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Short communication

# Isolation of *Toxoplasma gondii* strains similar to Africa 1 genotype in Turkey

Mert Döşkaya [a,*], Ayşe Caner [a], Daniel Ajzenberg [b,c], Aysu Değirmenci [a], Marie-Laure Dardé [b,c], Hüseyin Can [d], Derya Dirim Erdoğan [a], Metin Korkmaz [a], Ahmet Üner [a], Çiğdem Güngör [e], Kürşat Altıntaş [e], Yüksel Gürüz [a]

[a] Department of Parasitology, Ege University Medical School, Bornova/İzmir 35100, Turkey
[b] Centre National de Référence (CNR) Toxoplasmose/Toxoplasma Biological Resource Center (BRC), Centre Hospitalier-Universitaire Dupuytren, Limoges 87042, France
[c] INSERM UMR 1094, Neuroépidémiologie Tropicale, Laboratoire de Parasitologie-Mycologie, Faculté de Médecine, Université de Limoges, Limoges 87025, France
[d] Department of Molecular Biology, Ege University Faculty of Sciences, Bornova/İzmir 35100, Turkey
[e] Ankara University Medical School, Department of Parasitology, Ankara 06100, Turkey

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ABSTRACT

*Introduction: Toxoplasma gondii* is a protozoon parasite that has a worldwide dissemination. It can cause serious clinical problems such as congenital toxoplasmosis, retinochoroiditis, and encephalitis. Currently, *T. gondii* genotypes are being associated with these clinical presentations which may help clinicians design their treatment strategy.

*Case reports:* Two *T. gondii* strains named Ankara and Ege-1 were isolated from newborns with congenital toxoplasmosis in Central and Western Anatolia, respectively. Ankara and Ege-1 strains were isolated from the cerebrospinal fluid of newborns. According to microsatellite analysis, Ankara and Ege-1 strains were sorted as Africa 1 genotype.

*Conclusion: T. gondii* strains isolated in Turkey were first time genotyped in this study. Africa 1 genotype has previously been isolated in immunosuppressed patients originating from sub-Saharan Africa. The reason of detecting a strain mainly detected in Africa can be associated with Turkey's specific geographical location. Turkey is like a bridge between Asia, Europe and Africa. Historically, Anatolia was on the Silk Road and other trading routes that ended in Europe. Thus, detecting Africa 1 strain in Anatolia can be anticipated. Consequently, strains detected mainly in Europe and Asia may also be detected in Anatolia and vice versa. Therefore, further studies are required to isolate more strains from Turkey.



## 1. Introduction

*Toxoplasma gondii* is a protozoon parasite that can infect all warm-blooded animals and humans. Toxoplasmosis is usually asymptomatic in healthy people however serious clinical presentations may occur in immunocompromised patients and in fetuses that were infected during pregnancy [1]. In the event of congenital toxoplasmosis, spontaneous abortion or death of the fetus and serious malformations may occur based on the starting time of infection. Currently, *T. gondii* genotypes, host genetic variability and immune response are being associated with severe clinical manifestations [2,3]. As a result of this association, data obtained from genotyping studies will ultimately help clinicians design their treatment strategy.

Three major clonal lineages of *T. gondii*, designated as Type I, II and III, were classified according to their genetic polymorphism. In addition, atypical and recombinant strains exist [4–6]. It has been shown that Type I and some of the recombinant or atypical strains are virulent in mice, whereas genotype II and III are avirulent [4].

Particular genotypes have been shown to have different geographical distribution. In Europe, genotype II, frequently isolated from human and animals, is an avirulent strain in mice. However, type II strains may cause various clinical presentations in congenital toxoplasmosis such as life-threatening neuro-ocular involvement in early maternal infections and retinochoroiditis or asymptomatic toxoplasmosis in late maternal infections [7]. Diverse clinical manifestations in newborns with identical genotype have been explained by different initiation time of infection [7]. Infection during the initial phase of the pregnancy is expected to cause major damage in fetus. Concurrently, few atypical strains, isolated from severe congenital toxoplasmosis cases in Europe, were observed in late maternal infections [4].

In sub-Saharan Africa, besides the three main lineages, non-archetypal genotypes named Africa 1, 2, and 3 have been isolated from humans and domestic animals [5,8,9]. In North Africa, the Middle East and the Arabic peninsula, type II and III strains predominantly appear in various animal species [10–12].

* Corresponding author at: Department of Parasitology, Ege University Medical School, 35100, Turkey. Tel.: +90 232 390 4734; fax: +90 232 388 1347.
*E-mail address:* mert.doskaya@ege.edu.tr (M. Döşkaya).

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In Far East Asia, most of genotyping data come from China where the three major clonal lineages have been identified in addition to the predominant atypical genotype, Chinese 1 [13,14]. Non-clonal genotypes have also been isolated in Indonesia and Vietnam [15]. In Iran, located in three spheres of Asia, genotypes II and III were described [16].

In North America, Haplogroup 12 which is formed through a recombination between Type II and a unique parental lineage was primarily detected in wild animals and occasionally in humans [17]. In Central and South America, there is high diversity within and between *T. gondii* populations. In a study conducted in Central America (Guatemala, Nicaragua, Costa Rica), South America (Venezuela, Colombia, Peru, Chile, and Argentina) and Caribbean (Grenada) district, ToxoDB PCR-RFLP #7, Type III and II were isolated from chickens and cats [18]. Interestingly, Type II was not isolated from cats, dogs, and chickens in Brazil however there were four common atypical clonal lineages (names as BrI, BrII, BrIII, and BrIV) [19]. Among these isolates, BrI seems to be similar to Africa 1.

This study aimed to genotype two strains isolated from newborns with congenital toxoplasmosis in Turkey. Microsatellite analysis with 15 markers has been used to determine the genotype of the strains [20].

## 2. Materials and methods

In the first case, only the Sabin Feldman dye test was used to detect the presence of anti-*Toxoplasma* antibodies in the mother of the new born [21–23]. In the second case, ELISA IgG, IFA IgG, Capture IgM ELISA (Radim, Italy) and Avidity Assay were used to investigate the presence of anti-*Toxoplasma* antibodies in the new born and the mother [24,25]. In addition, Real Time PCR was performed to detect *B1* gene of *T. gondii* from cerebrospinal fluid of second case. The bio-assays were conducted in *outbred* Swiss Webster mice for both strains. After the isolation of strains, a single multiplex PCR assay detecting 15 microsatellite markers of *T. gondii* was used to determine the genotype.

### 2.1. Virulence assay

To isolate the *T. gondii* strains, cerebrospinal fluids obtained from both cases during autopsy were administered intraperitoneally to at least three female 6–10 weeks old *outbred* Swiss Webster mice as described [26]. As symptoms of acute toxoplasmosis emerged in mice, they were sacrificed and tachyzoites were isolated from mouse ascitic fluid.

### 2.2. Real Time PCR

Real Time PCR detecting the *B1* gene of *T. gondii* was performed as described [22]. Isolation of DNA from the cerebrospinal fluid sample was performed by using High Pure PCR Template Preparation kit according to the manufacturer's protocol (Roche Applied Sciences, Germany). During Real Time PCR, the primers used for amplifying 126 bp B1 gene (GenBank no. AF179871) fragment were 5′-GGAGGACTGGCAACCTGG TGTCG-3′ (23nt, TOX B1 F, forward primer) and 5′-TTGTTTCACCCGGAC CGTTTAGCAG-3′ (25nt, TOX B1 R, reverse primer). The hybridization probes were 5′-CGGAAATAGAAAGCCATGAGGCACTCC-FL (27nt, TOX B1 FL, labeled at the 3′ end with fluorescein) and 5′-640-CGGAAATAGAA AGCCATGAGGCACTCC-3′ (27nt, TOX B1 LC, labeled at the 5′ end with LC-Red 640) (TIB Molbiol, Germany). 20 μl final volume PCR reaction included 5 μl purified patient DNA template or controls, 1× LightMix (TIB Molbiol), 1× FastStart mix (Roche), and 4 mM $MgCl_2$. The PCR amplification reactions were performed using the following calculated control protocol: 10 min preincubation step at 95 °C, followed by 45 cycles of 10 s at 95 °C, 5 s at 60 °C, and 5 s at 72 °C. As positive controls, *T. gondii* genomic DNA serially 10-fold diluted ranging from 5000 to 0.5 parasites per μl (TIB Molbiol) and one negative control prepared by replacing template DNA with distilled water were used. Melting curve analysis was performed using the following calculated protocol: 20 s denaturation step at 95 °C with temperature transition rate of 20 °C/s followed by 20 s annealing step at 40 °C with temperature transition rate of 20 °C/s and extension step gradually increasing temperature to 85 °C with temperature transition rate of 0.2 °C/s. The parasite quantification and melting curve analysis were performed by a 1.2 LightCycler Real Time instrument using LightCycler software, Version 3.5 according to the manufacturers protocol (Roche).

### 2.3. Genotyping analysis with microsatellite markers

During microsatellite analysis of Ankara and Ege-1 strains, a single multiplex PCR assay detecting 15 microsatellite markers (*TUB-2*, *W35*, *TgM-A*, *B18*, *B17*, *M33*, *IV.1*, *XI.1*, *M48*, *M102*, *N60*, *N82*, *AA*, *N61*, *N83*) located on 11 different chromosomes of *T. gondii* was used as described [20]. Briefly, 25 μl amplification reaction included 1 μl DNA extracted from tachyzoite containing mouse ascitic fluid, 15 pairs of primers (5 mM each) and 12.5 μl multiplex PCR master mix (Qiagen). The PCR amplification reaction was performed using the following calculated protocol: 15 minute initial denaturation step at 95 °C, followed by 35 cycles of 30 s at 94 °C, 3 min at 61 °C, and 30 s at 72 °C, and a final extension of 30 min at 60 °C. After amplification reaction, PCR products were 1/20 diluted in deionized formamide. Thereafter, 1 μl of diluted PCR product was mixed with 0.5 μl dye labeled DNA standard ROX 500 (Applied Biosystems) and 23.5 μl deionized formamide. Mixture was denatured for 5 min at 95 °C and electrophoresed using an automatic sequencer (ABI PRISM 3130xl; Applied Biosystems). The sizes of the microsatellites were assessed using GeneMapper analysis software (Version 4.0; Applied Biosystems). During the analysis, 12 reference strains belonging to type I (ENT), type II (Me49), and type III (NED) as well as atypical strains from Africa (DPHT, GAB3-2007-GAL-DOM014, GAB5-2007-GAL-DOM001, GAB3-2007-GAL-DOM002, FOU, CCH002-2004-NIA, and GAB2-2007-GAL-DOM002) and South America (TgCatBr5, VAND, and GUY-CAN-FAM001) were studied in parallel with Ankara and Ege-1 isolates [5,9,20,27,28].

## 3. Results

### 3.1. Isolation of Ankara strain

The strain isolated from the cerebrospinal fluid of a severe congenital toxoplasmosis case in Ankara (capital city of Turkey located in Central Anatolia) in 1972 was named Ankara [21]. After delivery, anti-*Toxoplasma* antibodies were detected in the mother and she was treated with Pyrimethamine-Sulfonamide during the last two months of pregnancy. There was no clinical data regarding the symptoms of the newborn. To isolate the *T. gondii* strain and diagnose toxoplasmosis, a cerebrospinal fluid sample of the newborn was administered intraperitoneally to the mice. The virulence assay showed that Ankara strain can kill mice in 4–5 days when inoculated with $1 \times 10^5$ tachyzoites intraperitoneally. Since then, this strain is being continuously passaged in the Department of Parasitology, Ege University Medical School.

### 3.2. Isolation of Ege-1 strain

In 2007, Ege-1 strain was isolated from a cerebrospinal fluid sample of a congenital toxoplasmosis case whose mother was living in Balıkesir located in Western Anatolia. Balıkesir has a coast to the Aegean (called as "Ege" in Turkish) sea. The mother was not screened for anti-*Toxoplasma* antibodies during or before pregnancy. During the neonatal physical examination of the newborn, hepatosplenomegaly, bilateral chorioretinitis and jaundice were noticed. Two weeks after the birth, the newborn died and the cerebrospinal fluid obtained during autopsy was administered intraperitoneally to the *outbred* Swiss Webster mice. The virulence