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High heterogeneity, mixed infections and new genotypes in human congenital toxoplasmosis cases in the mega-metropolis of Central Mexico

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ABSTRACT

Mexico presents high prevalence of *Toxoplasma gondii* infection, including the congenital form, but there are few data about the genetic diversity of the parasite, so we attempted parasite isolation and genotyping in nine mother/children pairs with congenital toxoplasmosis (CT), living in the Valley of Mexico, who were part of a 30 cases cohort that started 12 years ago. They were recruited through research projects which included pre- and postnatal screening of congenital infections or directly CT, and cases referred to INP for management because they had clinical abnormalities. Genotyping was performed by PCR-RFLP of *SAG1*, *SAG2*, *SAG3*, *BTUB GRA6*, *c22-8*, *c29-2*, *L358*, *PK1* and *Apico* markers, followed by sequencing. Sixty seven percent of samples were typed for the *SAG3* locus, 39% for *Apico* and 33% for *BTUB*, while Alt. *SAG2*, *GRA6* and *c29-2* types could be labelled in less cases. Type I alleles predominated, followed by II and III. We isolated the first strain obtained from humans in Mexico and found three genotypes not previously found in the world. The presence of ToxoDB#10 clonal type was documented in one pair, as well as mixed infections in five mothers. No relation of genotype or parasite load with clinical signs was found. In conclusion, we encountered great genetic diversity and mixed *T. gondii* infections among mother/children pairs with congenital toxoplasmosis in the mega-metropolis of the Valley of Mexico.

1. Introduction

Toxoplasma gondii is a cosmopolitan pathogen, due to its multiple hosts and ways of transmission, including the vertical route (Weiss and Kim, 2013). Classically thought to be of low diversity, this parasite is now grouped into 278 genotypes, pertaining to 15 haplogroups (Su et al., 2012; ToxoDB.org). Recent studies provided evidence that in the Northern hemisphere the population is mainly clonal, while a pandemic dispersion is taking place in the South (Shwab et al., 2014). Although Mexico is in the Northern hemisphere, it presents a quite high prevalence of *T. gondii* infection, both acquired and congenital, as well archetypal and atypical genotypes, with an intermediate diversity between North and South America (Vela-Amieva et al., 2005; Cedillo-Peláez et al., 2011; Caballero-Ortega et al., 2012; Dubey et al., 2004, 2009, 2013; Rico-Torres et al., 2012; 2015; Martínez-Flores et al., 2017). This may be of global significance since Mexico is an area located between the Nearctic and the Neotropical zones separated by the "Sierras Madre Oriental y Occidental" (long and high mountain ranges). Also, there is a great variety of wild, feral and domestic hosts freely roaming in the sylvatic, rural, semi-urban and urban areas, so there is large opportunity for *T. gondii* differential distribution and diversification in the country (Khan et al., 2007; Su et al., 2012; Rico-Torres et al., 2015). Mexico City, one of the largest urbanities of the world, is located at high altitude (2250 masl) and has a mild weather.

Regarding congenital toxoplasmosis (CT), it is recognized that besides pregnancy time at infection, parasite burden and maternal/fetal immune response, *T. gondii* type seems to play an important role on clinical outcome, being type I variants more frequent among infections of the first half of gestation and closely related to clinical severity (Rico-Torres et al., 2016). In Mexico, there were no data about genetic diversity of *T. gondii* in congenital toxoplasmosis, except for a short article in which we reported four perinatal cases infected with type I-related

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variants in neonates with moderate to severe damage, using three genetic markers (Rico-Torres et al., 2012). In the present work, we show the results of *T. gondii* diversity analysis among congenitally infected children of a group of cases of the metropolis of the Valley of Mexico, which is located at high altitude, using a larger panel of markers (Su et al., 2010).

2. Material and methods

2.1. Patients and congenital toxoplasmosis diagnosis

In the flowchart depicted in the supplementary figure we show the origin of cases of congenital toxoplasmosis that were included in a cohort that is composed by 30 cases nowadays. They came from three main sources along 12 years: one project of pre and postnatal congenital infections screening (including but not exclusive of *T. gondii*, project INP 002/2008), another project about immune response in CT (project 060/2011) and cases referred to INP because they had clinical suspicion of CT. In all cases the mother/newborn (child) pair was included after CT confirmation by clinical and laboratory methods.

Briefly, diagnosis included clinical examination by specialists, and when needed, ocular fundoscopy, Computed Tomography and MRI scanning, and electroencephalogram. Laboratory tests included indirect ELISA for IgG antibodies adapted for titration and avidity, mother/newborn comparative Western Blot (WB) for both IgG and IgM, bioassay in mice and real-time PCR. The patients were classified according to severity, in mild vs moderate/severe, and to localization in neurologic, neuro-ophthalmic and disseminated, following the suggested criteria based on international literature (Martín-Hernández, 2004; Baquero-Artigao et al., 2013; Wilson et al., 2016; Cortes et al., 2017). Mothers and newborns received treatment according to international recommendations for prenatal and postnatal schemes.

From this cohort, nine pairs (mother/newborn) were selected due to positive PCR or bioassay in mice and enough DNA to perform genotyping. Four of them were recruited during prenatal or postnatal screening projects and five were clinical cases, referred to INP for clinical management (Supplementary figure).

2.2. Parasite load and genetic characterization

Parasite load was determined with the Applied Biosystems fluorescent real time PCR as previously reported (Cedillo-Peláez et al., 2011). To genotype, we performed the PCR-RFLP method reported by Su et al. (2010) for markers *SAG1*, *SAG2*, *SAG3*, *BTUB*, *GRA6, c22-8*, *c29-2*, *L358*, *PK1* and *Apico*. The restriction patterns were compared with type I (RH), II (ME49) and III (VEG) strains included in each nested assay. We considered as classical types I, II or III when the genotype was I, II or III in all loci analysed and recombinant when there were different genotypes at different loci (I,II; I,III or I,II,III). We encountered mixed infections in unique samples & loci (see below); they were labelled as I + II or I + III.

From 54 amplicons tested by RFLP, 36 could be purified in enough amount to sequence them, which was performed at the Instituto Nacional de Medicina Genómica (INMEGEN) Mexico. Sequences were analysed with SnapGene viewer 4.0.5 and Chromas Lite 2.1.1 softwares; they were aligned with those reported in Toxo.DB.org and GenBank web pages by Clustal W2 and NCBI/BLAST websites.

2.3. Bioassay in mice

Isolation was attempted by i.p. inoculation of amniotic fluid (one case) and peripheral blood of all cases into Balb/c mice, as reported previously (Rico-Torres et al., 2015). Two of these animals seroconverted (pairs two and nine, Table 1). A pool of brain, heart, liver, lung and spleen samples of each of these animals was inoculated into Balb/c mice again. Living tachyzoites were recovered from the peritoneal cavity of the mice from case two. These parasites and PCR positive tissues (spleen or lung) of the mouse of pair nine, were used to genotype *T. gondii*. All other genotypes were gathered from clinical samples directly.

3. Results

From thirty pairs of congenital toxoplasmosis recruited from prenatal/postnatal screening projects or because they were clinical cases, we could genotype nine mother/newborn (child) pairs, since the mother, the newborn or both were PCR positive or suspicious, or because we had the isolate or *T. gondii*-positive mice DNA (in total 18 samples; Table 1). From these, twelve could be genotyped for at least one marker, and three for all loci (the isolate and two clinical samples). For the *SAG3* locus, twelve samples were typed (67%), followed by *Apico* with seven (39%), *BTUB* with six (33%) and Alt. *SAG2*, *GRA6* and *c29-2* with five. Three clinical samples revealed mixed infections (Fig. 1): the newborn from pair five had I + II alleles at *Alt. SAG2*, *GRA6* and *Apico*; the baby from pair six had I + II at the *Apico* locus as well; and the newborn from pair nine had I + III alleles at *SAG3*. Furthermore, two mixed infections were found in pairs two and seven, since the maternal samples presented type II and I *SAG3* alleles, respectively, while the isolate of pair two was type III and the baby's sample of pair seven harbored the type II. So, five mother/newborn pairs presented mixed infections; those of cases N5 and N9 were further corroborated by sequencing (see example in Fig. 2).

We observed a predominance of type I alleles, followed by type II and III, but there is a great heterogeneity in terms of pattern: even though pairs four and nine shared allele I at *SAG3*, and thus they could harbor the same *T. gondii* type (for one of the infections of pair nine), this could not be proven in the present work. The three pairs with complete genotype pattern, confirmed heterogeneity in this group of patients. The clinical samples and the isolate of pair two did not match to any of the 278 genotypes known up to date; besides, the sample of the mother correlated with the results from the isolate (obtained from the newborn) at Alt. *SAG2* and *BTUB* (type I and III respectively) but differed at *SAG3*. Moreover, none of the eight possible combinations built up from the pattern of pair five, corresponded to a reported genotype (ToxoDB.org). Finally, pair four harbored clonal/classical type I-ToxoDB #10- parasites.

The genotype described for pair three could be ToxoDB #112 or #142, found in one cat from Puerto Rico and a chicken of Brazil respectively (ToxoDB.org). Despite lacking results in six markers of pair eight, we determined this case was due to a new variant, since the four loci-pattern typed did not match any previously reported genotype. One of the infections of pair nine could be ToxoDB #10 (clonal Type I) or #27, previously reported in chickens from Nicaragua, but the other, harboring type III *SAG3* allele could correspond to ToxoDB #225, typed in chickens of China (Rajendran et al., 2012; Wang et al., 2013). Either individual loci alleles or partial genotypes shown here have been reported in congenital toxoplasmosis cases from America, Africa and Europe (Rico-Torres et al., 2016): type I *SAG3* was found in Brazil and Tunisia (expanded to other loci here, see case nine; Table 1); type II *SAG3* was observed in Poland, Serbia and Tunisia (Nowakowska et al., 2006; Boughattas et al., 2010; Markovic et al., 2014). Finally, the combination of type I *SAG3* with type III *Apico* has been reported in Brazil (Carneiro et al., 2013).

4. Discussion

In Mexico, there is a high prevalence of *T. gondii* infection −around 43%- with frequencies over 70% in tropical and coastal regions- but there are few reports about genotypes (Caballero-Ortega et al., 2012). Studies performed in the Northern state of Durango and the Coastal state of Colima have demonstrated I, II, III, recombinant and atypical strains in animals (Alvarado-Esquivel et al., 2011; Dubey et al., 2009;