Recent studies of *Toxoplasma gondii* isolates from animals in Brazil have revealed high genetic diversity. Many of these isolates are virulent to mice. It is speculated that these isolates may also be virulent to humans. However, there is very limited data regarding *T. gondii* strains from human infection. Therefore, it is not clear whether there is any association between parasite genotypes and disease phenotypes. In this study, a total of 27 *T. gondii* strains were isolated from newborns with congenital toxoplasmosis in Minas Gerais state, Brazil. The genetic variability was assessed by restricted fragment length polymorphism in 11 loci (SAG1, 5’ plus 3’ SAG2, alternative [alt.] SAG2, SAG3, BTUB, GRA6, c22-8, c29-2, L358, PK1, and Apico). Genetic analysis of 24 strains revealed 14 different genotypes, including 7 previously identified from animals and 7 new types. The widespread genotype BrII accounted for 29% (7/24) of the isolates and was the dominant genotype involved in this study. This is the first report of genotyping of *T. gondii* isolates obtained from blood samples from newborns with congenital toxoplasmosis. Genotypic characterization of these isolates suggests high genetic diversity of *T. gondii* in this human population in Brazil. Future studies are needed to determine the source of contamination of this human population.

*MATERIALS AND METHODS*

**Study population.** This study was part of an investigation on neonatal screening for congenital toxoplasmosis conducted by a multidisciplinary research group (Universidade Federal de Minas Gerais [UFMG]-Brazilian Congenital Toxoplasmosis Group) in the Minas Gerais state in southeastern Brazil. From 1 November 2006 to 31 May 2007, a total of 146,307 newborns were tested for anti-*T. gondii* IgG antibodies in dried blood samples on filter paper using the Toxo IgM kit (Q-Preven; Symbiosis, Leme, Brazil). Confirmative serologic tests were carried out for 220 infants with positive or undetermined screening results. These infants were tested for anti-*T. gondii* IgG, IgA, and IgM by enzyme-linked fluorometric assay ELFA-VIDAS (bioMérieux SA, Lyon, France). Out of these 220 infants, 178 tested positive for the persistence of anti-*T. gondii* IgG antibodies in serum at the age of 12 months. IgM tests (Q-Preven and ELFA-VIDAS) showed a moderate level of discordance. However, this was expected, since the collection of blood samples on filter paper for the initial screen-
ing (Q-Preven) was conducted for children 7 to 10 days after birth. Confirmatory tests (ELFA-VIDAS) were performed after 31 to 86 days after birth (mean, 55.6 days). It is likely that, at this time, the levels of IgM previously detected by Q-Preven had decreased in some children and were no longer detected by ELFA-VIDAS. Ophthalmologic examinations were performed for all 220 children according to the method described previously (3). The protocols used in this study were approved by the local Human Research Ethics Committee (COEP-Federal University of Minas Gerais, protocol 298/06).

Toxoplasma gondii isolates. Peripheral blood samples from children from 31 to 86 days after birth (average age of 55.6 ± 16.6 days) were collected. Heparinized peripheral blood samples (0.5 ml) were centrifuged at 2,000 × g for 15 min, and the blood cell sediments containing erythrocytes and leukocytes were resuspended in 0.2 ml of phosphate-buffered saline solution (PBS), pH 7.2. For parasite isolation, 0.1 ml of this cell suspension was inoculated intraperitoneally (i.p.) in each one of two 6- to 8-week-old female Swiss mice. Thirty days after inoculation, each surviving mouse was bled via retro-orbital plexus. The blood (0.1 ml) was centrifuged, and the plasma was used to perform enzyme-linked immunosorbent assay (ELISA) for anti- T. gondii IgG antibodies (15). Animals that died before 30 days postinoculation were examined for the presence of tachyzoites in the peritoneum or cysts in the brain. All surviving mice were sacrificed by cervical dislocation. The brains of ELISA seropositive mice were removed and macerated, and 1.0 ml of PBS, pH 7.2, was added. Ten-microliter samples of brain lysates were used to search for the presence of tissue cysts by light microscopy. To determine the virulence of T. gondii isolates in mice, tachyzoites were first produced from the peritoneal cavities of five Swiss mice i.p. inoculated with 500 to 1,000 brain cysts as described previously (16). Five to 7 days after inoculation, the parasites were collected and washed from the peritoneal cavity with PBS, pH 7.2, and used in the virulence assay (fresh tachyzoites) or stored as frozen pellets at -20°C, until genomic DNA extraction. In the case of virulent strains, the infected Swiss reservoirs were treated with sulfadiazine for 10 days after infection to obtain cysts (17). The protocols conducted in this study were approved by the local Animal Ethics Committee (CETEA-Federal University of Minas Gerais, protocol 013/2007).

Parasite virulence. The same criteria adopted previously (18) were applied to determine the virulence of the isolates of T. gondii. Five female BALB/c mice were i.p. inoculated with 10^6, 10^5, 10^4, or 10^3 tachyzoites of each isolate in 0.2 ml of PBS (pH 7.2). Five animals inoculated i.p. with PBS were maintained as negative controls. Mice mortality was observed over a 30-day period. The survivors were bled via retro-orbital plexus, and the sera were tested by ELISA. The mice that did not seroconvert were excluded from the experiment. All the surviving mice were sacrificed by cervical dislocation to search for tissue cysts in the brain. For comparison, RH (virulent) and ME49 (nonvirulent) strains were used as references. Isolates killing 100% of the infected mice were classified as virulent. Isolates with 100% lethal dose (LD50) greater than 10^3 tachyzoites were classified as nonvirulent, and isolates with an intermediate pattern between the two extremes were classified as having intermediate virulence (6).

DNA extracts and multilocus PCR-RFLP genotyping of T. gondii. DNA extraction of the tachyzoites was performed using the Promega Wizard genomic DNA purification kit following the manufacturer’s instructions. The genotyping of isolates obtained from newborns with congenital toxoplasmosis was determined by PCR-RFLP patterns of 11 DNA segments, including SAG1, 3′ SAG2 plus 5′ SAG2, alternative (alt.) SAG2, SAG3, BTUB, GRA6, c22-8, c29-2, L358, PK1, and Apico, as described previously (19). However, we performed a PCR in a single stage using only the internal markers, without preamplification by multiplex PCR using external primers. The amplification reactions were carried out in a final volume of 10 μl, containing 2 μl of 5× green buffer (Promega), 25 mM MgCl2, 2.5 mM each deoxynucleotide (dATP/dTTP/dGTP/dCTP; Invitrogen), 5 U/μl of Taq DNA polymerase (Promega), 5 pmol of each primer, and 1 μl of DNA. A negative control, without DNA, was included in each reaction mixture. Strains RH88 (type I), ME49 (type II), and VEG (type III) were used as controls. The first amplification step consisted of 4 min of denaturation at 95°C, followed by 35 cycles, with 1 cycle consisting of denaturation at 94°C for 30 s, annealing at 60°C for 60 s, and extension at 72°C for 90 s. The extension step in the final cycle was extended to 5 min. The PCR products were visualized in 5% polyacrylamide gel stained with silver nitrate. The amplified products were digested using the appropriate restriction endonucleases by a previously published method (19). The digestions were conducted at a final volume of 10 μl, containing 3 μl of the PCR product, 1 μl of the corresponding buffer, and 2.5 U (0.25 μl) of the enzyme, at 37°C for 3 h, according to the manufacturer’s instructions. The DNA of the digested products was purified by extraction with an equal volume of phenol-chloroform (1:1), submitted to polyacrylamide gel (5%) electrophoresis, stained with silver nitrate, and photographed.

Data analysis. The profiles found after digestion with restriction endonucleases were compared with the profiles of the reference strains in a virtual database, the ToxoDB database (www.toxodb.org). The study database included serological results, demographic data (age, gender, place of birth) of each child, as well as bioassay, virulence, genotyping results, and clinical signs. To reveal the genetic relationship of all the parasite isolates, the composite data set of multilocus PCR-RFLP genotyping was analyzed by SplitsTree4 (20, 21). The composite data set was based on the most recent genotyping results from Brazil (22). The results are presented as a reticulated network to describe complex relationships of these T. gondii strains.

RESULTS

Strain isolation and virulence determination of the T. gondii isolates. One hundred seventy-eight infants with anti-T. gondii IgG antibodies when they were 12 months old were selected for this study. We tested one blood sample from each child by bioassay. Twenty-seven isolates of T. gondii were obtained from 27 newborns with congenital toxoplasmosis, demonstrating parasitemia in 15.2% (27/178). The isolates were designated TgCTBr1 to TgCTBr27 (in the isolate designation TgCTBr, Tg stands for T. gondii, CT stands for congenital toxoplasmosis, and Br stands for Brazil, and the isolates were numbered according to the chronological order in which isolation was performed). Only one of two mice inoculated with blood from newborn infants was infected with T. gondii with one exception. Both mice inoculated with TgCTBr9 became infected. The TgCTBr6 isolate was lost before the production of tachyzoites and the DNA extraction and virulence experiment.

Table 1 presents data on 27 newborns from which T. gondii was isolated. It shows the age of the children at the time blood was collected for the bioassay, which ranged from 31 to 86 days (average age of 55.6 ± 16.6 days), gender, major clinical signs, and confirmative serologic results. The newborns from which T. gondii was isolated came from different regions of the state of Minas Gerais in Brazil (Fig. 1), with isolates being obtained from 10 out of the 12 regions in the state. The isolates were divided into three groups according to the virulence phenotype for BALB/c mice. The time between inoculation of blood from a newborn in two Swiss mice and isolation of T. gondii are in Table S1 in the supplemental material. Fourteen isolates (54%) were characterized as having intermediate virulence, 10 isolates (38%) were characterized as virulent, and only two isolates (8%) were characterized as nonvirulent (Table 1).

Genotyping analysis of T. gondii. The complete genotype was obtained in 25/27 (92%) isolates (Table 2). It was not possible to carry out the complete genotyping in one sample (TgCTBr16) due to the nonamplification of some markers and the occurrence of
extremely polymorphic digestion products. Isolate TgCTBr6 was lost before DNA was obtained. A representative result of PCR-RFLP analysis (BTUB marker) is shown in Fig. S2 in the supplemental material. Tables 1 and 2 present the isolates grouped according to the genotypes identified.

From the 25 isolates typed, 14 different genotypes were identified, and one isolate (TgCTBr19) had mixed infection, in which a combination of two alleles was observed in four of the 11 loci analyzed, i.e., 5′ plus 3′ SAG2, alt. SAG2, BTUB, and c29-2. Of the 14 genotypes found, 7 are considered new types that were not reported previously, and 7 (ToxoDB PCR-RFLP genotypes 8, 11, 36, 41, 67, 108, and 162) were previously identified (Tables 1 and 2). The 7 new genotypes were designated following the scheme of ToxoDB PCR-RFLP genotype numbers. The new genotypes are 206 (TgCTBr01, -03, and -25), 207 (TgCTBr10), 208 (TgCTBr13), 209 (TgCTBr21), 210 (TgCTBr22), 211 (TgCTBr24), and 212 (TgCTBr26). Seven isolates (TgCTBr02, -08, -09, -11, -14, -20, and -27) exhibited genotype 11 (also known as type BrII). This genotype was previously identified from domestic animals in southeastern Brazil (12, 23, 25, 26, 27). The geographic distribution of genotype 11 in Minas Gerais, Brazil, was clearly widespread among 6 of 10 regions in which positive isolation of T. gondii from patients with congenital toxoplasmosis was achieved (Fig. 1). Isolate TgCTBr05 exhibited genotype 8 (also known as type BrIII), which was previously identified from domestic animals in other geographic regions of Brazil (12, 23, 25, 26, 28, 29). Isolate TgCTBr18 exhibited genotype 36, which was previously identified from chickens in Rio de Janeiro, Brazil (23). Isolates TgCTBr15 and TgCTBr23 exhibited genotype 41, which was previously identified from chickens in Amazon, Brazil (23) and capybaras in São Paulo state, Brazil (26). Isolate TgCTBr07 exhibited genotype 67, which was previously identified in dogs in São Paulo city (25) and cats in São Paulo state (12). Isolates TgCTBr04 and TgCTBr17 exhibited genotype 108, which was previously identi-
fied from cats in São Paulo state (12). Isolate TgCTBr12 exhibited genotype 162, which was previously identified from capybaras in São Paulo state (26). The genetic relationship of the 24 isolates of T. gondii obtained from newborns (excluding isolate TgCTBr19, due to its mixed infection origin), together with previously published data were compared using SplitsTree4 software (20, 21). The results are presented in Fig. 2. The 14 genotypes identified from the 24 congenital cases showed high diversity and are scattered in the network.

Regarding virulence in mice, isolates with identical genotypes exhibited similar phenotypes. A descriptive analysis did not show any association between the genotypes of the isolates and retinochoroiditis in the newborns. Likewise, no association between the genotypes and the sites of origin of the isolates was observed.

**DISCUSSION**

Most of the isolates obtained and genotyped in Brazil are from domestic animals, including free-range chickens, cats, dogs, sheep, and goats; little is known about the genetics of T. gondii isolates from humans (22). To understand the epidemiology of toxoplasmosis, it is important to determine how and from which sources the parasite is transmitted from animals to humans. In the present study, we isolated and genotyped T. gondii from peripheral blood of newborns. The high positivity observed in the bioassay experiments confirms the presence of viable tachyzoites in the blood of these transplacentally infected infants, even after a period of 2 to 3 months. Isolation of T. gondii in infants up to 86 days old can be explained by the immunoimmaturity of the newborns, which allows parasitemia to occur longer than in immunocompetent adults (30). The 27 isolates were obtained from children from different regions in Minas Gerais, Brazil, showing that T. gondii is widely distributed in the state.

We observed variability in the virulence in mice of the T. gondii isolates from cases of congenital toxoplasmosis. Ninety-two percent (24/26) of the isolates are intermediately or highly virulent to mice. A previous study analyzed the virulence of T. gondii isolates obtained from domestic animals in the state of Minas Gerais in Brazil, showing a higher prevalence of samples with high/intermediate virulence phenotype (31). Several factors interfere in the virulence of T. gondii, including parasite stage, infection route, infective dose, inoculation mode, mouse lineage, and intrinsic characteristics of the isolate (32). Although few studies have been carried out with isolates of T. gondii derived from cases of congenital toxoplasmosis, most of the isolates obtained and genotyped in Brazil are from domestic animals, including free-range chickens, cats, dogs, sheep, and goats; little is known about the genetics of T. gondii isolates from humans (22).

### TABLE 2 Multilocus genotyping of Toxoplasma gondii isolates from newborns in Minas Gerais state, Brazil

| Isolate(s) | SAG1 | SAG2 | SAG3 | BTUB | GRA6 | c22-8 | c29-2 | L358 | PK1 | Apico | PCR-RFLP genotype | Reference |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| RH88 | I | I | I | I | I | I | I | I | I | I | 10 | 10 |
| ME49 | II/III | II | I | I | I | I | I | I | I | I | 1 | 10 |
| VEG | II/III | III | III | III | III | III | III | III | III | III | 2 | 10 |
| TgCTBr01, -03, -25 (n = 3) | u-1 | I | II | III | III | III | II | III | I | III | 206 (new) | This study |
| TgCTBr02, -08, -09, -11, -14, -20, -27 (n = 7) | I | I | II | III | III | II | II | III | I | III | 11 (BrII) | 12 |
| TgCTBr04, -17 (n = 2) | I | I | I | III | III | III | II | I | I | I | 108 | 12 |
| TgCTBr15, -23 (n = 2) | I | I | I | III | I | I | I | I | I | I | 41 | 23 |
| TgCTBr05 (n = 1) | I | III | III | III | I | I | I | I | I | I | 8 (BrIII) | 12 |
| TgCTBr07 (n = 1) | I | III | III | III | III | I | I | I | I | I | 67 | 12 |
| TgCTBr10 (n = 1) | I | I | u-1 | III | III | I | I | I | I | I | 207 (new) | This study |
| TgCTBr12 (n = 1) | I | III | III | III | III | III | I | I | I | I | 162 | 26 |
| TgCTBr13 (n = 1) | I | I | II | III | III | III | I | I | I | I | 208 (new) | This study |
| TgCTBr16 (n = 1) | I | ND | III | ND | III | III | ND | ND | ND | ND | ND | ND |
| TgCTBr18 (n = 1) | I | I | I | III | II | II | II | II | II | II | 36 | 23 |
| TgCTBr19 (n = 1) | I | I/III | I/III | III | I/III | II | I/III | I/III | I/III | I/III | Mixed |
| TgCTBr21 (n = 1) | u-1 | I | III | III | III | II | I | I | I | I | 209 (new) | This study |
| TgCTBr22 (n = 1) | u-1 | I | II | III | III | III | II | II | II | II | 210 (new) | This study |
| TgCTBr24 (n = 1) | I | I | I | III | III | III | I | I | I | I | 211 (new) | This study |
| TgCTBr26 (n = 1) | I | I | I | III | III | III | I | I | I | I | 212 (new) | This study |

* I, II, and III, clonal type I, II, and III alleles, respectively. II/III, clonal type II or III. u-1 is a new allele that is different from the clonal type I, II, and III alleles. ND, no data.

* ND, no data.
ital toxoplasmosis (33), it is known that, in general, the isolates obtained in Brazil are virulent (10). Of the 24 isolates completely genotyped in this study, 20 originated from newborns with retinochoroiditis.Comparing Brazilian and European infants with congenital toxoplasmosis, it was observed that \textit{T. gondii} causes more severe ocular disease in the former than in the latter (34). These authors concluded that the differences in frequency, size, and multiplicity of the retinochoroidal lesions in the two populations may have been due to infections with more-virulent strains of the parasite that predominate in Brazil and are rarely found in Europe.

In this study, we completely genotyped 24 \textit{T. gondii} isolates from peripheral blood from newborns with congenital toxoplasmosis. Analysis of the multilocus PCR-RFLP revealed 14 genotypes, suggesting high diversity of the parasite isolated from the human population in Minas Gerais, Brazil, similar to results reported by other authors in studies of parasites isolated from other host species (12, 23, 28). Of the 14 genotypes identified, four were identified in two or more isolates (genotypes 11, 41, 108, and 206), while the other 10 genotypes were found in only one isolate. Among the 14 genotypes, 7 were previously reported in animals and 7 were described for the first time in the literature (Table 2), with six found in only one isolate. This result reconfirmed the high diversity of \textit{T. gondii} strains in Brazil (23, 28, 29).

Although the \textit{T. gondii} population is highly diversified in Brazil, some clonal genotypes circulate in the hosts. The previously identified clonal isolates BrII (genotype 11) and BrIII (genotype 8) were found in this study. These genotypes had already been described in several hosts such as sheep, chicken, and cats (12, 23, 29), but this is the first report of these two genotypes in humans. The isolation of strains with identical genotypes in distinct geographic regions (genotypes 11 and 8) suggests the widespread distribution of clonal genotypes of \textit{T. gondii} in Minas Gerais, Brazil.

In this study, the TgCTBr19 isolate presented mixed profiles in four of the 11 markers used. This mixed infection may have occurred due to simultaneous and sequential infections (reinfections), with parasites of different genotypes, acquired from oocysts directly from the environment or through ingestion of tissue cysts in the intermediate hosts infected with two distinct isolates of the parasite, possibilities already discussed in the literature (35, 36). Studies show evidence of mixed infections mainly associated with congenital cases (36). The occurrence of mixed genotypes in Brazil corroborates the hypothesis that several genotypes of \textit{T. gondii} must be widely disseminated and circulating simultaneously (12, 23, 26, 28, 29). In Brazil, overall, isolates are recombinant and virulent (10, 12, 37). Isolate TgCTBr05, the only isolate classified as type BrIII, was shown to be nonvirulent to mice, corroborating a previous study (12). However, the seven isolates identified as genotype 11 (BrII) showed variable virulence. Biological differences between the isolates of the same genotype must not be neglected, and it may be possible that the genetic markers used in this study are incapable of reflecting possible phenotypic differences between the isolates under question (28). Thus, there is no clear correlation between the genotypes of the

FIG 2 NeighborNet phylogenetic network of \textit{Toxoplasma gondii} isolates from Brazil. TgCTBr1 to TgCTBr27 are \textit{T. gondii} isolates from newborns from Minas Gerais, Brazil.
isolates of *T. gondii* obtained from newborns in the state of Minas Gerais in Brazil and virulence to mice. A recent study in Brazil has also not shown any concrete evidence of correlation between virulence of *T. gondii* in mice and different genotypes (38).

No association between the parasite genotype and retinocochroiditis in the newborns was observed, with further studies being necessary to define the role played by phenotypic and genotypic characteristics of *T. gondii* in the development of ocular lesions.

The results of this study indicate that a significant proportion of cases of congenital toxoplasmosis (62.5% [15/24]) were caused by *T. gondii* genotypes previously reported from animals (Table 2). In particular, 29% (7/24) cases were caused by the common *T. gondii* genotype 11 (BrII) that was previously reported from a variety of animals in Brazil (12, 23, 24, 25, 26, 27). Nine *T. gondii* isolates were grouped into 7 new genotypes that were not reported before, indicating high diversity of the parasite in the population.

Further studies of sampling of *T. gondii* in animals from Minas Gerais, Brazil, are needed to better understand the population structure of *T. gondii* and transmission of the parasites among animals and humans. This is the first report of genotyping of isolates obtained from blood from newborns, providing important technical and scientific information on the epidemiology of congenital toxoplasmosis in Brazil.

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REFERENCES


