	I	II	u-1	I	I	I	I	Outbreak
TgCkBr93	U_I	II	…	I	III	III	III	II	I	III	I	II	I	Santa Isabel 1
TgCkBr94	U_I	II	…	I	III	III	III	II	I	III	I	II	I	Santa Isabel 1
TgCkBr95	U_I	u-2	U_{III}	I	III	III	III	III	I	I	I	III	III	Santa Isabel 2
TgCkBr96	U_I	u-2	III	u-1	II	III	III	III	II	I	I	III	I	Santa Isabel 3
TgCkBr97	U_I	u-3	U_{III}	I	II	III	III	III	I	III	I	II	III	Santa Isabel 4
TgCkBr99	U_I	u-1	U_{III}	I	III	III	III	II	u-1	I	I	II	I	Santa Isabel 5
TgCkBr100	U_I	II	U_{III}	I	III	III	III	II	u-1	I	I	II	I	Santa Isabel 6

NOTE. I, II, III, *Toxoplasma gondii* archetypal clonal strain types; NA, not available; PCR, polymerase chain reaction; RFLP, restriction fragment–length polymorphism; TgCKBr, *T. gondii* isolates from chickens in Brazil; U_{I-III}, unique alleles that are closely related to types I, II, and III, respectively; u-1, -2, and -3, unique alleles that are related to, but substantially divergent from, types I, II, and III, respectively.

[a] PCR-RFLP data are from Dubey et al. [6, 11].

[b] Isolates recovered from water taken from the cistern that was epidemiologically implicated as the source of the outbreak.

[c] Isolate recovered from a cat living in the area of the cistern.

J Infect Dis. Author manuscript; available in PMC 2017 December 07.

Table 3

Serotype Determination Based on the Immunoglobulin G Reaction to 5 Genotype-Specific Peptides in Mice Infected with Isolates Associated with an Outbreak of Toxoplasmosis in Santa Isabel do Ivai, Brazil

	Genotype-specific peptides							
Isolate	**6 I/III**	**6 d-I/III**	**6 II**	**6 d-II**	**7 II**	**SAG1**	**Serotype**	**GRA6 epitope**
Outbreak[a]	4.5[b]	0.8	1.2	1.0	0.9	6.9[b]	I/III	LHP**E**R**V**N**V**FD**Y**
TgCkBr 98	3.5[b]	1.4	0.8	0.7	1.4	4.8[b]	I/III	LHP**E**R**V**N**V**FD**Y**
TgCkBr 97	6.2[b]	1.1	1.3	1.0	0.7	7.3[b]	I/III	LHP**EH**V**V**FD**Y**
TgCkBr 95	3.0[b]	1.5	3.8[b]	1.9	1.2	2.7[b]	Atypical	LHP**GS**V**V**FD**Y**
TgCkBr 94	1.3	1.0	4.7[b]	3.9[b]	1.6	5.4[b]	II	LHP**GS**V**E**FD**F**
TgCkBr 100	1.0	1.0	6.8[b]	4.1[b]	1.0	9.2[b]	II	LHP**GS**V**E**FD**F**
I	14.5[b]	1.2	1.3	0.9	1.0	19.3[b]	I/III	LHP**E**R**V**N**V**FD**Y**
II	1.1	1.1	3.4[b]	2.3[b]	3.7[b]	21.2[b]	II	LHP**GS**V**E**FD**F**
Nil	1.4	1.5	1.4	1.4	0.9	1.0	Negative	…

NOTE. Enzyme-linked immunoassay data are presented as an optical density (OD) index as follows: the OD value obtained at 405 nm for each of the 5 serotyping peptides (6-I/III, d6-I/III, 6-II, d6-II, and 7-II) was divided by the mean of OD readings for 2 control peptides, and the result is expressed as an arbitrary unit. I, II, III, *Toxoplasma gondii* archetypal clonal strain types; TgCkBr, *T. gondii* isolates from chickens in Brazil.

[a] An isolate recovered from water in the cistern epidemiologically linked to the outbreak.

[b] Values that are greater than cutoff thresholds and that represent positive results. Cutoff values were determined by averaging the normalized OD ratio from 16 immunofluorescence assay–negative samples and adding 2 standard deviations (SDs), as described in Table 1, which appears only in the electronic version of the *Journal*, using methods previously published elsewhere [5].

Table 4

Serotype Determination Based on the Immunoglobulin G Reaction to 5 Genotype-Specific Peptides and the Reaction to SAG1 among Individuals Infected during an Outbreak of Toxoplasmosis in Santa Isabel do Ivai, Brazil

	Genotype-specific peptides[b]						
Specimen[a]	6 I/III	6 d-I/III	6 II	6 d-II	7 II	rSAG1[b]	Serotype
B21	1.2	0.9	1.0	0.9	0.9	8.7[c]	NR
B23	1.4	1.0	1.0	1.1	1.5	18.6[c]	NR
B33	1.2	0.9	1.0	1.0	1.0	4.4[c]	NR
B05	3.7[c]	1.0	1.2	1.0	1.7	12.6[c]	I/III
B24	10.8[c]	1.0	1.7	1.0	1.4	15.5[c]	I/III
B25	13.1[c]	0.8	2.1	0.9	0.9	16.7[c]	I/III
B27	8.1[c]	0.9	1.0	1.1	1.5	22.4[c]	I/III
B28	9.5[c]	0.9	2.1	0.9	1.0	17.6[c]	I/III
B29	2.3[c]	0.8	1.0	1.0	1.4	15.9[c]	I/III
B32	4.3[c]	0.9	1.3	1.1	0.9	5.8[c]	I/III
B34	4.9[c]	0.9	1.4	1.0	1.0	8.5[c]	I/III
B35	3.7[c]	0.7	1.0	0.7	1.2	12.8[c]	I/III
B37	3.2[c]	0.9	1.0	0.9	1.1	11.3[c]	I/III
B38	5.7[c]	0.9	1.1	1.0	1.6	10.5[c]	I/III
B39	3.1[c]	1.0	1.7	1.0	1.1	12.2[c]	I/III
B40	9.1[c]	1.1	1.9	1.0	1.1	12.0[c]	I/III
B10	7.8[c]	0.9	1.0	0.9	2.3[c]	4.3[c]	Atypical
B36	9.3[c]	2.7	3.0[c]	1.0	1.6	12.1[c]	Atypical
B22	17.3[c]	8.7[c]	9.2[c]	1.0	1.7	15.3[c]	Atypical
B30	14.6[c]	3.7[c]	9.5[c]	1.4	1.2	19.1[c]	Atypical
B31	10.0[c]	3.3[c]	4.9[c]	1.0	0.9	16.3[c]	Atypical

Specimen[a]	Genotype-specific peptides[b]					rSAG1[b]	Serotype
	6 I/III	6 d-I/III	6 II	6 d-II	7 II		
B26	8.0[c]	6.1[c]	5.5[c]	3.8[c]	2.4[c]	4.0[c]	Atypical
Type II[d]	2.2	1.0	4.2[c]	3.6[c]	5.5[c]	14.1[c]	…

NOTE. NR, nonreactive. Cutoff values were as follows: for 6 I/III, 2.2; for 6 d-I/III, 2.8; for 6 II, 2.4; for 6 d-II, 2.3; for 7 II, 1.7; and for rSAG1, 1.2. The cutoff value was defined as the mean value ± 2 standard deviations for assay results using serum from a group of uninfected individuals (from Table 1, which appears only in the electronic version of the *Journal*).

[a] Anonymized serum specimen number.

[b] For serum samples, values are expressed as arbitrary units, on the basis of methods previously described elsewhere [5, 12], and are summarized in Table 1, which appears only in the electronic version of the *Journal*.

[c] Values that are greater than cutoff thresholds and that denote positive results.

[d] Serum from a control individual known to be infected with a type II strain.