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Short communication

Isolation of *Toxoplasma gondii* strains similar to Africa 1 genotype in 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].

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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 1×10^5 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

assay showed that Ege-1 strain can kill mice in 4–5 days when inoculated with 1×10^5 tachyzoites intraperitoneally.

According to the results of the serological assays, the sera of the new born and the mother after delivery were positive for IgM and IgG and the avidity indices were 42% and 44%, respectively indicating late infection (>6 months). The PCR assay identified *T. gondii B1* gene in cerebrospinal fluid.

3.3. Microsatellite genotyping

According to microsatellite analysis, Ankara and Ege-1 strains were both highly similar to strains belonging to the Africa 1 genotype (Table 1). Genetic structures of these strains are closely related, but minor genetic polymorphisms can be detected in highly polymorphic microsatellite markers such as *M48*, *N60*, *AA*, *N61*, and *N83*.

4. Discussion

The "Africa 1" genotype designation has been given to this atypical strain because it is overrepresented in sub-Saharan Africa, especially in immunocompromised patients who reactivate a past infection acquired in different African countries [5] or in domestic animals collected in Gabon [9]. The reason of isolating a strain mainly detected in Africa can be associated to Turkey's specific geographical location. Turkey is like a bridge between Asia, Europe, and Africa and it is surrounded by seas. Historically, Anatolia was on or very close to many historic overland (such as Silk Route) or maritime (along the coast of Mediterranean sea) trade routes that ended in Europe. There was also a trans Saharan trading route that linked sub-Saharan Africa with North Africa [29]. Overall, sub-Saharan Africa, North Africa, Europe and Anatolia were linked to each other through several trading routes.

In addition to commercial items, numerous diseases also traveled along the trade routes by rodents and their ectoparasites. Cats, definitive host of *T. gondii*, were possibly kept in trade ships or caravans because they were petted and excellent rodent hunters. Therefore, toxoplasmosis may be one of the other diseases that traveled along the trading routes. Thus, detecting Africa 1 genotype in Anatolia can be anticipated. In addition, strains detected mainly in Europe and Asia may also be detected in Anatolia and vice versa.

As depicted in the Introduction, Africa 1 genotype has not been defined in Asia and Middle East. In sub-Saharan Africa, in addition to the archetypal clonal lineages, atypical or recombinant genotypes have been reported from Nigeria, Uganda, and Ghana [8,30,31]. Ajzenberg et al. detected Africa 1 genotype in nine immune-compromised patients who were mostly from sub-Saharan Africa (Ghana, Benin, Côte d'Ivoire, Cameroon, Uganda, Senegal, Guinea) [5]. Due to wide distribution in western and central Africa, Africa 1 genotype has been described as an African clonal lineage candidate [5]. Previously, Africa 1 genotype has only been detected in Europe mainland in a French renal transplant patient who has acquired toxoplasmosis by transplant (the donor of unknown origin was seropositive and recipient seronegative) [5]. In this case, the origin of the donor may be related to sub-Saharan Africa. In another study conducted in Gabon, located in western Africa, Type III, Africa 1 and Africa 3 genotypes were isolated from animals. Among these isolates, the most virulent strain in mice was Africa 1 [9]. Interestingly, BrI isolated from Brazil, seems to have similar clonal and virulence properties with Africa 1 genotype [19]. Consequently, Africa 1 genotype may be transported from sub-Saharan countries to North Africa and finally to Europe or Anatolia mainly through trans Saharan trading routes. Thus, Africa 1 and BrI connection may have been established by slave ships that transported slaves between Africa and Americas. Cats were possibly kept in these ships to hunt the rodents. A possible way of intercontinental transport may be through long distance migratory birds which have not been intensely investigated in genotyping studies yet.

Table 1
Genotyping results with 15 microsatellite markers of the Ankara and Ege-1 strains described in this study and 12 reference *T. gondii* strains.

Isolate (type)	Microsatellite markers (size range in bp)														
	TUB-2 (287–291)	*W35* (242–248)	*TgM-A* (203–211)	*B18* (156–170)	*B17* (334–366)	*M33* (165–173)	*IV.1* (272–282)	*XI.1* (354–362)	*M48* (209–243)	*M102* (164–196)	*N60* (132–157)	*N82* (105–145)	*AA* (251–332)	*N61* (79–123)	*N83* (306–338)
Ankara (Africa 1)	291	248	205	160	342	165	274	354	227	166	147	111	295	91	310
Ege-1 (Africa 1)	291	248	205	160	342	165	274	354	227	166	149	111	289	91	310
DPHT (Africa 1)	291	248	205	160	342	165	274	354	225	166	147	111	271	89	306
GAB3-2007-GAL-DOM014 (Africa 1)	291	248	205	160	342	165	274	354	229	166	142	111	271	95	306
GAB5-2007-GAL-DOM001 (Africa 1)	291	248	205	160	342	165	274	354	231	166	149	111	277	87	306
GAB3-2007-GAL-DOM002 (Africa 1)	291	248	205	160	342	165	274	354	223	166	147	111	269	89	306
FOU (Africa 1)	291	248	205	160	342	165	274	354	227	166	147	111	281	89	306
CCH002-2004-NIA (Africa 2)	289	248	205	160	336	165	274	354	225	166	145	111	273	89	308
GAB2-2007-GAL-DOM002 (Africa 3)	291	242	207	160	342	165	278	354	223	166	142	111	277	97	310
ENT (Type 1)	291	248	209	160	342	169	274	358	209	166	145	119	267	87	306
Me49 (Type 2)	289	242	207	158	336	169	274	356	215	174	142	111	265	91	310
NED (Type 3)	289	242	205	160	336	165	278	356	209	190	147	111	267	91	312
TgCatBr5 (Atypical)	291	242	205	160	362	165	278	356	237	174	140	111	265	89	314
VAND (Amazonian)	291	242	203	162	344	167	276	356	217	170	142	113	277	91	308
GUY-CAN-FAM001 (Caribbean 1)	291	242	205	162	342	165	278	356	213	164	142	109	265	87	312

In the present study, *T. gondii* strains isolated in Turkey were first time analyzed and sorted as Africa 1 genotype. In Europe, the Africa 1 genotype is usually isolated in immunocompromised patients originating from sub-Saharan Africa. Interestingly, Africa 1 was isolated from two newborns that lived in Turkey located in Eastern Europe. The predominant Type II strain in Europe and Type III strain have not been isolated in Turkey yet. However, due to its specific geographical location linking historical trading routes, Type II, III, and other atypical strains can be isolated in Turkey. Therefore, further studies are required to isolate more strains from animals and humans in Turkey. The results of these studies will reveal the variety of *T. gondii* genotypes present in Turkey.

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