# An atypical strain associated with congenital toxoplasmosis in Tunisia

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**SUMMARY**

We report the identification and typing of a congenital toxoplasmosis case in a diabetic pregnant young woman living in Tunis. The *Toxoplasma* DNA extracted from amniotic fluid was detected by Real Time PCR and subjected to a multilocus genetic characterisation of the strain at markers: 3'SAG2, 5'SAG2, New SAG2, SAG3, GRA6, BTUB, APICO, PK1, KT850 and UPRT1. An atypical genotype of *T.gondii* with unusual genetic composition was revealed. It is the first time that an atypical strain has been associated with congenital toxoplasmosis in Africa. Atypical strains are associated with severe clinical manifestations so systematic genotyping should be investigated with the amniocentesis.

*KEY WORDS:* Congenital toxoplasmosis, Multilocus genotyping, Atypical strain

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Congenital toxoplasmosis (CT) contamination occurring early during pregnancy can lead to severe fetal damage. Clinical manifestations depend mainly on when the infection was acquired in utero. Infectivity is highest during the later stages of pregnancy; however, the earlier in gestation an infection occurs, the greater the likelihood of severe postnatal sequelae (Garcia-Méric *et al.*, 2010). The genotype of the parasite is also incriminated in disease severity. *Toxoplasma gondii* has a clonal genetic structure: most *T.gondii* isolates have been grouped into three archetypal lineage types I, II and III (Howe and Sibley, 1995). In Europe and North America, type II is predominant (Ajzenber *et al.*, 2002) whereas in Asia, an apparent dominance of type III is showed (Zhou *et al.*, 2009). In South America, a high level of allelic variation and non-archetypal lineage is reported (Dubey *et al.*, 2007). In Africa there is a high frequency of mixed clonal types (Lindström *et al.*, 2008). Here, we extend investigations in Tunisia (North Africa) and report on the presence of a non-archetypal strain.

A diabetic 29-year-old woman living in Tunis (North Tunisia) pregnant with her first baby showed a toxoplasmic seroconversion during the first semester of pregnancy. To verify fetal contamination, the amniotic fluid was sampled and subjected to DNA extraction (DNA Mini Blood Kit-Qiagen). A positive amplification reaction was obtained by real time PCR TaqMan targeting the repetitive genes B1 and Rep529 (Siala *et al.*, 2007). The isolate was called AF20/04. The amniotic fluid was not cultured on mice; genotyping analysis was performed directly on DNA extracted from the amniotic fluid sample.

Multilocus genotyping analysis was carried out by multiplex PCR using ten markers: 3'SAG2 (Howe *et al.*, 1997), 5'SAG2 (Howe *et al.*, 1997), new SAG2 (Su *et al.*, 2006), SAG3 (Grigg *et al.*, 2001), GRA6 (Khan *et al.*, 2005), BTUB (Khan *et al.*, 2005), APICO (Su *et al.*, 2006), PK1 (Su *et al.*, 2006), KT-850 (Howe and Sibley, 1995), and UPRT1 (Khan *et al.*, 2005). Primers PK1Fo (AGCTGTCCACCCTGAAACAG), PK1Ro (CCCATAAGCTGCAAGAATGA) and KT-850 Fo (AGAACTCCTGGACGCTGCT), KT-850 Ro (AGTGACGAAGCAAGCCAAGT) were designed in this study as

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outer primers of PK1 and KT850 genes respectively. For the new SAG2 marker, primer combinations were used. Outer primers were SAG2.F4 (Howe *et al.*, 1997) and SAG2.Ra (Su *et al.*, 2006) whereas inner primers were SAG2.Fa (Su *et al.*, 2006) and the new designed SAG2.Rin (CTCGGGAGTACCGTCGTAAG). Amplification and enzymatic restriction conditions were in agreement with methods described elsewhere (Boughattas *et al.*, 2010). Several control measures were taken to avoid contamination: separate spaces to set up PCRs, electrophoresis and RFLP, filter tips, and UV radiation. Different negative controls (no DNA, uninfected sample, and extracted no DNA) were also used. Positive controls were carried out from RH, Prugniaud and NED strains kindly provided by Pr. Marie Laure Dardé. Sequencing was also investigated whenever there were unclear enzyme digestion results and at the polymorphic uracil phosphoribosyl transferase (*UPRT*) intron 1 marker as described previously. Phylogenetic network analysis was performed for UPRT1 locus using the program SplitsTree 4 (Huson and Bryant, 2006). All sequences were submitted to GenBank, their accession numbers are cited below.

The multilocus genotypic characterization of the *Toxoplasma gondii* strain yielded a new genotype with unusual combination of type I, II, III and nonarchetypal alleles.

Both digestions in 3' and 5' extremities of SAG2 marker concluded for a mixture of types II and III. For the new SAG2 marker, final amplification size, with the new association of primers, was 486bp. Its RFLP pattern revealed type II allele.

The restriction profile of SAG3 product suggested a mixture of types I and III which was confirmed by sequencing. In fact, superposed picks were observed at the level of polymorphic sites generating two sequences: one with both NciI restriction sites (Type I: GU249509) and the other with just one site (Type III: GU249510). Restriction profiles of Apico and PK1 markers were identical to type I, whereas restriction profiles of GRA6 and KT850 were identified as type II. For this last marker, sequences of reference strains were unavailable in GenBank. So, RH (GU249505), Prugniaud (GU249506) and NED (GU249507) strains were subjected to sequencing in addition to AF20/04 (GU249508). The alignment of different sequences showed 15 nucleotide positions polymorphism. It also confirmed type II of our isolate.

By BTUB marker, amplification product gave a band with unexpected size of 400pb instead of 411pb. Restriction of the product gave a unique profile (u-1), different from those of the reference strains (Figure 1). This result was reproduced in at least ten experiments.

Analysis of sequences of the Intron 1 revealed double peaks at known polymorphic sites thus giving two sequences for the strain (GU249511, GU249512). The presence of multiple alleles was observed as well as the presence of an exotic nucleotide which is well demonstrated by phylogenetic network analysis (Figure 2).

Primary maternal *Toxoplasma* infection exposes the fetus to the risk of parasite transmission, leading to congenital toxoplasmosis (Garcia-Méric *et al.*, 2010). In this situation, it is important to check for the presence or absence of the parasite in the amniotic fluid by amniocentesis. DNA amplification provides a fast and sensitive result. A positive reaction indicates parasite transmission to fetus. Amniocentesis is usually practised in early pregnancy, and it has been established that the toxoplasmic fetal transmission rate is lower when the infection is in this period. However, when ac-

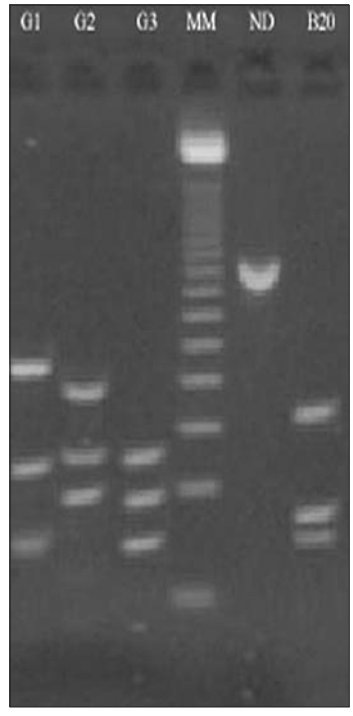


FIGURE 1 - *3% Agarose gel electrophoresis of BTUB RFLP products. G1 corresponds to RH strain, G2 to Prugniaud strain, G3 to NED strain, MM to molecular DNA ladder 50bp (Invitrogen), ND to 411bp undigested control and B20 to AF20/04.*

quired then, the congenital toxoplasmosis is more severe, ranging from in utero death to severe neuro-ophthalmic involvement. The damage could increase drastically in the presence of atypical/exotic strains of the parasite (Delhaes *et al.*, 2010). In our case, due to the early and specific parasite detection, others analyses were not required and the patient's pregnancy was terminated. In retro-

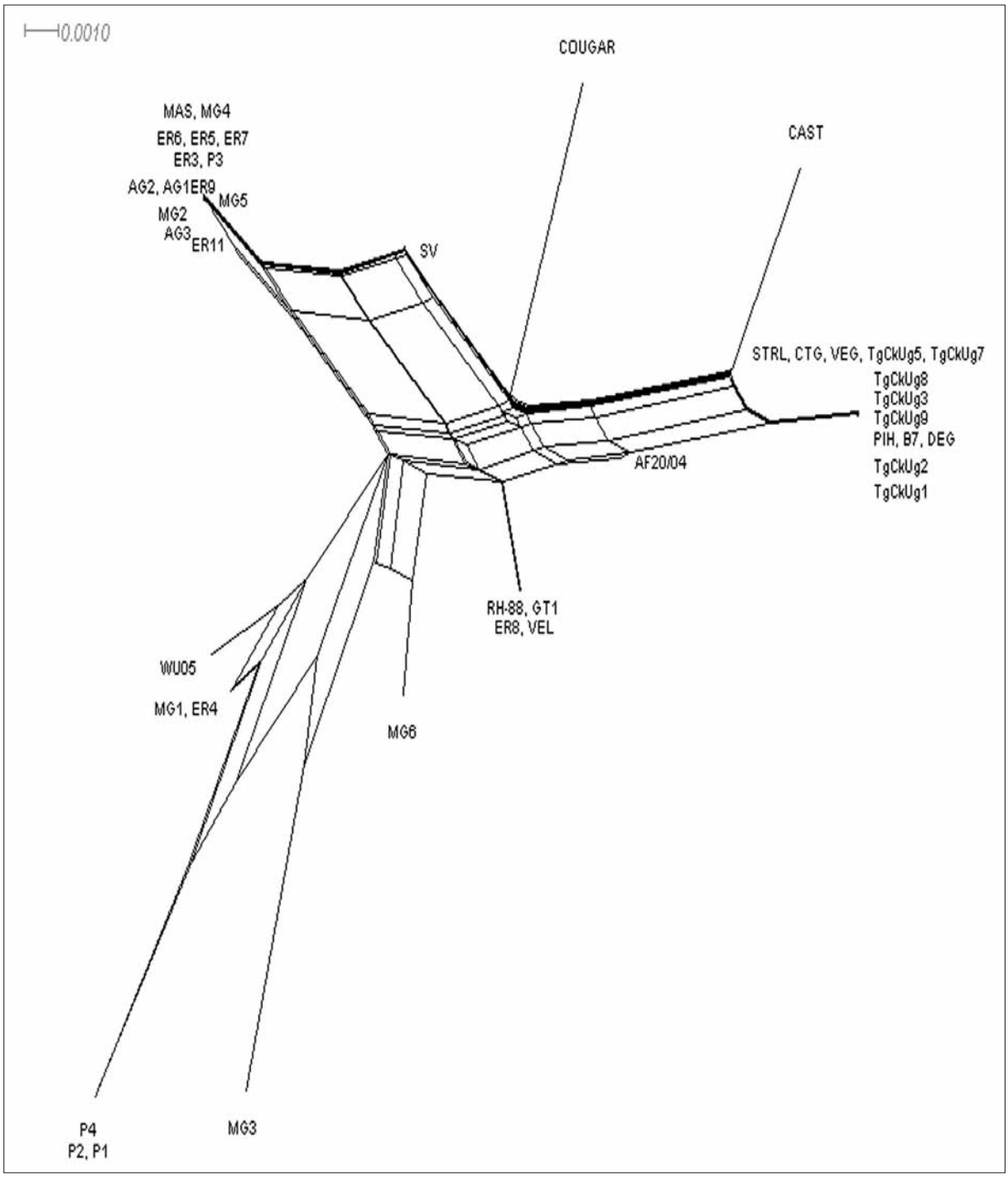


FIGURE 2 - *Phylogenetic network (NeighborNet) of Toxoplasma gondii isolates analyzed at the locus UPRT1.*

spect, this seems to have been the best taken especially with the identification of the atypical strain. Such strains are often isolated from exotic areas, however in Africa, as far as we know, it is the first time that an exotic strain has been associated with congenital toxoplasmosis.

Different methods have been described to type toxoplasmic strains. Microsatellites one is the most polymorphic, however it requires at least 50parasites/sample (Ajzenberg *et al.*, 2005). RFLPs shows a good sensitivity level (5parasites/sample) permitting direct typing on clinical samples which is why we used it (Khan *et al.*, 2005). To enhance the polymorphism information we used 10 independent markers. Some markers were adapted to our multiplex PCR by primers design. Sequencing analysis improved our result. This was not as described in our previous study in which mixed infections were frequent without any observation of exotic atypical strains (Boughattas *et al.*, 2010). This pattern could indicate that African strains of *T.gondii* undergo more frequent sexual recombination, resulting in more unique genotypes. Such emergent epidemiological data concerning circulating of atypical *T. gondii* strains in Africa needs special awareness on the part of physicians. A systematic genotyping of the parasite strain should be investigated with amniocentesis.

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